Long-term follow-up study in experimental animal model: exploration of circadian and extracircadian rhythms

The effects of altered exterior environment on the rhythms of the endocrine systems

Ph.D thesis

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1 Introduction

Most of the biological rhythms in all living organisms are adaptive oscillations with respect to the major periodicities (day, month, year etc.) of the physical enviroment. The biorhythms are genetically coded but are influenced by enviromental factors. Of the several possible cues (temperature, electromagnetic radiation, nutrition, chemical factors, etc.), called Zeitgebers (synchronizers), which can contribute to entrainment, bright light is by far the most effective. In general, three factors define the biological rhythms: the exterior stimuli (the Zeitgebers) and their intensity, the inner biological clock and the inner states of the body (Takahashi et al. 1979). According to their duration, different biological rhythm known, ultradian (fast, under seconds passing, eg. heart rhythm), circadian (cca. 24 clocks repetitive), semicircadian rhythm (cca. 12 hourly), and infradian rhythms, like the circasemiseptan, circaseptan, circannual (several daily/weekly/yearly or seasonal) rhythms. The same organs of the same species might have more rhythms with different period times. At cellular levels, the rhythms are controlled by the so-called clock genes. The most potent signal for circadian synchronization in the vast majority of species, including humans, is the daily light-dark cycle. In most lower vertebrates, both the retina of the eye and the pineal gland in the brain are also capable of generating circadian rhythms. In mammals, the suprachiasmatic nucleus (SCN) is the central pacemaker, the major internal rhythm-generating area. The SCN receives inputs from specialized photoreceptive retinal ganglion cells via the retinohypothalamic tract. Destruction of this area in rodents abolishes nearly all circadian rhythms (free running rhythym) (Liu et al. 1997).

The SCN contains several cell types and several different peptides and neurotransmitters and interacts with many other regions of the brain (hypothalamus, vegetative neural centres, pineal gland, pituitary). Neurons in the *ventrolateral SCN* (vlSCN) have the ability for light-induced gene expression, neurons in the *dorsomedial SCN* (dmSCN) are believed to make an endogenous 24-hour rhythm that can persist under constant darkness (in humans averaging about 24h 11 min). The SCN sends information to several hypothalamic nuclei and also the pineal gland, modulates several functions, among others the body temperature, food–intake, the production of hormones such as cortisol and melatonin. The role of melatonin in circadian organization is very important, it serves to reinforce and elicit physiology and behaviour associated with darkness. Melatonin can also act on the SCN itself, producing 'feedback'

resetting of the clock. In the regulation of the circadian rhythms the light has the primary role, however other factors (body temperature, electromagnetic radiation, nutrition, chemical factors, etc.) play also a considerable role (Oláh et al. 2008, Morin et al. 2007).

The examination of the rhythms and the rhythm disturbances has a great importance among other things, because of in everyday life the plan of work alteration related rhythm disturbances connected to the workplace practises an effect more than a one third of the employees, that often lead to various diseases.

In everyday life one of the best-known rhythm disturbances is the melatonin-rhythm disturbance in the case of jet-lag, and in those working in shift work. Not only the rhythm of the formation and release of melatonin, but the other circadian rhythms controlled by it, suffer disturbance in several neurodegenerative processes and in the case of endocrine disturbances. In those working in shifts it is very common that sleep- and general condition disorders develop (Donald et al. 1999), in whose background there are mostly non-organic reasons, but rhythm desynchronization resulting from external factors and lifestyle changes. The shift work sooner or later may lead to the attendance of different psychosomatic changes. Several data in the literature document the higher frequency of the diseases of GI-tract, heart circulatory system (Andres Knutsson 1999) and tumourous diseases (Scott Davis et al. 2001), and the higher risk of the premature labours (Marazzi et al. 2003) in people working in shifts Theese projects are long-term follow-up studies carried out in several countries, which follow the incidence of to disturbances in employees working longer time in shift work, focusing of the populations of a variety of countries.

Both in the health care system as well as in other fields of the economy there is increasing demand on the prevention of a number of diseases by means of early diagnosis of disturbances in biological rythms. Joining this area of research, the present study seeks the answer to the question how altered lighting regimens characteristic of shift work and chronic stress lead to disturbances in the biological rythms and which rythm parameters offer a chance for an early recognition of such disturbances well before alterations in the organs may start, using long-term follow-up methods. The present study was the first to develop such an experimental animal testing model, which may serve as a basis for a complex examination of various rythm parameters.

