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A CHARACTERISTIC OF THE EFFECTS OF MELATONIN AND EPITHALON ON THE STATE OF C-FOS GENE IN THE NEUROSECRETORY NUCLEI OF THE HYPOTHALAMUS IN AN EXPERIMENT

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ХАРАКТЕРИСТИКА ЕФЕКТІВ МЕЛАТОНІНУ ТА ЕПІТАЛОНУ НА СТАН ГЕНА C-FOS У НЕЙРОСЕКРЕТОРНИХ ЯДРАХ ГІПОТАЛАМУСА СТРЕСОВАНИХ СВІТЛОМ ЩУРІВ

Резюме. Досліджено вплив мелатоніну і синтетичного біорегулятора епіталону з метою корекції стрес-індукованих змін активності гена "надранньої відповіді" c-fos в латеральних великоклітинних субядрах паравентрикулярного ядра (лвПВЯ) гіпоталамуса щурів у різні проміжки доби (удень і вночі). Експресія продукту цього гена – білка c-Fos – у тварин, яких утримували за нормальних умов чергування освітлення й темряви, демонструвала чіткий циркадіанний характер (з більшим рівнем удень). За умов світлового стресу денний показник індексу вмісту c-Fos у лвПВЯ тварин на 33,0 % нижчий, а нічний наближався до контрольних величин. Ін'єкції мелатоніну (0,5 мг/кг) стресованим світлом тваринам віддзеркалилися о 14.00 год підвищенням індексу вмісту білка c-Fos у лвПВЯ майже вдвічі порівняно з даними експерименту на стресованих щурах без введення гормону, а також нормалізацією циркадіанної динаміки експресії досліджуваного гена. При застосуванні тетрапептиду епіталону (0,5 мкг/кг) виявлено збільшення індексу концентрації білка c-Fos у структурі вночі відносно особин з епіфізарною гіпофункцією без проведення експериментальної терапії епіталоном. Удень такого ефекту препарату не зафіксовано.

Ключові слова: c-fos, паравентрикулярні ядра, стрес, мелатонін, епіталон.

A study of the place and role of the neuroendocrine structures in the central mechanisms of the circadian rhythms is one of the pressing issues of modern chronophysiology [1, 2]. Changes of the duration of the principal photoperiod, as a stress factor, desynchronize the rhythms of the somatic and visceral functions, as well as the coordination and modulation of the organism's adaptation mechanisms to the effect of different factors [3, 4].

The paraventricular nuclei (PVN) of the hypothalamus are involved in a neuroendocrine response, in the first place in case of stress reactions, they are a vegetative centre of coordinating the functions and are made up of a number of neuronal populations – subnuclei that vary by structural – functional specific characteristics and the character of neuronal correlations with different portions of the nervous and neuroendocrine systems [5].

While studying stress reactions and the action of stress-limited factors (specifically, melatonin), it is significant to investigate the indicated subpopulations of neurons of the hypothalamic PVN which synthesize the stress-releasing hormones that initiate stressor reactions of the organism [6]. One of the basic peptides, exerting an effect in the regulation of the ACTH secretion is vasopressin (VP). The VP – immunoreactive marker is revealed mainly in the lateral macrocellular subnucleus (lm PVN) [7]. It is of interest to ascertain the effect of the light stressor on the state of the mentioned subnuclei of the hypothalamic PVN. It is important at that to study changes of the expression of the gene of a pre-early response of c-fos in the structure as well as to analyze possibilities of enhancing the adaptation of the neurosecretory cells to the damaging action of the stressor.

An epiphysial tetrapeptide – epithalon has been designed and synthesized on the basis of an

analysis of the findings, dealing with the amino-acid composition of the pineal peptides at St-Petersburg Institute of Bioregulation and Gerontology. Preliminary studies have demonstrated that it prosesses an oncostatic, antioxidant and heroprotective action [8]. There is no information, as to the effects of epithalon in relation to the expression of the c-fos gene during a prolonged exposure to light.

The object of the research: to ascertain the effect of permanent lighting (a light stressor) on the condition of the c-fos gene in the lateral macrocellular subnuclei of the hypothalamic PVN of rats during different periods of a 24-hour period; to analyze changes of the activity of the said gene, when using melatonin and the synthetic bioregulator-epithalon against a background of the impact of the light stressor.

