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Search for Seasonal Rhythmicity of Pineal Melatonin Production in Rats Under Constant Laboratory Conditions: Spectral Chronobiological Analysis, and Relation to Solar and Geomagnetic Variables

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Earlier we reported that in a number of experiments pineal melatonin production in rats under constant laboratory conditions displayed seasonal rhythms but subsequently were not always able to confirm this. Since there was no indication under which conditions such rhythms may be present, we performed four consecutive identical experiments with untreated female Sprague–Dawley rats within the same animal room during 1997–2006. Nocturnal urine samples (19–23, 23–3, 3–7 h) were collected at monthly intervals over 494–658 d with 12 animals each in experiments I and II (1997–1999, 1999–2000), 30 animals in experiment III (2002–2004), and 15 in experiment IV (2005–2006). 6-Sulfatoxymelatonin (aMT6s) was measured by ELISA. The excreted aMT6s at each time interval as well as total nocturnal aMT6s-excretion (19–7 h) was submitted to standard statistical analyses as well as to a spectral chronobiological analysis to determine the period lengths of the components involved which was followed by processing with the single cosinor method. Seasonal rhythm components (circannual period length: 360 ± 60 d) were detected in experiment III (2002–2004) for the overall nocturnal excretion as well as for two sub-intervals (23–3 and 3–7 h) and in one night interval of experiment II (23–3 h). Multiple components with mostly short period lengths of around 100 d and some long ones of 500–650 d were found in the other experiments. Systematic MESOR and amplitude variations were observed during the experiments, being highest in experiment II (19–7 h, also 23–3 h and 3–7 h) and lowest in experiments I and IV. These results illustrate that seasonal melatonin rhythms are not a general phenomenon in female laboratory rats indicating an involvement of unknown environmental cues. As an extension of our earlier hypothesis regarding a seasonal *Zeitgeber* function of the horizontal intensity *H* of the geomagnetic field showing circannual variations, we assume further modulation by the 11-yrs' sunspot cycle which leads to geomagnetic disturbances and could facilitate seasonal aMT6s rhythmicity during specific years. (Author correspondence: christian.bartsch@uni-tuebingen.de)

Keywords: Circannual, Pineal, Seasonal Geomagnetic field, 6-Sulfatoxymelatonin, Sunspots, Urine

INTRODUCTION

During the last two decades of our research dealing with the link between the pineal gland and cancer, we repeatedly observed that pineal activity of rats showed seasonal variations even under controlled laboratory conditions. Such variations were found for the so-called pineal anti-tumor activity of yet unknown chemical nature (Bartsch et al., 1990, 1993) as well as for nocturnal plasma melatonin (Bartsch & Bartsch, 2007) and the nocturnal production of melatonin estimated by the urinary excretion of the metabolite 6-sulfatoxymelatonin (aMT6s) in female F344 Fischer (Bartsch et al., 1994) as well as in BDII/Han-rats (Bartsch et al., 2001). Karasek et al. (1988) reported precise annual changes of pineal synaptic ribbons and spherules in the rat which are thought to possess important, yet incompletely understood, intra-pineal functions (Reuss et al., 2010). To explain such temporal phenomena under controlled laboratory conditions we developed the hypothesis that seasonal variations of the daily profiles of the horizontal intensity *H* of the geomagnetic field act as *Zeitgeber* in case of pineal melatonin secretion (Bartsch et al., 1994). In subsequent studies, however, we could not always fully verify our earlier observations (Jost, 1994) and therefore decided to perform multiple repetitive studies with

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the same strain of rats kept within the same animal room. We are now able to present a detailed chronobiological evaluation of the longitudinal nocturnal production of melatonin as estimated by urinary aMT6s in the course of four consecutive experiments performed during 1997–2006 for the overall nocturnal excretion (19–7 h) as well as for three sub-intervals (19–23, 23–3 and 3–7 h).

MATERIALS AND METHODS

Animals and Housing Conditions

The studies were approved by the Animal Care Committee of the regional council of Tübingen and were in accordance with the standards set forth in the *Guide for the Care and Use of Laboratory Animals* (published by the National Academy of Science, National Academy Press, Washington, DC) as well as with the standards of this journal (Portaluppi et al., 2010). Sixty-nine female Sprague–Dawley rats, obtained from Charles River Wiga (Sulzfeld, Germany), were studied in four experiments. In the first experiment (I), 12 animals (born April 27/28, 1997) were investigated in the same manner as in the second experiment (II: born April 28, 1999). In the third experiment (III), 30 animals were used which were born on October 9, 2002. Finally, 15 animals (born May 4, 2005) were studied in the fourth experiment (IV).

In all experiments, 12 animals were housed per cage receiving tap water and food *ad libitum* (pellets, ssniff from RIMH, Soest, Germany). The cages made from transparent polymethacrylate (Plexiglas) with a ground area of .4 m^2 and a height of 20 cm were equipped with three stainless steel nipples giving access to drinking water. They were covered by Plexiglas lids perforated with holes. One cage each was placed at the bottom of a special chamber constructed for mobile phone exposure of freely moving animals as described earlier (Bartsch et al., 2002). The animals studied served as sham-exposed controls. The chambers were made from sheet steel (height approximately 190 cm, with ground dimensions 90 cm × 90 cm) and were equipped with wire grids at the bottom, front, and back as well as at the top to allow ample circulation of air but preventing high-frequency radiation to enter. These chambers were not magnetic so that the natural geomagnetic field present within the animal facility could permeate the chambers as well as animal cages. At the very top of each chamber, outside of the wire grid of 8 mm diameter, four energy saving bulbs of 8 W each (energy saver E27, 420 Lumen of Philips) were installed to illuminate all animal cages at their bottom with a variation of 28–35 Lux light intensity (for more details regarding the chambers: see Bartsch et al., 2002). The lighting regimen comprised 12 h of illumination per 24 h, with lights turned on at 0700 h Central European Summer Time throughout the year. Air temperature (mean ± standard deviations) was very similar in all four experiments (I: 21.8 ± .4°C, II: 22.4 ± .6°C, III: 21.8 ± .7°C, and IV: 22.2 ± .5°C) and so was the relative air humidity with an average of 60 ± 8% standard deviation. The experiments were performed within the same animal room of the Interfaculty Institute of Biochemistry of the University of Tübingen, which has no window and is located at the center of the basement of a three-storied building in which mobile phone communication is impossible, even after repeated upgrading of the surrounding basestations over the years.

Details of Urine Collection

Animals were transferred to individual metabolic cages for 12 h during the scotophase once per month starting from 7 (experiments I and II) or 9 wk of age (experiments III and IV) until the age of 23 (experiment I), 18 (experiments II and III), or 20 mo (experiment IV). In experiment I, urine was collected between June 1997 and April 1999 (over a total period of 658 d), in experiment II between June 1999 and October 2000 (over 494 d), in experiment III between December 2002 and April 2004 (over 503 d), and in experiment IV between June 2005 and December 2006 (over 542 d). Four metabolic cages that were located within one sham-exposure chamber were used for urine collection at three nocturnal intervals (19–23, 23–3, and 3–7 h). The samples of the first interval (19–23 h) were collected at 2300 h in complete darkness (to avoid suppression of melatonin by light) and immediately aliquoted and frozen at –25°C. Urine of the other two intervals were collected shortly after 7 h and frozen. The volume of each sample was quantified with measuring pipettes at a precision of 100 µl. Storage time at –25°C was 2 yrs until aMT6s assays were performed thus lying well within the reported time range of 5 yrs to ensure reliable determinations of this variable (Travis et al., 2003).

