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Detection of Changes in the Mouse Circadian Rhythm Induced by Stress

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Mice were subjected to three types of acute stress (cold, forced swimming and tail hanging) in order to investigate the effects of stress on the motor activity circadian rhythm. This rhythm was studied using the cosinor method and spectral analysis. A statistically significant decrease in the amplitude and the power content of the circadian harmonic was found after stress application. These decreases could be due to a desynchronization of the circadian oscillators which drive the rhythm. The use of the power content of the circadian harmonic is proposed for the detection of the alterations due to stress.

Key words: Circadian rhythm, Motor activity, Acute stress, Mouse, Power spectra.

It has been suggested that there is a relationship between stressful life events and depression (12). Based on this theory, many investigators, attempting to study depression using laboratory models, have applied different kinds of stress to animals. It has been reported, for example, that when mice are forced to swim in a restricted space, they rapidly cease moving and become lethargic. This immobility is reduced by administering antidepressant drugs (14). In addition, in female rats with a regular cycle of spontaneous running activity, which peaks every 4-5 days corresponding to the estrous cycle, it has been found that when these rats are exposed repeatedly to forced running stress, they show an inactive state accompanied by impairment of the biological rhythms. In this case, imipramine has been proven to cause a rapid resumption of the spontaneous activity and its cyclicity (9), Moreover, chronic unpredictable stress produces a syndrome in the rat which closely resembles endogenous depression in humans, and only clinically effective antidepressant treatments reverse the behavioural and physiological changes induced by chronic stress (10).

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Another depression model is maternal separation. In infant monkeys, this stress can alter the level, the amplitude and the phase of circadian vegetative rhythms followed by behavioural depression. It is important to note that the onset of this behavioural depression becomes evident on the second or third morning of separation, while the circadian alteration is evident on the first night of separation (15).

Considering the fact that there are circadian alterations in depressed patients (5, 11, 17) as well as in animals subjected to certain types of stress, the study of circadian rhythms in stressed animals takes on a great importance in order to understand the mechanisms of depression. The use of stress animal models may be helpful in the study of certain kinds of mental disorders and their physiological consequences as it mimics, in a certain way, some human anxious situations.

This work has two related purposes: first, to study the alterations produced by acute stress on the circadian rhythm of motor activity in mice; and second, to find the parameters which change in the most significant way and to develop a specific methodology to detect these alterations efficiently, even in the first stages.

Materials and Methods

Male albino mice weighing about 30 g were used. Animals were randomly put into three groups of 6, 5 and 5 mice respectively. Each group was subjected to a different type of stress. Another group of 6 mice was used as a control.

Animals, before the experiment, were living collectively, according to the groups chosen for the experiment, under constant environmental conditions: temperature 20 ± 1 °C and light-dark cycles (12L: 12D), with lights on at 9.00. Mice had free access to food and water. Two days before the beginning of the experiment mice were housed individually in white opaque cages (25 × 25 × 14 cm).

An open field (8) was used to measure some emotivity and activity parameters before and after the stress application.

Motor activity was detected automatically by 6 inductive sensor units (Actisystem, Panlab S.L., Barcelona). Data countings were printed every 15 minutes.

Experimental design.— The experiment lasted five days for each kind of stress. On the first day, mice were tested, one by one, by the open field test, during the last hour of the darkness period. Then, they were returned to the individual cages and put on the sensor units. Lights-on was 15 minutes later, coinciding with the first print-out of data, which was eliminated.

Motor activity was registered for 48 hours until 9.00 on the third day. On this day, animals were subjected to stress, a different kind for each group. On the 4th day, the same operations were carried out as on the first day: The open field test and the registration of spontaneous motor activity for another period of 48 hours.

Group 1 (cold stress): animals were subjected to a temperature of −21 °C for 45 min.

Group 2 (swimming stress): mice were forced to swim three times for 15 min. in water at 20 °C, interrumped by two rest-breaks of 105 min.

Group 3 (hanging stress): mice were hung by the tail for a period of 7 h.

Group 4 (control): these animals, on the stress day, were only transported to the room where the stress was carried out.

Calculations.— Data recorded by the printer was saved on a magnetic tape. Rhythms of motor activity were studied by means of Fourier analysis (18) applied to motor activity data corresponding with the two observation periods of 48 hours (preceding and following stress). In this way, the power spectrum of the motor activity function of each animal was found

(2, 7) and the power content of each harmonic was expressed as a percentage of the main power of the activity function.