2 Objectives

- To develop an experimental animal model making possible the long-term follow up of the rhythm parameters.
- To examine the body core temperature and the corticosteron as a rhythm marker.
- To examine the rhythm parameters of corticosteron, prolactin, endothelin-1 and melatonin.
- To examine the effects of chronic mild stress and various lighting regimens on the endocrine system.
- To examine the rhythm parameters of the melatonin levels in the pineal gland and in the duodenum following starvation.
- To examine the effects of the electromagnetic field of the Earth on biological rhythms.

3 Materials and method

In the present study adult inbred Wistar (Amsterdam) rats of identical age were used. The animals were kept in two rooms with contrasting lighting regimens and were left undisturbed for 1 month. In one room the lighting regimen corresponded to the time of the day, i.e. a 12 h period of light (L) was followed by a 12 h period of darkness (D), (LD regimen), while in the other room reversed lighting regimen (DL regimen) was introduced.

Body temperature and blood corticosteron levels as rhythm markers (4-hour samples for 24 hours) were measured prior to the experiments to check wheter the adaptation to the lighting regimen occurred. After obtaining evidence of the adaptation the experiments were conducted for 7-14 days.

Blood samples were taken by way of cardiac puncture (2 ml per animal) under ether anesthesia. The blood was centrifuged for 20 min at 4 °C (4000 rpm) and the plasma was separated. Following exsanguination and cervical dislocation, the brain, the pituitary gland and, in some cases, the duodenum were removed. The samples were stored at –70 °C. Corticosterone, melatonin, prolactin and testosterone concentrations were determined with radioimmunoassay (RIA). Endothelin-1 levels were determined with ELISA method. For data analyses the cosinor software and parameter tests were used.

As part of the present study, the most frequent nursing shift regimens were also modelled to examine how chronic mild stress (CMS protocol) on its own and combined with repetitive altered lighting regimens influences the rhythm parameters in the case of various hormones.

4 Results

4.1 The development of an experimental animal model facilitating long-term follow up study of the rhythm parameters, and the results obtained by using the model

4.1.1 The development of the model

The purpose of the new animal test model is to facilitate long-term follow up examination of the whole circadian profile during the working hours. According to the theory detailed in Materials and Methods above, the animal test model counts as usable, if the sampling from animals on two different lighting regimens can take place in a way that the whole daily profile can be covered without sampling during the night. Considering that the aim of this study was a long-term observation, it would have been technically difficult to stay awake for one week ore a fortnight.

A round-the-clock i.e 24 h follow up - which requires sampling once in every 4 hours, i.e. 6 times in 24 hours - could be carried out during the working hours only, if sampling takes place 3 times a day (in every 4 hours) and samples are taken from animals in both groups at the same time, using 2 animals on every occasion, one from the LD group and the other from the DL group. Before placing the animals in the two rooms with different lighting regimens, the acrophase of their body core temperature circadian rhythm was found identical, then, following the synchronization period, a 12-hour postponement could be observed in animals kept in DL.

4.1.2 The examination of the rhythm of the body temperature in the developed anilam test model

The circadian rhythm in core temperature was tested using six timepoints. With this the presence of the known circadian rhythm could be confirmed in animals kept in each room (P<0,05), and so could the dislocation of the acrophase in the animals kept in DL. The result of the body temperature justified the validity of the method as well, therefore this examination was carried out before all further experiments in 24 animals, with 6 measurment, 4 hourly, during 24 hours in animals kept on the LD and DL lighting regimens, as well.