Quantification of Urinary 6-Sulfatoxymelatonin

aMT6s was measured in duplicates with ELISA-kits of IBL International GmbH, Hamburg, using the same batch of kits for each experiment to minimize interassay-variations. Incubations were performed at highly controlled temperatures (±.1°C) using a Variomag Thermoshake (Inheco, Martinsried, Germany). In this way, the interassay coefficient of variation could be limited to 8%, the intra-assay coefficient of variation to 5%. aMT6s concentrations were multiplied with the corresponding urine volumes to calculate the respective quantity for each collection interval. In addition, the total nocturnal excretion (19-7 h) for each animal in each month of collection was calculated summing up aMT6s quantities of all three nocturnal intervals (19–23, 23–3, and 3–7 h).

Basic Calculations, Plots, Statistics and Spectral Chronobiological Analysis

As a first step, the arithmetic means (±standard errors of the mean) of the total nocturnal excretion of aMT6s (19–7 h) as well as all sub-intervals (19–23, 23–3, and 3–7 h) were calculated of each month of collection for all four

experiments. These results were graphically depicted with Sigmaplot (version 8.0 of SPSS Science Software GmbH, Erkrath, Germany).

Standard statistical comparisons were carried out within (i, ii) and between (iii) the four experiments, followed by chronobiological analyses of the longitudinal profiles of aMT6s excretion (iv).

(i) To verify the obvious differences of the aMT6s-excretion levels during the different time intervals of each experiment seen in the Sigmaplot graph, average values of the sampling intervals were calculated for the total sampling periods of 17–22 mo for each animal and submitted to ANOVA for repeated measurements, followed by pairwise Tukey HSD tests.
(ii) For the analysis of longitudinal month-to-month changes of aMT6s within the different experiments, the monthly values of each animal at each nocturnal interval as well as the total nocturnal excretion, were submitted to ANOVA for repeated measurements followed by pairwise Tukey HSD tests using Statistica 7.1 of Statsoft (Tulsa, OK, USA).
(iii) The comparison of monthly averages of aMT6s excretion between the four experiments at the different nocturnal collection intervals (19–23, 23–3, and 3–7 h, as well as 19–7 h) was carried out by ANOVA followed by pairwise Tukey HSD tests using Statistica 7.1 of Statsoft (Tulsa, OK, USA).
(iv) The chronobiological analysis of the longitudinal profiles of the total nocturnal excretion of aMT6s (19–7 h) as well as of the three sub-intervals (19–23, 23–3, and 3–7 h) of all four experiments was performed with the help of the software TSA Cosinor 6.3 (Expert Soft Technologie, Richelieu, France) applying the single cosinor method (Bingham et al., 1982; Nelson et al., 1979) together with a spectral analysis to determine the different period lengths of the components involved. For this purpose, all permissible periods were tested between 60 d (twice the sampling distance of 1 mo – Nyquist–Shannon sampling theorem) and the maximal observational times of 494–658 d in the different experiments. A central part of the spectral analysis by the applied software is the so-called reverse elliptic periodogram (REP; Gouthière et al., 2004) allowing the detection of statistically significant period lengths. The REP depicts the area of the 95% confidence ellipse surrounding the cosinor vector as a function of the corresponding period. The ellipse areas are calculated according to Nelson et al. (1979) for the single cosinor regression model. Those parts of the REP showing a minimum of the curve (reverse peak) indicate possible significant fits by the cosinor model for the corresponding periods provided the zero ellipse test can be rejected. If this applies, those single or multiple regions of the periodogram are color-coded to indicate significant period ranges. From the upper and lower limits of the color-coded regions, the corresponding 95% confidence range for each period is determined (zero ellipse test: $p < .05$; Bingham et al., 1982; Nelson et al., 1979). A color-coded reverse peak of the plot marks a period at which not only the ellipse area is relatively lowest but where ellipse as well as amplitude test yield highest statistical significance within the corresponding period range. The corresponding p-values were not corrected for multiple testing. For such a period, the rhythm parameters are recorded, namely MESOR (arithmetic mean of the measured values within the respective longitudinal collection period), amplitude (i.e. half of the rhythmic variability), and acrophase (peak time of the cosine function used to approximate the rhythm component); furthermore, the so-called percent rhythm as well as the relative amplitude expressed as percent of the MESOR (Bingham et al., 1982; Nelson et al., 1979). It is pointed out that those components with circannual, respectively, seasonal periods (360 ± 60 d) which were searched for and thus anticipated *a priori* are designated in the following as rhythms or rhythmic components, whereas those components with other than circannual periods, and which corresponded to a spectral peak accounting for a statistically significant portion of the overall variance, are referred to as oscillations.

Sunspot Numbers, Solar Radioflux as well as Index of Geomagnetic Disturbances and Melatonin Production

The daily number of sunspots, the corresponding solar radioflux (10.7 cm wave, 2.8 GHz; expressed in radioflux units), and the so-called daily averaged planetary A-Index (Ap, estimating geomagnetic disturbances in response to solar activity) were downloaded from the ftp site of the National Oceanic and Atmospheric Administration's (NOAA)'s National Geophysical Data Center of the USA (NGDC) (ftp://ftp.ngdc.noaa.gov/STP/GEOMAGNETIC_DATA/APSTAR/, e.g. for Ap-Index) and subsequently averaged over periods of 30 d between January 1, 1995 and February 28, 2008. These three sets of heliophysical respective geophysical data were plotted to allow comparisons among them and to visualize the course of Solar Cycle 23 (1996–2008). Subsequently, the Ap-Index (in logarithmic form) was depicted together with the overall nocturnal excretion of aMT6s (19–7 h), as an estimate of total melatonin production, during Experiments I–IV (1997–2006).

RESULTS

Graphical Depiction of aMT6s Excretion and Results of Basic Statistical Tests

In Figure 1, the total nocturnal excretion of aMT6s (19–7 h) is shown for experiments I–IV expressed as monthly arithmetic means with standard errors of the mean (SEMs). These values were calculated from the corresponding quantities of the three nocturnal sub-intervals (see Figure 2).