Material and methods. *The experiments were carried out on 60 sexually mature male albino rats. The animals were kept under the standard conditions of a vivarium at a fixed air temperature and humidity and free access to water and food. The rats were divided into five groups, each of them, in its turn, consisted of two subgroups (table 1).*

The experiment lasted seven 24-hour periods. The animals of the first group (intact) stayed under the conditions of a regular photoperiod (lighting from 08.00 to 20.00 the level of illuminance in the cages with the animals – 500 lx). The animals of group 2 stayed under the conditions of permanent lighting of similar intensity (an induced hypofunction of the pineal gland). The animals of series №3 were under the same conditions of the experiment as the rats of series №1, however, they received an injection of 1,0 ml of a solvent (0,9 % solution of ethanol in a physiological solution) intraperitoneally, at 19.00 daily. The animals of series № 4 were under the same conditions of the experiment as the rats of series № 2. They were injected melatonin (Sigma, USA, the degree of purification is 99,5%) ntraperitoneally in a dose of 0,5 mg/kg in 1,0 ml of a solvent at 19.00 daily. The animals of series № 5 were kept under the same experimental conditions as the rats of series № 2, however, they received a subcutaneous injection of epithalon at 19.00 daily (Russia) calculated on the basis of 0,5 μg/kg, in 0,5 ml of the physiological solution).

Table 1

Runs of trials	
1	(12.00 L – 12.00 D)
2	(24.00 L – 24.00 D)
3	(24.00 L – 00 D) + solvent
4	(24.00 L – 00 D) + melatonin
5	(24.00 L – 00 D) + epithalon

On the 8th day at 02.00 p.m. and 02.00 a.m. the animals were withdrawn from the experiment, implementing a one stage decapitation under phenobarbital narcosis (40.0 mg/kg, i/p). The brain of the animals was immediately removed and placed into a 10% solution of formalin on a phosphate buffer (0,1 M, pH 7,2) for 20 hours at a room temperature. After the standard procedure of dehydration and impregnating with chloroform and paraffin specimens were embedded in paraffin. In order to indetify c-Fos in histologic sections of the hypothalamus the indirect immunofluorescense method was used. Rabbit antibodies were used as primary antibodies (immunoglobulin-IGG) to c-Fos ("Sigma-Aldrich", USA).

The identification of c-Fos in the neurons of the hypothalamus and the determination of the content of this protein were realized by using the computer system of the digital analysis of imaging – VIDAS – 38 G (Kontron Elektronic, Germany) in the ultraviolet spectrum.

An image analysis was performed in an automatic regime by means of a package of applied programs VIDAS-2,5 (Kontron Elektronic, Germany), in the process of an automatic processing of the images of the hypothalamic PVN subnuclei the area of the material, immunoreactive to c-Fos; the concentration and the content of the protein of c-Fos in a neuron; the density of c-Fos -iimmunopositive neurons; the total content of the c-Fos protein in the structure were evaluated. Belonging of identified hypothalamus was mapped according to the stereotaxic atlas of the rat brain [9].

The obtained experimental findings were processed, using a package of applied and statistical programs VIDAS-2,5 (Kontron Elektronik, Germany).

The reliability of differences of values in the experimetntal and control animals was determined on the basis of Student's test (t). The values for which $p<0,05$ were considered trustworthy.

The results of the research and their analysis. A trustworthy decrease of the area of the immunopositive sites of the structures by 19,4 % ($p<0,05$) was revealed at night compared with the day-time measurements via identifying the product

of the expression of the gene of a prearly response of c-Fos by means of the immunofluorescence method in the lateral macrocellural subnuclei of the paraventricular nucleus (lm PVN) of the hypothalamus of intact animals. The average values of the areas of such immunopositive sites of the subnuclei varied also in the subgroup of rats that were under the conditions of a light stimulation and where lmPVN samples were taken for an investigation at 02.00 p.m. and 02.00 a.m., however, intergroup differences did not reach the level of reliability.