The circadian rhythm was demonstrated (P<0,05). Parameter tests revealed that female rats had a higher MESOR of core temperature than male rats when the analyses were based on six timepoints (LD: 37,2° vs. 36,8°, F=3,189, P=0,096; DL: 37,5° vs. 37,0°, F=5,547, P=0,038). A test of the equality of acrophases adjusted to the respective lighting regimens (HALO time) was statistically significant for males (F=5,812, P=0,035) but not for females (F=0,289, P=0,600). This indicates that males became completely synchronized to their lighting regimen. The individual variation of the acrophase was wider in the case of females, although the synchronization to the two different lighting regimens was present at the group level. The explanation of the vider variation is presumably that females are less sensitive to the external effects than males. In the case of both lighting regimens (LD and DL) the rhythm middle value (M, MESOR) was slightly higher. Data also show that there was a phase displacement (ø) in the LD and DL values, so the time of the highest body temperature shifted 12 hours. In the case of males the difference in LD was -206°, in DL: -69°. At the same time, in females the difference in LD was -234°, in DL -62°. The body temperature of female animals was higher than that of the males (LD: P=0,060). The mean values of the body core temperature resulted in a characteristic sinus curve, the values being the highest in the period of the activity, in the dark section.

4.1.3 Using the newly developed animal test model for the examination of the extracircadian rhythms in addition to the known daily rhythm of corticosteron

4.1.3.1 Confirming the circadian rhythm of orticosteron

Prior to several-day-long follow up the adaptation to the opposite lighting regimen was checked by way of measuring the circadian rhythm of corticosteron in both lighting regimens. The examination of the circadian rhythm parameters in the individual cases yielded significant results (P<0,05) in 3 of the 4 groups in both lighting regimens, with the exception of female animals in the LD group.

The round-the-clock analyses showed higher corticosterone levels in females than in male rats. A sex difference was also found when considering all animals in the study (LD12:12: 530 ± 64 nmol/ml (female) vs. 235 ± 27 nmol/ml (male), t=4,226, P<0,001; DL12:12: 826 ± 87 nmol/ml (female) vs. 474 ± 51 nmol/ml (male), t=3,541, P<0,001).Parameter tests confirmed the MESOR difference between males and females (F=34,65, P<0,001). A circadian rhythm was demonstrated as statistically significant in both male and female rats. The lowest corticosteron values were found on moving from the dark phase to the light phase, and the highest values on moving from the light phase to the dark phase (i.e. at the time of the beginning of the activity).

4.1.3.2 The infradian rhythm of corticosteron

As part of the long term follow up study the following examination was performed: for 11 days according to the protocol (between 4 and 15 April, 2004; M: n=52, F: n=51). Separate analyses of male and female rats indicate the presence of a prominent circadian rhythm (P<0,001) complemented by another 12-hour-long harmonic period (M: P=0,048; F: P=0,058) and a circaseptan component showing borderline statistical significance (P=0,057). The model accounts for 65% of the variance in males (P<0,001) and for 38% of the variance in females (P=0,002).

Nonlinear analyses of the pooled log10-transformed (relative) data confirmed the presence of both the circadian rhythm, with an estimated period of 24.03 h, and that of an infradian

component with an estimated period of 103.8 h (4.3 days) (95% CI: 79.5–128.1 h). These results were similar for male and female rats: for males, the circadian and infradian periods were estimated as 24.00 and 108.2 hours; for females, the respective periods were estimated as 24.03 and 101.9 hours.

4.1.4 Using the newly developed animal test model for the examination of the extracircadian rhythms in addition to the known daily rhythm of prolactin

Prolactin levels in male (M) and female (F) animals (M: n=52, F: n=51) were examined in the samples from animals kept on the two different lighting regimens (LD resp. DL), in both groups much higher values were found in females than in the group males of the same age, irrespectively of the kind of lighting regimens the animals were kept on. The difference of the mean values between the sexes was staistically significant in both rooms of the opposite lighting regimens. (LD12:12: 158,4 ± 38,4 ng/ml (female) vs. 48,3 ± 4,1 ng/ml (male), t=2,852, P=0,006; DL12:12: 124,4 ± 17,3 ng/ml (female) vs. 50,8 ± 4,4 ng/ml (male), t=4,204, P<0,001). Irrespectively of the animals being on normal or reverse lighting regimens, there was no significant difference between the male female groups (LD vs. DL).

An analysis of the rhythm parameters revealed that in the background of the variance in female animals primarliy there were circadian changes (P=0,079), while in male animals the 12-hour variation (ultradian rhythm component) was dominant, which accounted for 16% of the variance (P=0,016). Further modeling of the data was performed in a complex cosinor model, in which the 1- and 3.5-day periods were compared. The statistical method verified the simulatneous presence of the circadian (24 h) rhythm component and the infradian rhythm component (3,5 days), which altogether accounted for 8% of the variance. A similar result was obtained for the log10-transformed data, accounting for 7% of the variance, where the 3.5-day component (P=0,140) and the 24-hour component (P=0,113) did not reach the value of statistical significance. The parameter test confirmed the difference in MESOR (F=20,132, P<0,001) and revealed a statistically significant difference in 24-hour amplitudes as well (F=4,440, P=0,040).