In order to investigate the possible changes in mice motor activity rhythm induced by acute stress, the following parameters have been studied: Power content of the circadian harmonic (PCCH), corresponding to the second harmonic in the power spectra; amplitude of the circadian rhythm; acrophase of the circadian rhythm, referred to the beginning of the light period; motor activity, expressed as the daily average of the countings recorded every 15 minutes.

In order to reduce the individual variations, the statistical analyses of the four parameters have been carried out with paired data. Differences between the values obtained in the periods before and after the stress application, have been expressed, in the case of PCCH, amplitude and motor activity, as a percentage of the first period values, but for the acrophase the absolute differences were used.

With the differences corresponding to each parameter, an ANOVA has been carried out on the basis of two linear models (6). One defined with two variables (Stress absence and Stress presence), and the other with four variables (Cold stress presence, Swimming stress presence, Hanging stress presence and Stress absence). In both models the y term means the differences corresponding to each parameter studied (PCCH, amplitude, etc.).

According to these two models, the following null hypothesis have been tested: *a*) There are no statistically significant differences between control animals and stressed animals. *b*) There are no statistically significant differences among the four groups of animals. *c*) There are no statistically significant differences between control animals and animals subjected to cold stress. *d*) There are no statistically significant differences between control animals and animals subjected to swimming stress. *e*) There are no statistically significant differences between control animals and animals subjected to hanging stress. *f*) There are no statistically significant differences among the effects produced by the three types of stress.

The global study of circadian rhythms was made by applying the mean-cosinor method (13) with $p = 0.05$, considering a period length of 24 hours.

With the purpose of performing paired comparisons before and after the stress application by means of this last method, the difference vector has been calculated. This vector results from the subtraction of the cosinor obtained before stress, from the one obtained after stress. With this, the difference vector will be defined as:

$$\phi_d = \mathrm{atn} \frac{A_1 \sin \phi_1 - A_0 \sin \phi_0}{A_1 \cos \phi_1 - A_0 \cos \phi_0}$$

$$A_d = [A^2{}_0 + A^2{}_1 - 2\, A_0\, A_1 \cos (\phi_0 - \phi_1)]\ {}^1\!/_2$$

Where A and ϕ are respectively the amplitude and the angle of cosinors corresponding to before and after the stress application (0 and 1 subindexes respectively). Taking the initial cosinor as a reference, ϕ_0 becomes 0 and ϕ_1 becomes $\phi_1 - \phi_0$. Thus, the formula is considerably simplified.

In order to obtain the confidence limits of these difference vectors, the confidence ellipse of the points corresponding to the vector extremes (4) has been calculated.

Results

Behavioural parameters measured by the open field test were not statistically significant due perhaps to the high number of animals that should be subjected to this kind of test. The results obtained have, therefore, been omitted.

Comparing the power spectra, before and after the application of each type of stress (fig. 1 and 2), a decrease in the pow-

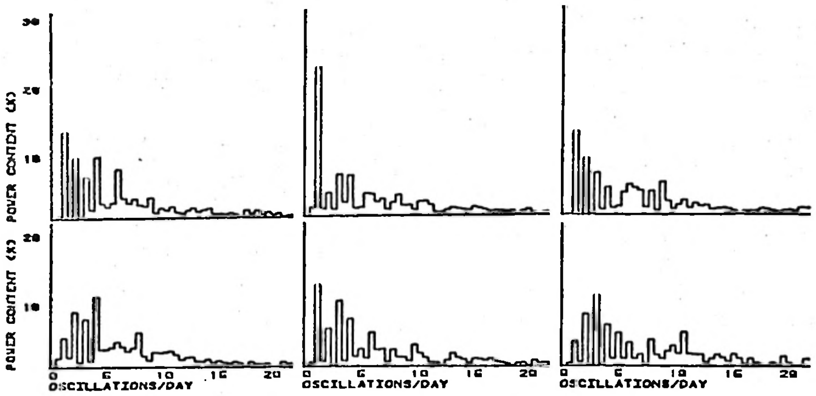

Fig. 1. *Average power spectra of the three types of stressed animals (from left to right: cold, swimming and hanging), obtained before (above) and after (below) the stress application.*
As the analysis was carried out over an observation period of 48 hours, the PCCH corresponds to the second harmonic.

er content of the circadian harmonic and, at the same time, an increase in the power content of the 6th and 8th harmonics, corresponding to 8 and 6 hour period rhythms respectively, are observed. A decrease in the power content of the circadian harmonic did not imply loss of circadian rhythm, which is deduced by observation of the predominancy of even harmonics (submultiple of the second harmonic) in all the spectra. The chronograms (fig. 3) show a fragmentation of the main activity phase after the stress application, supporting earlier findings.