The averaged values for the entire group, on the whole, (ignoring the period of a 24-hour period) of the cross section area of the subnuclei of the lm PVN neurons in the animals that were simulated a epiphysial hypofunction, are, probably, larger (by 7.8%) than the corresponding values in the intact group. The circadian dynamics of variations of the section area of the subnuclei is also different. The area in the intact rats is smaller at 02.00 a.m. than at 02.00 p.m. The average values of the area of the cross section are almost identical at night and the day-time in the group of animals that were kept under the conditions of permanent lighting. By a pair-wise comparison of the corresponding values measured in different runs at 14 00 hours it was disclosed that the area of the section of a subnucleus in light stressed rats almost coincided with that observed under the physiological conditions. Analyzing the value in the specimens selected at night in case of a pineal hypofunction, the indices are considerably higher (by 12.3%) as far as the intact group is concerned (table 2).

Simulating an epiphysial hypofunction exerted an essential effect on the concentration of the c-Fos protein in the subnuclei of the lm PVN neurons. The index of the c-Fos concentration under light stress conditions is lower by 29.4% in the day-

Table 2

A characteristic of c-Fos - immunopositive neurons in the lateral macrocellular subnucleus of the rat hypothalamic paraventricular nucleus under a diverse duration of the photoperiod and with experimental therapy (x+Sx)

Series of experimental animals	The area of the material, immunoreactive to c-Fos, μm²	The concentration of the c-Fos protein in a neuron UIF	The content of the c-Fos protein in a neuron, UIF	The density of the c-Fos immunopositive neurons (mm²)	The total content of the c-Fos in the structure, UIF / mm²
Intact 02.00 p.m.	130,88±9,933	0,330±0,0229	44,40±5,132	190±39	8436±1731
Intact 02.00 a.m.	105,53±4,969 p=0,046	0,236±0,0105 p=0,004	24,65±1,599 p=0,004	204±27 p=0,774	5029±665 p=0,096
Permanent lighting 02.00 p.m.	129,27±10,461 p=0,913	0,233±0,0198 p=0,009	29,73±3,474 p=0,039	127±23 p=0,194	3775±684 p=0,031
Permanent lighting 02.00 a.m.	124,25±7,683 p=0,068 p_1=0,707	0,197±0,0128 p=0,040 p_1=0,158	23,43±1,359 p=0,574 p_1=0,122	120±25 p=0,046 p_1=0,841	2811±586 p=0,031 p_1=0,310
Permanent lighting+melatonin, 02.00 p.m.	124,48±11,992 p_2=0,770	0,467±0,0212 p_2<0,001	57,11±5,548 p_2=0,002	120±22 p_2=0,830	6854±1257 p_2=0,051
Permanent lighting+melatonin, 02.00 a.m.	111,57±15,883 p_2=0,489 p_1=0,531	0,279±0,0110 p_2<0,001 p_1<0,001	30,96±4,317 p_2=0,144 p_1=0,004	132±12 p_2=0,674 p_1=0,642	4087±372 p_2=0,096 p_1=0,061
Permanent lighting+epithalon, 02.00 p.m.	137,74±7,251 p_2=0,521	0,255±0,0061 p_2=0,313	36,31±2,229 p_2=0,142	92±6 p_2=0,172	3341±218 p_2=0,559
Permanent lighting+epithalon, 02.00 a.m.	106,33±8,103 p_2=0,140 p_1=0,016	0,258±0,0152 p_2=0,012 p_1=0,858	23,69±1,652 p_2=0,906 p_1=0,001	180±37 p_2=0,209 p_1=0,041	4265±877 p_2=0,198 p_1=0,331

Footnote: p – reliable changes concerning the parameters of the animals that were under the conditions of the standard photoperiod of the same time interval; p_1 – pertaining to the parameters of the animals of the previous time interval within the range of the series; p_2 – pertaining to the animals that were exposed to the action of permanent lighting.

time, whereas at night – by 16.5% in comparison with the similar values in the intact group.

Under such experimental conditions the index of the c-Fos protein content in the subnuclei of the lm PVN neurons in the intact group at 02.00 a.m. is reliably lower (by 44.5%, $p<0.01$) than in the day-time. The daily value of the index of the c-Fos content is lower by 33% in individual animals that were under light stress conditions compared with that of the intact group, whereas the nocturnal one approximated to the value in the indicated group of comparison. The diurnal dynamics of the parameter in question also turned out to be similar, however, a considerable difference between the diurnal and nocturnal levels was not marked in a series of animals stressed by light (table 2).