4.1.5 Using the newly developed animal test model for the examination of the extracircadian rhythms of endithelin-1 in blood samples and pineal gland of the rat. Comparison with literature data on human samples

In this experiment covering the 24 hour cycle, samples from male and female animals on LD and DL lighting regimens (n=34) were taken in every two hours in a way described in the Materials and Methods. In the case of the concentration of endothelin-1 there was no circadian rhythm in the plasma in rats, but a cca. 4.8-hour-long circasemidian rhythm (P=0,024) was demonstrable. The peaks measured in the pituitary showed coincidence with the plasma peaks in the dark period, in the light period a small-scale but not significant phase delay could be observed. The ultradian rhythm of the ET-1 in the pitutary was not significant, despite that the concentratioon change showed linear correlation with the endothelin-1 levels measured in the plasma (r=0,376; P=0,084).

4.1.6 The daily rhythm of melatonin in the pineal gland, in the plasma and in the GI-tract

The greater portion of melatonin is produced in the pineal gland, but recent studies using immunhistological and RIA methods demonstrated melatonin production in other organs, such as the retina and in the GI-tract, as well (Bubenik et al. 1980). With regard to this, the aim of our examination was to compare the daily rhythm of melatoin levels in the pineal gland, the plasma and the duodenum (M: n=52, F: n=51).

Initially the pineal gland circadian rhythm of the melatonin known from the literature was verified with sampling in every four hours during 24 hours (Poeggeler et al. 1992). The values gained in the course of $\log_{10}$-transformation much better approached the characteristic circadian patterns reaching their peak in the dark period (HALO 20). The plasma melatonin concentration showed a lesser elevation in the light period in the case of female animals, but the highest values could be found in both sexes in the dark period (between HALO 16-20).

The hypothalamus had minuscule concentrations of melatonin as compared to to those in the plasma, which, in turn, was less than 1/10 of the concentration in the pineal gland. Despite that the 24-hour rhythm component was justifiable with a cosinor program and also that the highest values were between HALO 16-20 (P<0,001).

A widely accepted way of describing acrophases is that they are expressed in negative degrees, so the 24-hour whole-cycle is considered as 360° and the beginning of the lighting is considered as a reference-value.The present study showed that the acrophase in the case of the duodenum was 252°, in the case of plasma it was 280° and in the case of the pineal gland it was 303°. The parameter test verified statistically significant differences between the plasma and the pineal gland (P=0,003), between the hypothalamus and the pineal gland (P=0,021), and, to a small degree, between the acrophases in the duodenum and the hypothalamus (P=0,100). The acrophase of the duodenum melatonin acrophase (P=0,015), and, slightly though, the hypothalamus melatonin-acrophase (P=0,021) exceeded the melatonin acrophase of the pineal gland.

The results of the present study confirmed that the melatonin circulating in the blood could originate from the intestinal tract, as well. On the other hand, these results confirm that the melatonin contentration measured in intestinal tissues comes not only from hormones stored in or releasing from the blood, but hormones produced locally, whose production and / or release is mostl likely influenced by other regulatory systems, similarly to the melatonin of the pineal gland. To our knowledge the present study is the first to provide evidence based on 24-hour follow-up and rhythm analysis demonstrating that the circadian rhythm of the duodenum melatonin and the pineal gland melatonin in rats show a phase delay. This also suggests that the production of the peripheral melatonin with may not exclusively be the function of the melatonin-release of pineal gland alone, in addition it is presumable, that other regulatory systems may also control the melatonin rhythm of the central and peripherical organs.

4.1.7 The effect of starving on the circadian rhythm of melatonin

A series of experiments (between April 8-14 2005.) was carried out to find an answer to the question weheter or not the withdraval of food, as a modified external factor influences the circadian rhythm of melatonin, and if so, whetherr or not it has a similar or a different effect on the central and peripheral organs.