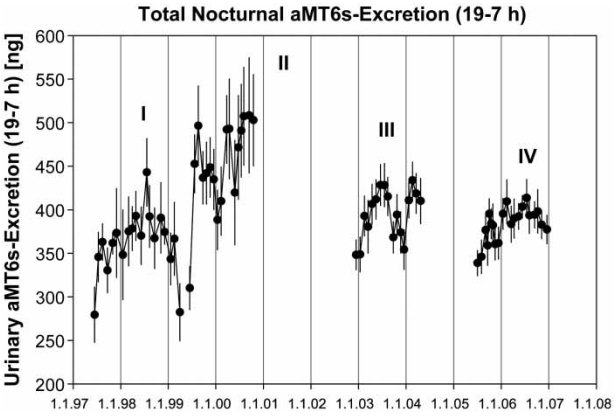

FIGURE 1. Total nocturnal (19–7 h) excretion of 6-sulfatoxymelatonin (aMT6s) in untreated female Sprague–Dawley rats during four experiments (I–IV) carried out within the same animal room (I: 12 animals during 1997–1999; II: 12 animals during 1999–2000; III: 30 animals during 2002–2004; IV: 15 animals during 2005–2006). Results are shown as monthly arithmetic means ± SEMs: II > I, III, IV (p = .0002); III > I (p = .0300). Excretion of aMT6s among the different months of collection did not differ in I and IV; II: August 2000 > January and March 2000 (p = .0098, p = .0345), June 1999 < July 1999 (p = .0406), June 1999 < January 2000 (p = .0259), June 1999 < March–May 2000 (p = .0028–.0002); III: June 2003 > December 2003 (p = .0281).

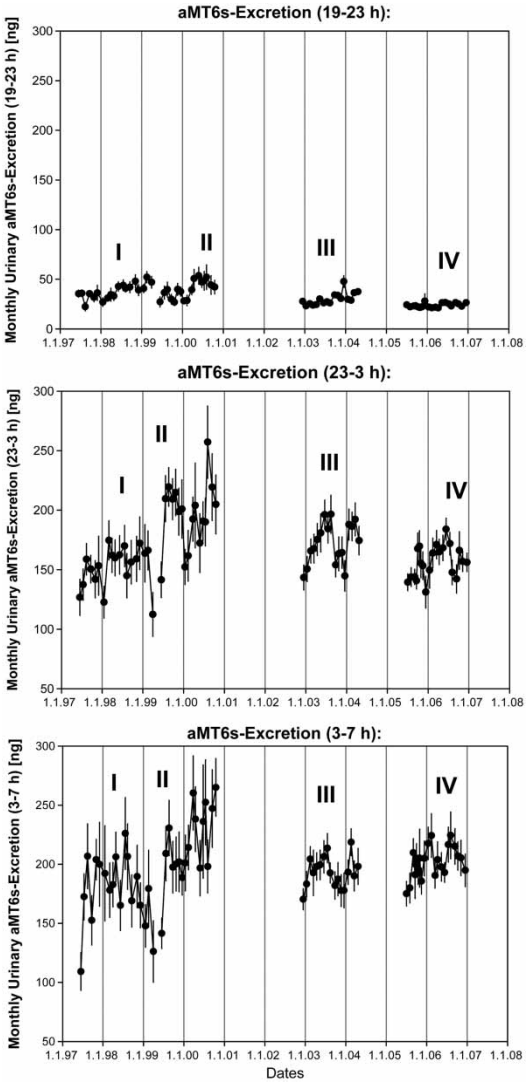

FIGURE 2. Excretion of 6-sulfatoxymelatonin (aMT6s) in untreated female Sprague–Dawley rats during three nocturnal intervals (upper panel 19–23 h, center panel 23–3 h, and lower panel 3–7 h) collected in the course of four experiments. Total nocturnal aMT6s excretion (19–7 h, see Figure 1) was calculated from the values of the three sub-intervals. Comparison of aMT6s among the three nocturnal collection intervals: I–IV: 23–3 h, 3–7 h > 19–23 h (p = .0001); III, IV: 3–7 h > 23–3 h (p = .0281 and .0001). Comparison of monthly average aMT6s excretion at the three nocturnal collection intervals (19–23, 23–3, and 3–7 h) between experiments I–IV: 19–23 h: I, II > III, IV (p = .0046–.0002); III > IV (p = .0162). 23–3 h: II > I, IV (p = .0002); II > III (p = .0029); III > I (p = .0156). 3–7 h: II > I, III (p = .0002 and p = .0263); IV > I (p = .0094). Longitudinal patterns of monthly aMT6s excretion at the three nocturnal collection intervals (19–23, 23–3, and 3–7 h) of experiments I–IV: 19–23 h: *experiment I:* November 1998, February 1999 > August 1997 (p = .0317 and .0436); *experiment III:* January–April 2003 < September, December 2003 (p = .0312–.0006), January–April 2003 < March 2004 (p = .0289–0.0089), August 2003 < December 2003 (p = .0454). 23–3 h: *experiment II:* June 1999 < August, September 2000 (p = .0074 and .0200); January, February 2000 < August, September 2000 (p = .0244–.0070); *experiment III:* December 2002 < April, June, August 2003 (p = .0147–.0031); December 2003 < April, August 2003 (p = .0405 and .0251); *experiment IV:* December 2005 < October 2005, June 2006 (P = .0308 and .0017), August 2006 < June 2006 (P = .0226). 3–7 h: *experiment II:* June 1999 < July 2000 (P = .0166); *experiment III:* February 2004 > November 2003, December 2002 (P = .0404 and .0132).

(i) Figure 2 clearly shows that the excretion of aMT6s is very low during the 19–23 h interval and rises during the second (23–3 h) as well as third collection period (3–7 h), being manifold higher than during the first nocturnal interval when the final steps of melatonin biosynthesis are initiated after lights are turned off at 1900 h (Vollrath, 2001). Statistical evaluation of aMT6s excretion between the three nocturnal collection intervals consistently shows that aMT6s is significantly higher in all four experiments during the 23–3 and 3–7 h interval compared with 19–23 h (p = .0001) and that during 3–7 h it is even higher than during 23–3 h (p = .0281, p = .0001 in experiments III and IV). The latter indicates that a circadian phase delay of nocturnal melatonin secretion may be present in the last two experiments compared with experiments I and II.

(ii) Within each experiment for each collection interval, variations between the different months were determined applying ANOVA plus pairwise Tukey tests. These results are given in full detail in the legends of Figures 1 and 2. They show a considerable number of differences which include possible seasonal variations. The total nocturnal excretion of aMT6s (19–7 h) is significantly higher in June 2003 than in December 2003 (p = .0281; experiment III) as well as in August 2000 compared with January and March 2000 (p = .0098, p = .0345; experiment II). Hints to seasonal variations were also detected for the 23–3 h interval (January, February 2000 < August, September 2000, p = .0244–.0070,

experiment II; December 2002 < April, June, August 2003, p = .0147–.0031, experiment III; December 2003 < April, August 2003, p = .0405, p = .0251, experiment III). These results in their complexity are difficult to evaluate and summarize stressing the need for a complementary statistical approach, e.g. in the form of chronobiological methods, to better understand the temporal structures involved in longitudinal melatonin secretion (see iv).