Power Content of Circadian Harmonic (PCCH). — In table I the mean values of the PCCH are shown. After stress application, a decrease in the PCCH is observed in all cases. The decrease in PCCH

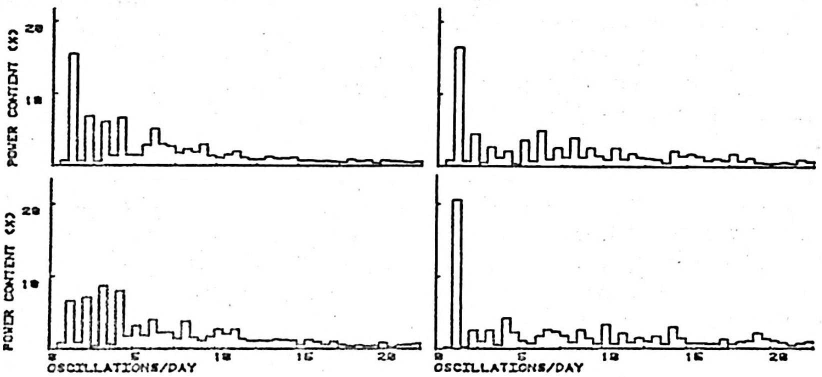

Fig. 2. *Average power spectra of the stressed animals (on the left and control animals (on the right), obtained before (above) and after (below) the stress application.*
As the analysis was carried out over an observation period of 48 hours, the PCCH corresponds to the second harmonic.

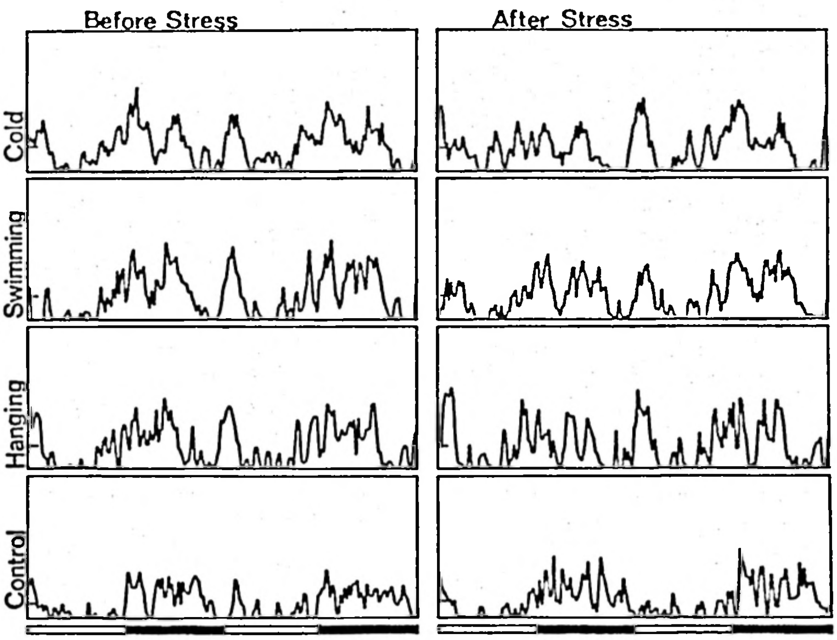

Fig. 3. *Average chronograms of the three types of stressed animals and control animals.* The vertical axis of each graph represents 3000 arbitrary units of motor activity.

produced by stress application in the two linear models, was statistically significant ($p < 10^{-6}$). The PCCH decreases produced by each type of stress, considered separately, were also statistically significant: cold stress, $p < 0.5 \cdot 10^{-5}$; swimming stress, $p < 0.5 \cdot 10^{-3}$; hanging stress, $p < 0.1 \cdot 10^{-4}$.

Although the most significant decreases have been obtained by using cold and hanging stress, it is not possible to distinguish among the decreases produced by each type of stress, as the «f» hypothesis cannot be rejected ($F_{2,16} = 1.906$, $p = 0.1081$).

When the PCCH variations obtained in the control and stressed animals are compared, the variation in PCCH due to the stress application was found to be different from the one produced in the control group, as it is inferred from testing the «a» hypothesis ($F_{1,18} = 58.178$, $p < 0.5 \cdot 10^{-6}$).