Before starting the experiment described in Materials and Methods, a 180° phase-change in animals in the DL group was verified. On the following morning food was withdrawn and

only free water consumption was allowed for 3 days. The sampling started on the morning of the fourth day, when the body weight of all animals was measured, at all times. This part of the experiment started on 11 April, 2005 (at 9, 13, and 17 hours) and continued through the following 6 days.

The melatonin circadian rhythm of the pineal gland, which is responsible for 66-78% of the variation, was verified in every group (P<0,001) on the basis of the original data and the relative data gained with a $\log_{10}$-transformation as well. A 7-day (circaseptan) rhythm close to the statistical significance border line could only be verified in female animals, both in the control (P=0,039) and in the starved group (P=0,044). Parameter tests did not justify significant difference between the sexes in the values of the acrophase.

The daily changes of the melatonin concentration measured in the blood are characterized by a prominent circadian rhythm in all groups, which is responsible for 51-78% of the variation. The 7-day component was also present but showed significance around the borderline value (P=0,077 only). Parameter test in this case also failed to verify significant differences between the sexes in the values of the acrophase.

Because of the technically demonstrable limits, the data of the duodenum melatonin concentration were analyzed with regard to the sexes. At the group level about the same amplitudes could be observed in the active (dark) period in both groups, while in the light period decreased values with significant difference between the groups were found.

The most considerable difference between the starved and control groups was that in the dark phase in the starved animals higher circadian amplitudes were caracterized by the levels of the plasma melatonin and in a small compass of the duodenum melatonin, while in the case of the pineal gland, as compared to the control, minor amplitudes (and lower MESOR) were typical.

The animal test model elaborated in the present study made studying the extracircadian variations possible. With the 7-day-long follow-up the complex component cosinor examination demonstrated the presence of the 24- and 12- hour components, which were responsible for the 66-78% of the variations. The existence of the circaseptan component could only be verified in the pineal gland (P<0,001).

4.1.8 The effect of the magnetic storm to the melatonin rhythm

Theese results were obtained from a series experiments, part of which coincided with the time of the second eruption maximum of a medium strength magnetic storm (M: n=52, F: n=51). According to subsequently obtained data that part of the experiment randomly coincided with the period of the magnetic storm. The Kp. index was 6,3 (the night of 3 to 4 April 2004), the Geomagnetic Equatorial DsT index (the gauge unit of the changes of the electromagnetic space) was -112 nT at 00.30 on 4 April, which decreased to a value of -81 nT at 19.30 on 5 April (http://www.sec.noaa.gov/SWN).

The magnetic storm had an effect on the function of the pineal gland, hypothalamus and adrenal glands. To our knowledge this is the first study to provide objective data concerning the disorders of the endocrine system caused by magnetic storms. Compared to the "peaceful" days before and after the same period with the magnetic storm, beside a slight acrophase (Φ) postponement in the circadian rhythm of melatonin concentrations, lower MESOR and amplitudes demonstrable in the pineal gland and higher MESOR values and amplitudes in the hypothalamus could be found. In the case of all comparisons $P<0,05$, except the acrophases(Φ) of the hypothalamus.

The MESOR and circadian amplitude were lower for pineal mealtonin and higher for hypothalamic melatonin on the stormy vs. quiet days. The corticosteron concentrations, MESOR and amplitude values showed greater variability on the stormy days.

4.1.9 The effects of chronic mild stress

Body Weight: The CMS procedure did not significantly alter body weight relative to control conditions ($P>0,05$).

Sucrose Consumption: We measured the consumption of the 1 % sucrose solution once in every week. The consumption of sucrose solution during the 4 weeks showed an increasing tendency in the control groups, while it slightly decreased, in the animals with a CMS but the difference between the two groups was not significat. Reference water intake in the CMS group did not differ from the control group's intake following 4 weeks of CMS ($P>0,05$). Sex

differences were observed in each week (week 1: P <0,001, week 2: P <0,001, week 3: P=0,013, week 4: P=0,001).

The connection between the sucrose consumption and the lighting regimen: When comparing the sucrose consumption, differences could be observed between lighting regimen and sucrose consumption from the second week on (week 2: P=0,004, weeks 3 and 4: P <0,05).