(iii) It is apparent that the longitudinal patterns of total nocturnal aMT6s excretion vary considerably among the four experiments: total nocturnal aMT6s (19–7 h) is lowest during experiment I (1997–1999) and rises steeply in the course of experiment II (1999–2000), being maximally 80% higher. The corresponding levels of aMT6s during experiment III (2002–2004) and experiment IV (2005–2006) are lower than in experiment II lying slightly above those of experiment I. Pairwise comparisons with Tukey-HSD tests show that the monthly arithmetic means of total nocturnal aMT6s are significantly higher in experiment II than in all other experiments (p = .0002). These values are also higher in experiment III than in the first experiment (p = .0300). Comparing the profiles of aMT6s at the different nocturnal collection intervals among the four experiments, it is evident that during 3–7 h, and particularly during 23–3 h, the highest levels are found in experiment II same as for total nocturnal aMT6s excretion (19–7 h). Pairwise Tukey tests show that the monthly averages of aMT6s are significantly elevated at the 23–3 h-interval in experiment II compared with all other three experiments (II > I, IV p = .0002; II > III p = .0029), and at the 3–7 h interval compared with two other experiments (II > I, III p = .0002 and p = 0.0263). aMT6s excretion in the first nocturnal interval (19–23 h) is significantly higher in experiments I and II than in III and IV (p = .0046–.0002), which also applies to experiment III compared with IV (p = .0162). This indicates that a circadian phase delay of nocturnal melatonin secretion may develop in the course of experiments I–IV over the 1997–2006 span.

(iv) The macroscopically observable variations of aMT6s for each experiment and collection interval were submitted to a spectral chronobiological analysis testing for the presence of components with period lengths between 60 d and the maximal observational periods (I: 658 d, II: 494 d, III: 503 d, and IV: 542 d) which was followed by single cosinor analyses. It is pointed out again that components with circannual, respectively, seasonal (360 ± 60 d) periods are designated as rhythms or rhythmic components, whereas those components with other than circannual periods are referred to as oscillations.

Results of the Spectral Chronobiological Analysis

Total Nocturnal Excretion of aMT6s (19–7 h)

The chronobiological results for the *total nocturnal excretion of aMT6s (19–7 h)* testing potential period lengths between 60 d and the maximal observational time of each experiment are given in Table 1, and the calculated period lengths are depicted in the upper panel of Figure 3. The following main results were obtained in the different experiments:

In *experiment I (1997–1999)*, two significant components were detected, one with a period of 658 d (i.e. maximal observational period; p = .0054) and another with 111 d (p = .0345). The amplitude was slightly higher for the oscillation of the longer period (34.8 ng vs. 30.3 ng) showing an acrophase on June 27, 1998. In

TABLE 1. The results of spectral chronobiological analysis: total nocturnal aMT6s excretion: 19–7 h

Experiment	Number of samples	Period [days]	Period range [days]	Rhythm detection (p)	Percent rhythm (%)	MESOR [ng] Mean	MESOR [ng] ± SEM	Amplitude [ng] Mean	Amplitude [ng] ±SEM	Amplitude [ng] % of MESOR	Acrophase (s) [date] Mean
I	183	658	498–658	.0054	5.6	363	8	34.8	11.2	9.6	27.6.98
		111	109–113	.0345	3.7	364	8	30.3	11.3	8.3	19.8.97
											8.12.97
											29.3.98
											18.7.98
											6.11.98
											25.2.99
II	180	180	179–181	.0497	3.3	453	11	40.2	16.9	8.9	14.9.99
											12.3.00
		111	105–120	.0155	4.6	451	11	43.9	15.7	9.7	14.8.99
											3.12.99
											23.3.00
											12.7.00
III	457	300	231–420	.0002	3.8	396	6	31.0	8.3	7.8	12.6.03
IV	317	542	455–542	.0063	3.2	385	4	18.6	6.2	4.8	3.6.06

Seasonal rhythm components with circannual period lengths (360 ± 60 d) are highlighted by shading.

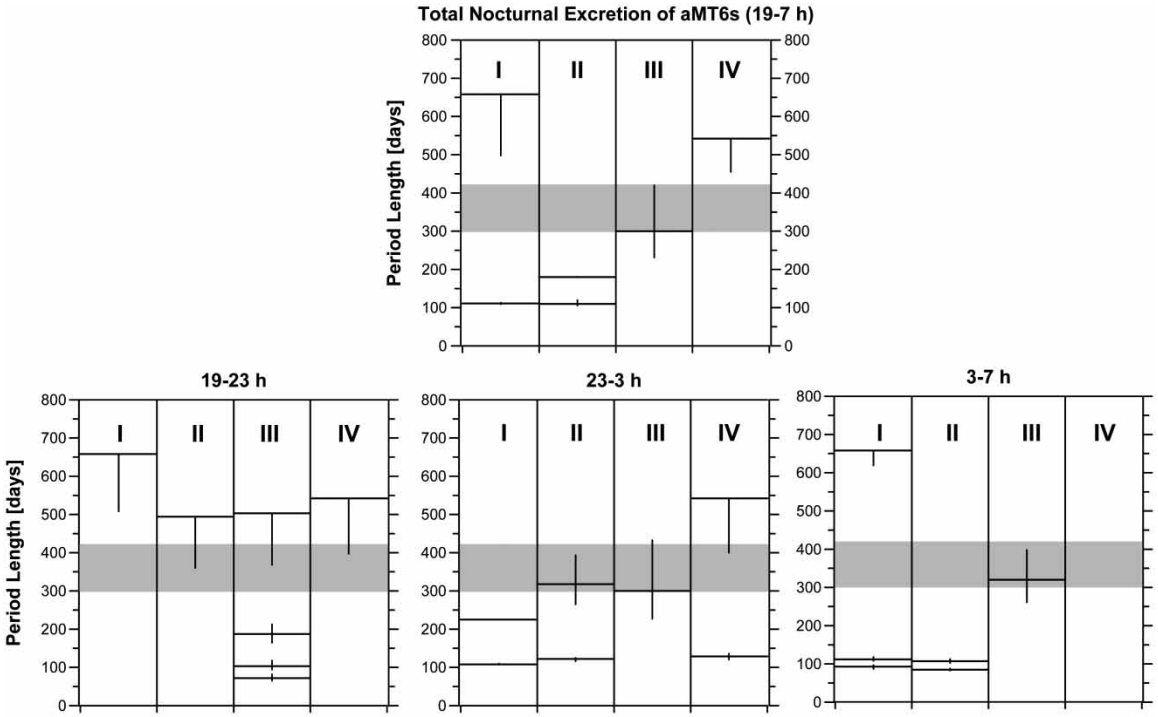

FIGURE 3. Period lengths (days) of the components of 6-sulfatoxymelatonin (aMT6s) excretion (horizontal lines) together with the corresponding 95% confidence ranges (vertical lines), according to the results of spectral chronobiological analyses. The results for the total nocturnal aMT6s excretion (19–7 h) are depicted in the upper panel, and for the three nocturnal sub-intervals in the lower panels (left: 19–23 h, center: 23–3 h, right: 3–7 h). Results are given for each of the four experiments (I: 1997–1999, II: 1999–2000, III: 2002–2004, IV: 2005–2006). The shaded areas mark the range of seasonal respective circannual rhythms (360 ± 60 d).

experiment II (1999–2000), two significant components were detected, one with 180 d (p = .0497) and another with 111 d (p = .0155), both with similar amplitudes. The oscillation with a period of 180 d showed acrophases in September 1999 and in March 2000. In case of *experiment III (2002–2004)*, only one, but statistically highly significant, rhythm component was found (period of 300 d, p = .0002) with an acrophase in late spring (June 12, 2003). This rhythm can be regarded as seasonal, respectively, circannual. Also *Experiment IV (2005–2006)*, possessed only one component, however, with a very long period of 542 d covering the maximal observational period (p = .0063), and showing an acrophase in late spring (3.6.2006).