The study of the «b» hypothesis demonstrates that at least one of the four groups shows changes in the PCCH different from at least one of the other groups ($F_{3,16} = 22.625$, $p < 0.1 \cdot 10^{-4}$).

The results of testing the «c», «d» and «e» hypotheses demonstrate that the effects produced by each type of stress are different from those observed in the control group ($F_{1,16} = 52.905$, $p < 0.5 \cdot 10^{-5}$) for cold stress; ($F_{1,16} = 29.384$, $p < 0.1 \cdot 10^{-3}$) swimming stress; and ($f_{1,16} = 47.103$, $p < 0.5 \cdot 10^{-5}$) hanging stress.

Amplitude. — Amplitude values have been treated in the same way as the PCCH values, using the two linear models described before (table I). A statistically significant decrease in amplitude is detected after the stress application ($p < 0.5 \cdot 10^{-4}$) while changes produced by each type of stress separately, were also statistically

Table I. *Circadian rhythm parameters before and after of stress.*
Motor activity is expressed in arbitrary units, PCCH as a percentage of the total power content, amplitude in the same units as the motor activity and acrophase in minutes. The acrophase reference is the moment the lights are switched on. Mean ± typical error. For acrophase, absolute differences were used. ● = $p < 0.05$, ●● = $p < 0.01$ and ●●● = $p < 0.001$.

	N	Before	After	Differences (%)
MOTOR ACTIVITY				
Cold	6	560 ± 56.32	445 ± 27.26	−18.67 ± 8.20●
Swimming	5	569 ± 47.08	467 ± 68.04	−19.09 ± 8.98●
Hanging	5	481 ± 40.63	474 ± 59.81	0.44 ± 8.98
Total	16	538 ± 28.61	461 ± 28.06	−12.83 ± 5.17●
Control	5	304 ± 51.06	334 ± 46.30	− 6.92 ± 8.98
PCCH				
Cold	6	12.63 ± 2.79	4.33 ± 1.57	−69.43 ± 9.13●●●
Swimming	5	21.67 ± 1.67	11.80 ± 2.63	−47.62 ± 9.99●●●
Hanging	4	12.04 ± 3.31	3.58 ± 1.38	−73.89 ± 11.17●●●
Total	15	15.48 ± 1.84	6.62 ± 1.44	−63.35 ± 6.05●●●
Control	5	16.47 ± 1.97	20.53 ± 1.48	29.01 ± 9.99●
AMPLITUDE				
Cold	6	300.8 ± 32.19	153.5 ± 29.03	−49.09 ± 11.23●●●
Swimming	5	414.2 ± 47.69	307.0 ± 60.06	−27.98 ± 12.30●●
Hanging	4	288.5 ± 24.35	150.0 ± 23.59	−41.73 ± 13.75●●
Total	15	333.7 ± 25.17	203.7 ± 72.00	−41.73 ± 7.08●●●
Control	6	235.8 ± 36.59	305.0 ± 36.14	39.11 ± 11.23●●
ACROPHASE				
Cold	6	890 ± 36.16	818 ± 35.70	−72.9 ± 34.00
Swimming	5	940 ± 28.79	950 ± 19.28	10.3 ± 37.24
Hanging	4	966 ± 24.10	912 ± 41.05	−54.4 ± 41.74
Total	15	927 ± 19.23	887 ± 23.63	−40.3 ± 22.00
Control	6	990 ± 70.00	1001 ± 47.71	11.2 ± 34.00

significant: Cold stress, $p < 0.5 \cdot 10^{-3}$; swimming stress, $p < 0.05$; hanging stress, $p < 0.005$).

As with PCCH, the most significant decreases were produced by cold and hanging stresses but as with PCCH, the three types of stress cannot be distinguished («f» hypothesis: ($F_{2,17} = 0.940$, $p = 0.41$).

A study of the «a» hypothesis proves that there is a statistically significant difference between the amplitude variations observed on control and stressed animals ($F_{1,19} = 37.247$, $p = 0.1 \cdot 10^{-4}$).

«b» hypothesis has been tested to prove that at least one of the four groups shows changes in amplitude different from at least one of the other groups ($F_{3,17} = 12.963$, $p < 0.5 \cdot 10^{-3}$).