As to the integral density of the material, immunoreactive to c-Fos, this parameter fluctuated from 120 to 204 neurons per 1 mm2 of the section area in the lmPVN tissue in the subgroups under study. It must be noted that higher values of the density of the localization of the c-Fos – positive neurons in the lmPVN of the intact rats were observed at night, whereas, in the group of the animals that were under hyperilluminated conditions the circadian dynamics of the mentioned index assumed a reverse character – the density was higher in the day – time. When determining the density of the mentioned neurons we didn't identify intergroup distinctions in the experimental series. However, with a light stimulation at night, the density of the localization of the c-Fos – positive neurons is reliably lower compared with that of the intact animals during the similar spells of a 24 – hour period (table 2).

An important effect on the index of the integral density of c-Fos in the tissue of the lmPVN was exerted by changes of concentration of the protein in question and the index of its content in the subnuclei of neurons. The index of the total density of the c-Fos protein in the rats that were under the conditions of a light stimulation was lower by 55.3% in the day-time and by 44.1% at night than the analogous value in the intact group (table 2).

The injection of a solvent (1.0 ml of a 0.9% solution of ethanol on the physiological solution of sodium chloride) to the rats exposed to the standard light conditions during seven circadian periods did not change essentially the characteristic of the c-Fos – immunopositive neurons in the lmPVN of the hypothalamus.

Melatonin injections (0.5 mg/kg of the body weight) to the animals which underwent the action of permanent lighting normalized the circadian rhythm of the index of the material square, immunoreactive to c-Fos. The effect of epithalon (0.5 μg/kg of the animals' body mass) turned out to be similar to that of melatonin, however it was more marked. The area of the material, immunoreactive to c-Fos in the subnuclei of the hypothalamic lmPVN reached 137.74±7.251 μm^2 in the day-time in this series of animals, considerably differing from the group of the animals whose samples were taken during the night period when it made up 106.33±8.103μm^2 (table 2).

While using melatonin against a background of permanent lighting, a sharp rise (0.467±0.0212 Uif of the protein concentration in the subnuclei of lmPVN of the hypothalamus was revealed during the diurnal and less pronounced (0.279±0.0110 Uif) during the nocturnal periods (table 2).

As to the effects of epithalon, no reliable difference in a circadian aspect was revealed, while it was used. Thereat, the concentration of protein in the day-time was by 9.4% higher, whereas at night – by 30.9% in relation to the indices of the individuals which were simulated a hypofunction of the pineal gland and correcting therapy was not performed (table 2).

Chronobiotic injections to animals reflected on the diurnal dynamics of the index of the c-Fos protein content in the subnuclei of the lmPVN. At 14 hours the index exceeded almost twice (192.1%) the findings of the experiment on the stressed animals without injecting the hormone, approximating it to the normal value (table 2). In addition, it is considerably higher in comparison with that in the sections taken at 02.00 a.m. While using tetrapeptide, an elevation of the investigated index was observed in the day-time, concerning individual animals with the epiphysial hypofunction without undergoing experimental therapy with epithalon. At night no such impact on the index of the protein content in the subnuclei of the lmPVN was registered (table 2).

The introduction of melatonin to the animals with an epiphysial hypofunction restored diurnal rhythmicity, however, at 02.00 p.m. it brought about an insignificant decrease (by 5.51%), and at 02.00 a.m. a moderate elevation (by 10%) of the integral density of the material, immunoreactive to c-Fos compared with the stressed animals without

correcting. The circadian distinctions between the indices of the samples of this series are also unreliable. While correcting by means of epithalon the density of the neurons of the lmPVN subnuclei of the hypothalamus was lower by 95.6% in the day-time than at night, and also almost half of the parameter of the intact animals during a similar diurnal period. Intergroup differences were also marked, when employing tetrapeptide (table 2).

The effects of melatonin are appreciable in regard to correcting disturbances of the c-Fos integral density of the lmPVN subnuclei caused by a hypofunction of the cerebral epiphysis. After a one week use of an indol the index constituted 6854±1257 Uif mm-2 in the day-time, at night 4087±372 Uif mm-2. Thus, melatonin restored the indices against a background of permanent lighting both at 02.00 p.m. and 02.00 a.m. in regard of those ones in the intact animals (table 2).

It should be noted that the introduction of tetrapeptide did not restore the changes of the index under study caused by the rats' staying under hyperilluminated conditions and led to an inversion of the circadian rhythmicity of the said index (table 2). The index of the c-Fos integral density in the subnuclei of the hypothalamic lmPVN decreased by 60.4% when measured in the day-time in contrast to the group of intact animals.