Comparing the components of the four experiments, it is apparent that considerable differences exist, in spite of the fact that animals of the same strain and sex as well as comparable age were studied within the same animal room. These differences pertain to period lengths, MESORs and amplitudes and can be highlighted as follows (see upper panel of Figure 3): only a single seasonal rhythm is found in experiment III (2002–2004) with a period length of 300 d, whereas in experiments I (1997–1999) and II (1999–2000) two oscillations each were found (I: 111, and 658 d; II: 111, and 180 d), and in experiment IV (2004–2006) a single oscillation with an extremely long period length is detected (542 d). In addition, clear differences in MESORs and amplitudes are observed; both being highest in experiment II (see Table 1). MESORs are 10–15% lower in experiments I, III, and IV compared with II, and the corresponding amplitudes are even 20–30% lower in experiments I and III relative to II and are depressed by even 60% in experiment IV. These differences are also clearly reflected by the results of the conventional statistical analysis of the original data mentioned above (see legend of Figure 1). These findings show that the longitudinal nocturnal production of melatonin (19–7 h), as estimated by the excretion of its main metabolite aMT6s, underlies profound alterations with respect to quantity as well as period lengths between the four experiments performed under identical conditions.

aMT6s Excretion During the Nocturnal Sub-Intervals (19–23, 23–3, and 3–7 h)

The total nocturnal aMT6s excretion (19–7 h) was calculated as sum of the corresponding sub-intervals during the scotophase (19–23, 23–3, and 3–7 h). It is, therefore, of interest to analyze the temporal structure of these intervals to understand in which way they may have contributed to the overall nocturnal excretion. The chronobiological results of all three intervals of each experiment are given in Table 2a (19–23 h), b (23–3 h), and c (3–7 h). The periods of the detected components are depicted in the lower panels of Figure 3.

TABLE 2. Results of spectral chronobiological analysis for the nocturnal aMT6s excretion during (a) 19–23 h, (b) 23–3 h, and (c) 3–7 h

Experiment	Number of samples	Period [days]	Period range [days]	Rhythm detection (p)	Percent rhythm (%)	MESOR [ng]		Amplitude [ng]			Acrophase(s) [date]
						Mean	±SEM	Mean	±SEM	% of MESOR	Mean
(a) *19–23 h*											
I	217	658	508–658	.0000	9.8	38.1	1.2	8.3	1.7	21.8	6.11.98
II	195	494	360–494	.0005	7.5	39.4	1.7	9.3	2.4	23.6	26.6.00
III	495	503	368–503	.0000	7.3	30.1	.6	5.6	.9	18.6	25.12.03
		187	164–213	.0041	2.2	30.4	.7	3.1	.9	10.1	12.5.03
											15.11.03
		103	94–118	.0004	3.2	30.4	.7	3.9	.9	12.7	21.2.03
											4.6.03
											15.9.03
											27.12.03
		72	65–82	.0056	2.1	29.9	.7	3.1	1.0	10.5	27.12.02
											9.3.03
											20.5.03
											31.7.03
											11.10.03
											22.12.03
											3.3.04
IV	321	542	397–542	.0005	4.6	23.5	.4	2.2	.5	9.4	19.9.06
(b) *23–3 h*											
I	211	225	213–237	.0307	3.3	153	3.9	14.8	5.6	9.7	28.9.97
											11.5.98
		108	107–110	.0435	3.0	153	3.9	14.2	5.6	9.3	17.8.97
											3.12.97
											21.3.98
											7.7.98
											23.10.98
											8.2.99
II	191	318	265–393	.0056	5.3	191	5.6	26.6	7.7	13.9	2.10.99
		122	116–125	.0406	3.3	197	5.5	18.3	7.6	9.3	9.8.99
											9.12.99
											9.4.00
											9.8.00
III	487	300	227–433	.0001	3.9	173	3.2	18.5	4.6	10.7	13.6.03
IV	320	542	400–542	.0177	2.5	158	2.4	9.5	3.5	6.0	17.5.06
		129	120–136	.0199	2.4	156	2.4	10.2	3.2	6.5	15.10.05
											21.2.06
											30.6.06
											6.11.06
(c) *3–7 h*											
I	195	658	619–658	.0371	3.4	179	5.9	20.8	7.9	11.6	22.4.98
		112	108–118	.0125	4.5	178	6.0	25.1	8.4	14.1	17.8.97
											7.12.97
											30.3.98
											20.7.98
											8.11.98
		93	87–97	.0025	6.1	177	5.9	28.8	8.0	16.3	4.8.97
											5.11.97
											6.2.98
											10.5.98
											11.8.98
											12.11.98
											13.2.99

II	183	107	102–113	.0138	4.7	215	6.5	27.7	9.2	12.9	16.8.99 1.12.99 17.3.00 2.7.00
		85	82–88	.0220	4.2	213	6.6	26.2	8.7	12.3	2.8.99 26.10.99 19.1.00 13.4.00 7.7.00
III	479	320	261–398	.0179	1.7	193	3.2	11.9	4.3	6.2	28.5.03
IV	317	—	—	—	—	—	—	—	—	—	—

Seasonal rhythm components with circannual period lengths (360 ± 60 d) are highlighted by shading.

In "Results (i)" it was shown that in all four experiments aMT6s excretion during the 23–3 h and the 3–7 h intervals was statistically significantly higher than during 19–23 h, allowing the conclusion that measurements of aMT6s during 23–3 h as well as 3–7 h principally reflect the peak secretion of nocturnal pineal melatonin thus justifying a focus on the two latter intervals.

aMT6s Excretion During 23–3 h

In *experiment I (1997–1999)*, two significant components are present, one with a period of 225 d ($p = .0307$) and another of 108 d ($p = .0435$) with very similar amplitudes (14.2–14.8 ng). In *experiment II (1999–2000)*, two components are detected, one with a period of 318 d ($p = .0056$) and another of 122 d ($p = .0406$). The 318 d rhythmic component is seasonal, respectively circannual, peaking in autumn (October 2, 1999). Amplitudes are higher for the seasonal (26.6 ng) than for the 122 d period component (18.3 ng). In *experiment III (2002–2004)* only one, but statistically highly significant, seasonal rhythm is found with a period of 300 d ($p = .0001$) and an acrophase in late spring (June 13, 2003). In *experiment IV (2005–2006)* two significant components exist, one with a period of 542 d (i.e. maximal observational period; $p = .0177$) and an acrophase in spring (May 17, 2006), and another of 129 d ($p = .0199$). The amplitudes of both oscillations are very similar (9.5–10.2 ng). The main results concerning the central aim of the spectral chronobiological analysis, namely to search for seasonal melatonin rhythmicity, is that such rhythms are present in the 23–3 h-interval in experiments II and III. For further details see Table 2b.