Finally, the «c», «d» and «e» hypotheses have been tested to prove that the amplitude variations in each type of stress are different from those of the control animals: ($F_{1,17} = 30.834$, $p < 0.5 \cdot 10^{-4}$) for cold stress; ($F_{1,17} = 16.219$, $p < 0.1 \cdot 10^{-2}$) for swimming stress; ($F_{1,17} = 24.009$, $p < 0.5 \cdot 10^{-3}$) for hanging stress.

Motor activity. — The study of motor activity has been performed by using the daily mean values of the data recorded every 15 minutes. The mean values corresponding to the days before and after the stress application and their differences expressed in percentage are shown in table I.

The statistical treatment, using the two described linear models, shows that there is a statistically significant motor activity decrease due to the stress application ($p < 0.05$). However, there is not a statistically significant difference between control and stressed animals ($F_{1,19} = 0.311$ $p = 0.584$ for «a» hypothesis).

According to the changes produced by each type of stress separately, statistically significant decreased have only been obtained in cold and swimming stress ($p < 0.05$ in both cases). Nevertheless, after a test with the «f» hypothesis it has not been possible to find any difference among the effects of the three types of stress ($F_{2,17} = 1.589$, $p = 0.233$).

From comparison of each group of stressed animals with control, statistically significant differences have not been observed, because the «c», «d» and «e» hypotheses cannot be rejected ($F_{1,17} = 0.934$, $p = 0.347$); ($F_{1,17} = 0.918$, $p = 0.351$); ($F_{1,17} = 0.336$, $p = 0.570$, respectively).

In the same way, the «b» hypothesis was tested to prove that there were no statistically significant differences among the four groups of animals ($F_{3,17} = 1.169$, $p = 0.351$).

Acrophase. — The analysis of the two linear models applied to this parameter (table I) did not find statistically significant differences between the acrophase values for at least the days after and before the stress application. In the same way, it is not possible to establish differences among the mean acrophase of the different groups, as no hypothesis can be rejected.

Cosinor. — The mean cosinors corresponding to the periods before and after the application of each type of stress show the presence of a motor activity circadian rhythm in all cases (figure 4). After the hanging stress application, as the origin of the vector touches the outer limits of the confidence ellipsis, the rhythm is not so evident.

In all cases, the confidence ellipses overlap. It means that there are no statistically significant differences between the circadian rhythms corresponding to the periods before and after the stress appli-

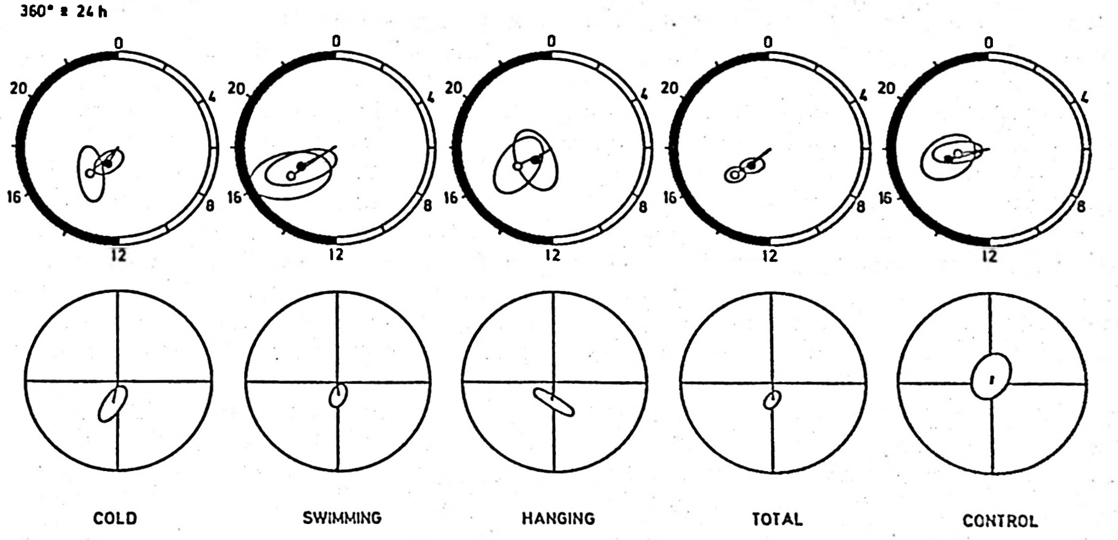

Fig. 4. *Cosinors corresponding to the periods before (○) and after (●) the stress application (above) and average of the difference vectors (below).*

cation. However, it must be noted that when the three types of stress are considered altogether the overlap region is less extensive.