Conclusions and prospects of further studies. 1. Analysing the diurnal expression of the gene pre-early functional activity – of c-Fos in the lateral macrocellular subnuclei of the paraventricular nucleus (lmPVN) of the hypothalamus, its reliable increase during the day-time hours is noted. The daily value of the index of the c-Fos protein content in individuals that were under the conditions of a light stress was lower by 33%, whereas at night it approximated to the control values. 2. Melatonin injections (0.5mg/kg of the body mass) to light stressed animals resulted in exceeding the index of the c-Fos protein content almost twice in the subnuclei of the lmPVN at 02.00 p.m. compared with the findings of the experiment on stressed animals without a hormone injection, as well a normalization of the circadian dynamics of the expression of the gene under study. 3. An increase of the index of the c-Fos protein concentration in the structure at night, while using epithalon tetrapeptide (0.5 mg/kg of the body mass) was revealed in regard to the individuals with an epiphysial hypofunction without performing experimental epithalon therapy; the effect of the agent was not registered in the day-time. 4. The results obtained may serve as a basis for a more profound understanding of the place and role of the subnuclei of the paraventricular nuclei of the hypothalamus in the mechanisms of the formation of circadian rhythms of the mammalian brain.

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ХАРАКТЕРИСТИКА ЭФФЕКТОВ МЕЛАТОНИНА И ЭПИТАЛОНА НА СОСТОЯНИЕ ГЕНА C-FOS В НЕЙРОСЕКРЕТОРНЫХ ЯДРАХ ГИПОТАЛАМУСА СТРЕСИРОВАННЫХ СВЕТОМ КРЫС

Резюме. Исследовано влияние мелатонина и эпиталона с целью коррекции стресс-индуцированных изменений активности гена "раннего ответа" c-fos в латеральных крупноклеточных субъядрах паравентрикулярного ядра (лкПВЯ) гипоталамуса крыс в разные промежутки суток (днём и ночью). Экспрессия продукта этого гена – белка c-Fos – у животных, содержащихся в нормальных условиях чередования освещения и темноты, демонстрировала чёткий циркадианный характер (с большим уровнем днем). В условиях светового стресса дневной показатель индекса содержания c-Fos в лкПВЯ животных на 33,0 % ниже, а ночной – приближался к контрольным величинам. Инъекции мелатонина (0,5 мг/кг) проявились в 14.00 ч повышением индекса содержания белка c-Fos в лкПВЯ почти вдвое, а также нормализацией циркадианной динамики экспрессии исследованного гена. При использовании тетрапептида эпиталона (0,5 мкг/кг) выявлено увеличение индекса концентрации белка c-Fos в структуре ночью относительно особей с эпифизарной гипофункцией без терапии эпиталоном. Днем такого эффекта препарата не зафиксировано.

Ключевые слова: c-fos, паравентрикулярные ядра, стресс, мелатонин, эпиталон.

A CHARACTERISTIC OF THE EFFECTS OF MELATONIN AND EPITHALON ON THE STATE OF C-FOS GENE IN THE NEUROSECRETORY NUCLEI OF THE HYPOTHALAMUS IN AN EXPERIMENT

Abstract. It has been established that the expression of the c-Fos protein in animals under the normal conditions of alternating lighting and darkness demonstrated a clear-cut circadian character (with a highly daily level). Under the conditions of a light stress the daily parameter of the index of the c-Fos content in the lateral macrocellular subnuclei of the paraventricular nucleus (lm PVN) of rats was lower by 33,0%, whereas the nocturnal one approximated tj the control values. Melatonin injections (0,5 mg/kg) to animals stressed by light manifested at 02.00 p.m. by an increase of the index of the c-Fos protein content in the lm PVN almost twice as much as compared with the fingers of an experiment on stressed rats without introducing the hormone and a normalization of the circadian dynamics of its expression. While using epithalon tetrapetide (0,5 mkg/kg), an increase of the index of the c-Fos protein concentrationwas detected in the structure at night relative to rats with an epiphysial hypofunction without epithalon therapy. Such an effect of the medication was not registered in the day-time.

Key words: c-Fos, paraventrivular nuclei, stress, melatonin, epithalon.

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