aMT6s-Excretion During 3–7 h

There are three significant components in *experiment I (1997–1999)* with periods of 658 ($p = .0371$), 112 ($p = .0125$), and of 93 d ($p = .0025$) possessing similar amplitudes (20.8–28.8 ng). In *experiment II (1999–2000)* two components with rather short periods of 107 and 85 d are detected ($p = .0138$ and .0220) with very similar amplitudes (26.2–27.7 ng). *Experiment III (2002–2004)* displays a single seasonal rhythmic component (same as during 23–3 h) with a period of 320 d ($p = .0179$) and an acrophase in spring (May 28, 2003). In *experiment IV (2005–2006)* no component is detectable. For further details, see Table 2c.

aMT6s-Excretion During 19–23 h

As mentioned before, aMT6s-excretion during this period is very low and thus its rhythmicity was unlikely to have contributed substantially to the overall rhythm components of the complete nocturnal collection interval (19–7 h), but it has to be stressed that seasonal respective circannual rhythm components do not exist here. Therefore the chronobiological results given in Table 2a are not dealt with in further detail here. However, it is relevant to point out that in all experiments oscillations of extremely long period length covering the whole observational time were detected in this collection interval. This, however, did not apply to the other two collection intervals (23–3 and 3–7 h).

Chronobiological Results of aMT6s for the Nocturnal Sub-Intervals in Relation to the Overall Nocturnal Excretion (19–7 h)

The primary aim of this report is to search for seasonal rhythms of urinary aMT6s-excretion. Only experiment III showed a pronounced circannual component in its overall nocturnal aMT6s-excretion (19–7 h) which can be explained by the presence of such components during both the 23–3 and 3–7 h-intervals coinciding with the peak of pineal melatonin secretion. It has to be mentioned that a seasonal rhythmic component was detected for aMT6s-excretion during the 23–3 h interval in case of experiment II as well which, however, was accompanied by an additional oscillation of substantially lower period length of 122 d. Furthermore, the absence of seasonal rhythmicity in the 3–7 h interval of this experiment explains why a circannual component was undectable for the overall nocturnal excretion (19–7 h).

Another important issue is in which way the variations of both MESORs and amplitudes for the total nocturnal excretion of aMT6s (19–7 h) among the four experiments may be reflected by those during the 23–3 h as well as 3–7 h-sub-intervals coinciding with maximal pineal secretory activity. A pattern of aMT6s excretion very similar to the one observed during 19–7 h is observed during the 23–3 h interval among the four experiments for both MESOR and amplitude and

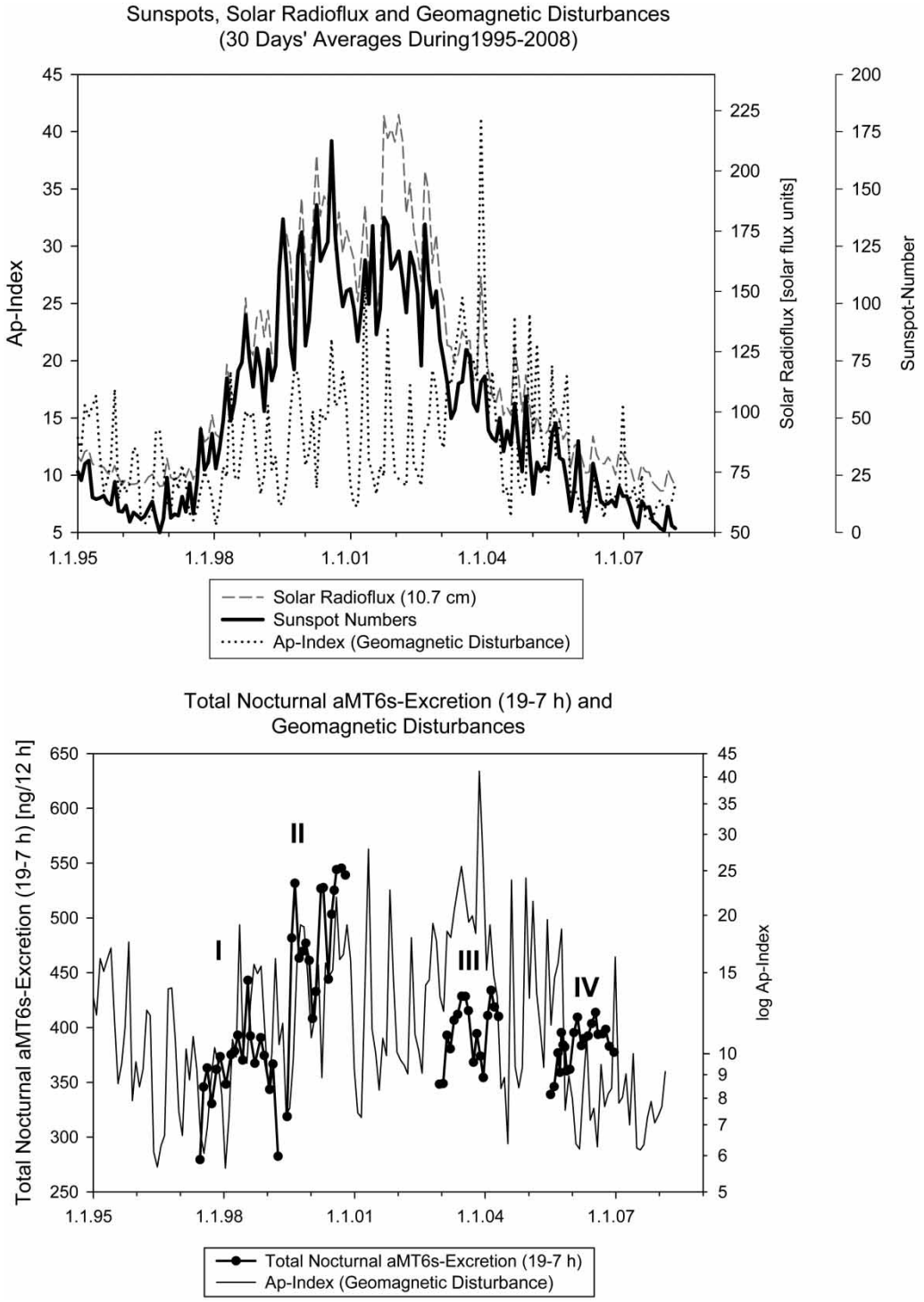

FIGURE 4. (a) Planetary A-Index (Ap), i.e. measure of geomagnetic disturbances, averaged over 30 d displayed together with the corresponding number of sunspots, as well as solar radio flux (10.7 cm wave, 2.8 GHz) for January 1, 1995 to February 28, 2008. Daily values were downloaded from the ftp-site of the National Oceanic and Atmospheric Administration's (NOAA)'s National Geophysical Data Center of the USA (NGDC) (ftp://ftp.ngdc.noaa.gov/STP/GEOMAGNETIC_DATA/APSTAR/ for Ap-Index). The period from May 1996 onwards constitutes the 23rd sunspot cycle with three main phases, an initial elevation (1996–2000), a plateau (2000–2002), and a gradual decline (2002–2008). (b) Planetary A-Index in logarithmic form (log Ap), as measure of geomagnetic disturbances, averaged over 30 d together with the monthly averages of the total nocturnal excretion of 6-sulfatoxymelatonin (aMT6s: 19–7 h) for the four experiments performed during 1997–2006 parallel to Solar Cycle 23 (1996–2008).

applies to a lesser extent to the 3–7 h interval (mainly MESOR). This indicates that the observed alterations of MESOR and amplitude for the total nocturnal excretion of aMT6s (19–7 h) during experiments I–IV seem to mainly stem from the variations during the 23–3 h interval. This assumption is supported by the results of the conventional statistical tests on the raw data of aMT6s excretion at different collection intervals and

experiments which were mentioned above and are given in the legends to Figures 1 and 2.