Corticosteron (CS) and testosteron: The plasma corticosteron levels slightly decreased in the light period and slightly increased in the dark period in female animals treated with CMS and kept in LD, compared to the control group kept in L (P=0,081), but not in males. Differences between the sexes in the case of corticosteron: corticosteron levels were higher in females, than in males (P<0,001). The testosteron level showed significant decrease in the animals treated with CMS compared with the controlls.

Rhythm components: After 4 weeks of CMS exposition corticosteron, testosteron and serotonin were determined. The circadian rhythm was statistically significant only in 21 cases. Surprisingly, instead of the circadian rhythm-component (P=0,091), the dominance of the 12-hour component (P=0,026) appeared to be resposnsible for the variations to a greater extent. On the other hand, obvious circadian rhythm-component (P=0,001) dominance was characteristic of the daily testosteron levels in the control males. Because for technical reasons only few samples could be taken, a sufficiently detailed examination of the serotonin rhythm was impossible. The data were pooled separately across groups 2-5 (subjected to CMS) and across groups 6-9 (not subjected to CMS). Corticosterone in male rats was characterized by a 24-hour component of borderline statistical significance (P=0,095) in the CMS-stressed animals, whereas non-CMS-stressed rats exhibited a 12-hour component of borderline statistical significance (P=0,071). Moreover, a circadian rhythm was demonstrated for testosterone for the CMS-stressed animals (P=0,004) but not for the non-CMS-stressed rats (P>0,20).

Animals in this stress study were exposed to two different kinds of load: the intended CMS and repeated shifts of the lighting regimen. Because schedule shifts are known to disturb circadian rhythmicity, the circadian rhythm characteristics were compared between animals kept on the same lighting regimen (groups 1, 2 and 6) and those undergoing shifts in schedule (groups 3-5 and 7-9). Only a small difference in the circadian amplitude of corticosterone was found by parameter tests for females (P=0,085) but not for males.

The peak value demonstrated in the dark period by the animals kept in LD was the highest, of which the values of the animals kept in LD and treated by CMS were lower and significantly lower than those in all other groups. There is no significant difference in the rest of the times. The remaining peak values in the dark period were the highest in control animals kept in LD, while those were lower in all the other groups. There was no significant difference at any one of the times. It was observable that the degree of the daily variance was small in the groups (CMS and/or only VF) kept in the alternate lighting regimens. Considering testosteron levels, it is observable that in the groups kept on alternating lighting regimens the alteration showed a converse tendency in the dark period as compared to animals kept in LD.

5 New results and practical utilization

1. The animal test protocol developed in the present study makes possible a long-term follow-up examination. Since then examinations have been proceeding in several laboratories taking over this protocol (BIOCOS international research team).

2. The present study demontrated that, beside the known circadian rhythm of the prolactin and the corticosteron, the circasemiseptan rhythm is also present, so the values measured at a given time of the day are added up by the two rhythm components combined (with the same or converse tendency)

3. The present study has shown that, simlarly to the samples from humans, there is no circadian rhythm in the case of endothelin-1 in rats, either. The rhythm can be characterzed by an 8-hour component in the plasma and int he pituitary.

4. The present study was the first we to verify, that the regulation of the melatonon rhythm produced in the GI-tract differs from the melatonin rhythm produced by the pineal gland, and, based on the rhythm curves, there is a phase delay in the rhythm of the pineal gland; and also that after withdraving the food the extent of the amplitude decreases.

5. The present study was the first to demonstrate the changes of the neuroendocrine system due the changes of the magnetic fields of the Earth (solar flare: Kp (geomagnetical index 6,3, Dst equatorial index -112 and -81 nT): the melatonin levels measured in the circulatory system and in the hypothalamus showed lower MESOR and smaller circadian amplitudes, than int he pineal gland int he samples measured during the solar flare. The corticosteron daytime values showed bigger instability compared to the 3 days following the eruption.

6. The experiments in the present study carried out with the application of chronic mild stress (CMS) and repeatrd alterations in the lighting regimens could serve as a basis for the creation of a new model. Further examinations including more animals are necessary to achieve bigger statistical certainty in order to verify what was demonstrated in this study as a tendency. It seems that the repeated alteration of the lighting regimens in itself results in a greater increase of load (rhythm disorder), at least in the time structure of the cotricosteron, than CMS does.

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