In order to interpret the graphics of the mean difference vectors an increase of the amplitude will be considered statistically significant when the confidence ellipsis is completely situated in the upper semi-circle. In the same way, an amplitude decrease exists when the ellipsis is completely situated in the lower semicircle. To detect an advance or delay in the acrophase, the same criteria is applied, but referring to the left and right halves of the circle, respectively.

Each type of stress application produces a statistically significant amplitude decrease (fig. 4). However, changes in the acrophase position are not observed.

All these observations, are more evident when the three types of stress are considered altogether.

Discussion

The overt rhythm of a particular parameter can be considered as the detectable result of the activity of all the oscillators, which drive the studied rhythm. However, the function caused only by the activity of the oscillators is modified by the environmental variations and the experimental conditions that change the studied parameter in an aleatory way.

By means of the cosinor method the most important characteristics of the circadian rhythm, such as the amplitude, the acrophase and the mesor can be determined. Similarly it is possible to establish how well the calculated function fits to the chronogram by means of specific tests. The variations in the overt rhythm, detected in a statistically significant way, can reflect changes occurring in the function due to the oscillators, or pristine function.

A study of the power spectra of a function gives a lot of information about the function shape, but only the statistically significant harmonics can be considered as components of the pristine function.

In this work, decreases in both the amplitude and the PCCH have been found after the stress application. This fact may suggest that a desynchronization among the circadian oscillators which drive the motor activity circadian rhythm of the mouse might be produced.

The present work makes it evident that at least some types of acute stress are capable of producing alterations in the circadian rhythm of the mouse. Several investigators have proposed the use of certain types of stress applications as an animal model of depression (3, 8, 9, 15), and some of them have found that there are also alterations in some biological rhythms.

The importance of all these findings lies in the fact that depressive patients show great alterations in the circadian system, and in the majority of cases, an advanced phase has been found. It has been proposed that manic depressives have circadian oscillators which uncouple and free-run fast even when the patients are in normal environments. That is to say, that both depression and mania may be caused by internal desynchronization (11).

The mechanism underlying the alterations in the circadian motor activity rhythm of the mouse, produced by stress application, might be a desynchronization among the population of oscillators which drive the rhythm. If this state of desynchronosis takes a longer period of time, it might produce alterations in the acrophase, which may be observed after a chronic stress application; the latter could be an indicator of a depressive state.

On the other hand, it should be noted that in the control animals statistically significant increases of the PCCH and the amplitude have been observed. These increases may be due to the mild stress to which the control animals have been subjected and this stress may have induced a general activation of the organism (16). This fact would explain why these alterations are contrary to those observed in animals which have been subjected to acute stress.

Referring to the power spectra method, it must be pointed out that it has provided a greater quantity of information than the cosinor analysis. This is due to the fact that the cosinor is defined by means of three parameters (acrophase, amplitude and mesor) whereas with the power spectra more than forty parameters (twenty harmonics) are calculated. Since the power contents are expressed as a percentage of the total power of the function, the power spectra is not changed by the variations either in the activity level of the animal or in the sensitivity of the detection methods. From all this it is evident that the information extracted from the power spectra is fuller than that obtained using only the cosinor method. Moreover, besides measuring the magnitude of the circadian oscillation, the PCCH is also dependent on its relationship with the rest of the power spectra. Because of this it becomes a good indicator of the circadian character of the rhythm in spite of the presence of possible spurious oscillations. Finally, we would like to emphasize that the use of PCCH has been very useful in the detection of changes in the pattern of the motor activity function, and has also been an important tool in the detection of a desynchronosis state in its initial phases, whithout manifest changes in the acrophase.

Resumen

Los efectos del stress sobre el ritmo circadiano de actividad motora en ratones fueron estudiados sometiendo a tres grupos de ratones macho a tres tipos distintos de stress agudo (stress por frío, natación forzada y suspensión por la cola). Este ritmo se estudió utilizando el método del cosinor y el análisis espectral. Después de la aplicación del stress se observó un descenso estadísticamente significativo en la amplitud y el contenido de potencia del armónico circadiano. Se sugiere que estos descensos puedan ser debidos a una desincronización entre los osciladores circadianos que rigen el ritmo de actividad motora. Se propone el contenido de potencia del armónico circadiano para el estudio de los cambios debidos al estrés.

Palabras clave: Ritmo circadiano, Actividad motora, Estrés agudo, Ratón, Espectro de potencias.

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