Solar and Geomagnetic Variables During the Observation Time and Relation to the Patterns of aMT6s Excretion

Figure 4a shows the number of sunspots, solar radioflux, and geomagnetic disturbances as 30 d averages during 1995–2008 thus covering Solar Cycle 23 and the complete course of all four experiments. These three variables display clear longitudinal variations, particularly the number of sunspots as well as solar radioflux, spanning a period of approximately 10 yrs. In Figure 4b, geomagnetic disturbances, expressed as the so-called planetary A-Index (Ap, in logarithmic form), are depicted along with the total nocturnal aMT6s excretion (19–7 h) which initially rises during experiments I and II (1997–2000) as Ap increases parallel to growing solar activity. Subsequent to that, during experiments III and IV (2002–2006), total nocturnal aMT6s excretion gradually declines as solar activity recedes again which, however, is accompanied by an intermediary phase of pronounced geomagnetic disturbances during 2002–2003. These observations will be discussed in further detail below.

DISCUSSION

The aim of this study was to monitor longitudinal patterns of nocturnal pineal melatonin production in rats during repetitive experiments performed under highly standardized laboratory conditions, and to analyze whether seasonal rhythmic components are detectable (Bartsch et al., 1994, 2001). The main result of the current investigations is that a seasonal rhythm was found in only one out of the four experiments (experiment III: 2002–2004) for the total nocturnal production of pineal melatonin (19–7 h). On the whole, longitudinal profiles in the different experiments were highly variable, each one displaying its own specific pattern which in most cases consisted of several non-seasonal oscillations with highly divergent period lengths (72–658 d), except for experiment III where a seasonal rhythm component was exclusively present. Despite divergent period lengths of the various oscillations in experiments I, II, and IV, amplitudes were similar within each experiment so that they can be compared, together with the corresponding MESORs, between all experiments. In experiment II, MESOR and amplitude were found to be highest for total nocturnal aMT6s excretion (19–7 h) and lowest in experiment IV (see Tables 1 and 2). It appears that systematic changes of both rhythm parameters occurred in the course of the four experiments showing a rise within experiments I and II, and a decline during experiments III and IV. This is particularly prominent for the total night period of 19–7 h as well as for the 23–3 h sub-interval (corresponding to the time of peak pineal melatonin secretion) which is clearly supported by the standard statistical tests on the raw data of the corresponding collection intervals. Thus, it appears that the changes of longitudinal aMT6s excretion throughout the four experiments (1997–2006) follows an overall variation with a period length of about one decade with an acrophase around 1999–2002 which was unfortunately incompletely covered by our investigations due to problems with funding. With respect to the occurrence of seasonal melatonin rhythmicity, it appears that this phenomenon occurs only around the peak of the postulated overall decennial rhythm of pineal activity.

On the basis of these observations and considerations, it is possible to re-approach our initial hypothesis regarding a general seasonal *Zeitgeber* function of the horizontal intensity H of the geomagnetic field (Bartsch et al., 1994) and to attempt a further refinement of the same. In our earlier publication presenting the initial hypothesis (Bartsch et al., 1994), the average 24 h profiles of H were plotted for each month of the year 1990 when those experiments were started. From this graph, it was apparent that each month of the year showed a typical daily profile with maximal values at sunrise and a trough at noon, with higher decrements in summer than in winter. Since our current experiments were performed over almost a decade, the daily profiles of H were plotted for each month of the years 1997–2006 and it became apparent that the profiles of comparable months of different years varied considerably showing smaller decrements between sunrise and noon in 1997 compared with the year 2000. For a systematic estimation of these monthly decrements, the maximal daily variations of H ($H_{(\text{Max–Min})}$) were calculated from the recordings of the Geophysical Observatory of the Ludwig-Maximilians-University of Munich at Fürstenfeldbruck throughout 1988–2007. These data are representative for South Germany and include our animal facility since measurements of H with a residual field strength probe connected to a magnetoscope of 1 nanoT sensitivity (Institute Förster, Reutlingen, Germany) placed inside of an animal cage showed a close parallelism over 24 h with the corresponding data recorded at Fürstenfeldbruck (Bartsch and Bartsch, unpublished observations; Bartsch et al., 1994). From a three-dimensional plot with two time axes corresponding to year and month, an overall trough for the monthly $H_{(\text{Max–Min})}$ values (recorded at Fürstenfeldbruck) was found to exist during 1995–1997, whereas pronounced peaks were present during 1988–1991 as well as 1999–2002. This indicated a further longitudinal modulation of $H_{(\text{Max–Min})}$ with a period length of about one decade (Bartsch et al., in press). From this, it was apparent that our initial experiments (1990–1992) reporting seasonal melatonin rhythms (Bartsch et al., 1994, 2001) were performed during a period of maximal $H_{(\text{Max–Min})}$ values. This also applies to experiment III of the current investigations (carried out during 2002–2004) where a seasonal melatonin rhythm component was clearly and exclusively detected for total nocturnal aMT6s excretion (19–7 h).

Since the geomagnetic field is known to be influenced by changing solar activity in the course of the approximately 11-yrs' sunspot cycle (Prölss, 2004), the number of average monthly sunspots were added to the $H_{(Max–Min)}$ plot and it became apparent that the annual variations of $H_{(Max–Min)}$ closely followed the changing number of sunspots during the sunspot cycle (Bartsch et al., in press) which is supported by Cornélissen et al. (1998).

Another and more common way to quantitate geomagnetic disturbances in response to changing solar activity is the so-called planetary A-Index (Ap) which is displayed in Figure 4a as 30 d averages together with the corresponding number of sunspots during Solar Cycle 23. In this figure, solar radio flux (2.8 GHz) is also depicted which closely follows the number of sunspots. A solar cycle can be divided into four main phases: a period of progressive increase (1996–2000), a plateau (2000–2002), a subsequent gradual decline (2002–2007), and finally, a phase of very low number of sunspots, respective radioflux, until the next cycle begins. Under natural conditions, solar radioflux reaches the surface of the earth if the corresponding frequencies lie within the so-called radio-window of the atmosphere of the earth (Prölss, 2004), which is thus an integral part of the natural terrestrial environment. For our experiments, however, this variable was most probably irrelevant since the animals were kept in special chambers (designed for the chronic exposure of high-frequency electromagnetic signals used for mobile telecommunication; Bartsch et al., 2002). They served as sham-exposed controls and were thus shielded from solar radiofrequency signals up to approximately 37 GHz. In contrast to this, the chambers in which the animals were kept did not possess magnetic properties so that they were exposed to the natural geomagnetic field and the respective disturbances due to changing solar activity. In Figure 4a, the variations of Ap during cycle 23 are also depicted which in their course apparently differ from the number of sunspots respective solar radioflux, the most prominent characteristics being single narrow spikes, separated by very low baseline values. Ap shows maximal spike values and does not return to low values between individual spikes during 2003–2004, 1–2 yrs *after* the number of sunspots peak. This is due to the fact that geomagnetic disturbances are primarily caused by charged particles that are emitted by the sun as the so-called coronal mass ejections (CME) which are more violent and frequent *after* the number of sunspots show their peak (Prölss, 2004; www.spacew.com./gic/guidance.pdf). Since the geomagnetic field is omnipresent, even within animal laboratories, and underlies CME-induced changes it is mechanistically understandable how solar activity can affect experimental animals, provided adequate receptor mechanisms exist (Wiltschko and Wiltschko 2005; Winklhofer, 2010).

In Figure 4b, variations of log Ap are plotted together with the overall nocturnal excretion of aMT6s (19–7 h) during experiments I–IV (1997–2006), which were performed during Solar Cycle 23 (1996–2008). As mentioned before, melatonin production is significantly higher in experiment II than experiment I, and subsequently declines during experiments III and IV. Increasing melatonin production during 1997–2000 is accompanied by rising Ap values suggesting a synergistic effect between progressive geomagnetic disturbances (due to elevated solar activity) and pineal melatonin secretion. Therefore, one might assume that declining levels of aMT6s, as observed during experiments III and IV (2002–2006), may be strictly accompanied by reduced geomagnetic disturbances. This seems to apply to experiment IV (2005–2006) when Ap values are very low but not to experiment III (2002–2004); here, Ap values are high or even extreme with the most pronounced single Ap spike observed during Solar Cycle 23 which occurred by the end of 2003 (Figures 4a and b). This event was accompanied by a phase of reduced aMT6s excretion and may thus have incidentally contributed to the phenomenon of melatonin circannual rhythmicity detected in these animals. From this point of view, it would appear that seasonal rhythms of pineal melatonin production are not basically endogenous in nature but rather cosmic (Bartsch et al., 2009). This assumption is supported by the observations of Burch et al. (1999) and Weydahl et al. (2000) in humans reporting an inhibitory effect of extreme geomagnetic disturbances on pineal secretion as well as by Cornélissen et al. (2009) suggesting a systematic effect of 5-mo solar activity cycles on circulating melatonin.

These considerations indicate that the biological effects of geomagnetic variations and disturbances are highly complex and critically depend on their extent as well as their timing relative to the overall phase of geomagnetic activity which, in turn, is a function of the state of solar activity during the 11-yrs' sunspot cycle. Acute spikes of Ap during the ascending phase of a sunspot cycle may thus exert a further stimulatory biological effect, whereas a similar spike occurring around the peak of the sunspot cycle leads to a paradoxical, i.e. inhibitory effect. Such complex and non-linear relationships between the environmental geomagnetic field (modulated by solar activity) and pineal secretory activity (with changing patterns of responsiveness) are a great hindrance in our current attempts to analyze mathematical correlations between these variables. Complex interactions between geomagnetic disturbances during the 11-yrs' sunspot cycle and circannual rhythms of melatonin as well as various other (patho)physiological parameters have also been reported by Halberg et al. (2004) as well as others (Hrushesky et al., 2011; Stoupel et al., 2007) strongly indicating that changing solar as well as geomagnetic activity may indeed profoundly affect human life and health. From this perspective, it will be very important to analyze in which way different patterns of longitudinal melatonin production in rats are connected with changing survival times (Bartsch et al., 2010): specifically, is seasonal melatonin rhythmicity connected with positive effects on survival or not?

From a basic science point of view, it will be of central importance to clarify whether seasonal pineal rhythms

(under constant photoperiods) may also be controlled by the endogenous calendar (Gwinner, 1986; Pengelley, 1974), which, in the absence of photoperiodic triggers, could use geomagnetic signals for synchronization (Bartsch et al., 1994), or whether such rhythms are passive events which are driven by changing geophysical signals in response to changing solar activity. Since it is technically very difficult to realize experiments under highly standardized geomagnetic conditions (up to the lowest nanoTesla-range) in which solar modulatory effects are totally absent (Machado, 1994), it will be indispensable to find out how geomagnetic perception could be blocked experimentally and to test how longitudinal melatonin rhythms, particularly seasonal, may respond. For this purpose, the underlying molecular mechanisms of geomagnetic field perception and processing as well as regulation (Winklhofer, 2010) require urgent elucidation. In this context, it will have to be clarified whether also the mammalian pineal gland may play a central role in such processes comparable to reptiles where (electro)magnetic fields appear to be perceived via this organ (Nishimura et al., 2010). It has to be pointed out that such investigations are of central current importance at a time when anthropogenic environmental electromagnetic fields of different type are becoming more and more prevalent and lead to many unanswered questions regarding their potentially hazardous or even carcinogenic nature (Baan et al., 2011). Recent findings indicate that there may be a mechanistic coupling between 50 Hz electromagnetic field-mediated as well as light-dependent effects on melatonin production in both mice (Kumlin et al., 2005) and humans (Juutilainen and Kumlin, 2006), which could involve retinal cryptochrome, and several experiments worldwide are ongoing or planned to test this hypothesis (Lagroye et al., 2011). In this context, it will be highly relevant to see whether chronic exposure to low-intensity high-frequency signals, as used for mobile telecommunication, may affect longitudinal pineal melatonin production. Respective data collected parallel to those presented here are being analyzed chronobiologically (Bartsch et al., in preparation). If effects of chronic radiofrequency exposure on longitudinal pineal melatonin production were detectable and would be modulated by the stage of the solar cycle, this would imply that a mechanistic coupling exists between geomagnetic and high-frequency electromagnetic perception (Fleissner et al., 2007; Kirschvink, 1996; Kirschvink et al., 1992) which, in this case, may not be mediated by cryptochrome but rather by ferromagnetic particle receptors (Wu and Dickman, 2012) as has been discussed by us before (Bartsch et al., 2010). Coupling between geomagnetic and electromagnetic perception of both extremely low and high frequencies would actually not be too surprising from an evolutionary point of view since geomagnetic as well as diverse solar electromagnetic fields have been integral parts of the natural terrestrial environment since the beginning of life. These complex and barely understood processes, however, may render plausible explanations why seasonal rhythms of pineal secretion are preferentially observed during certain phases of the solar cycle and why multiple non-seasonal oscillations are present at other times. Since the pineal gland with its main hormone melatonin seems to underlie such complicated longitudinal variations and at the same time is involved in the temporal control of various subsystems of the body affecting numerous physiological as well as pathological processes including cancer (Bartsch and Bartsch, 2007), it is plausible why it is currently so very difficult to perform reproducible long-term experiments under in vivo conditions (Bartsch and Bartsch, 2007; Bartsch et al., 2001, 2010; Bruguerolle et al., 1988; Kubatka et al., 2002; Löscher and Fiedler, 1996, 2000; Löscher et al., 1997).

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