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DYNAMICS OF GENE C-FOS ACTIVITY IN PARAVENTRICULAR NUCLEI OF THE HYPOTHALAMUS OF RATS AT LIGHT STRESS

Keywords: gene c-fos, immune specific c-Fos protein, paraventricular nucleus of hypothalamus, constant lighting, light deprivation.

Abstract. Stress influence (light deprivation and stimulation) on gene c-fos state of early functional activity in subnuclei of paraventricular nucleus (PVN) of hypothalamus of rats in various periods of twenty-four hours (day and night) has been elucidated. Product expression of this gene - c-Fos protein - in animals which were hold under normal conditions of light and darkness alternations showed rather distinct circadian character. Simultaneously, change of duration of the light-darkness cycle results in evident desynchronization.

Introduction

Elucidation of the place and role of neuroendocrine structures in the mechanisms of circadian rhythms is one of the topical questions of modern chronophysiology [1, 7, 10]. Paraventricular nuclei (PVN) of the hypothalamus are the vegetative centre of functional coordination and consist of a number of neuronic populations -subnuclei which differ by structural - functional peculiarities and character of the nervous connections with different parts of the nervous and neuroendocrine systems [4, 6].

Duration changes of the main pacemaker-photoperiod, as stress factor, desynchronize rhythms of somatic and visceral functions, as well as coordination and modulation of adaptive mechanisms of the organism to the influence of various factors [1, 13]. Investigation of the indicated subpopulations of PVN neurons of the hypothalamus synthesizing stress-releasing hormones, which initiate stressor reactions of the organism is of great importance while studying stress reactions and action of stress-limiting factors (especially, melatonin) [5,12].

Corticotropin-releasing factor (CRT) is one of the basic factors showing a pronounced effect in regulation of ACTH. CRT-immune reactive trace was detected, for the greater part, in medial small-cellular subnuclei (sc PVN) [6]. Elucidation of light stress influence on the state of the mentioned PVN subnuclei is the subject of interest. For all this, it is important to study changes of morphofunctional activity and the level of c-fos gene expression previous response in the structures and also to analyse possible adaptive increase of neurosecretory cells to the damaging action of the stress factor as well.

Photoperiodicity, among a wide complex of medium parameters, is the most reliable and stable synchronizing factor for homoiothermic animals, including a person [1-3]. Derangements of the light regimen (long lighting, constant darkness) cause immediate changes of c-fos gene expression in PVN [8, 11]. Reinforcement of its expression intensifies synthesis of the corresponding immune specified c-Fos protein [9, 14]. Peptide, mentioned above, takes part in the mechanisms of synchronization of the given activity by external cyclic influences, in particular, circadian, connected with alternation of light and darkness [8, 10].

At the same time, information concerning influences of steady lighting or darkness on the activity of the indicated subpopulations of PVN neurons of hypothalamus, involved in the formation of circadian rhythms mechanisms, remains relatively limited.

Purpose of the research

To elucidate c-fos gene activity of previous response in medial small-cellular subnuclei of paraventricular nucleus (scPVN) of hypothalamus as to the changed duration of the light-darkness cycle.

Material and methods

Experiments were carried out on 36 sexually mature males of non-breed white rats weighing 150-180g. Animals were hold in vivarium standard conditions at a steady temperature and air humidity and free access to water and food. Experimental rats were divided into three groups, and each of them, in its turn, consisted of two subgroups (six animals each).

Animals of the first group (intact) were held during 7 days under conditions of usual light regimen (light-darkness every 12 hours, LD, lighting from 8.00 till 20.00 by means of fluorescent lamps, the level of lighting in cages with animals was 500 lux.). Rats of the second group were kept under conditions of steady lighting of the same intensity (LL, induction of epiphysis hypofunction). The animals of the third group were under conditions of a constant darkness (light deprivation, DD, induction of epiphyseal hyperfunction) during the same period.

Next day following the completion of the 7 days

period at 14.00 p.m. and 02.00 a.m. the animals were taken out from the experiment, fulfilling simultaneous decapitation under pentobarbital narcosis (400mg/kg, intraperitonealy). The brain of the animals was immediately taken out and put into 10% formaldehyde solution on phosphate buffer (0,1M, pH 7.2) for 20 hours at a room temperature. Samples were embedded in paraffin following the standard procedure of dehydration and impregnation with chloroform and paraffin. All stages of the experiment were carried out keeping the basic requirements of the European convention concerning humane care of animals [Strasbourg, 1986].

Indirect immune fluorescent method was used to identify c-Fos in histological sections. Sections of 14 mcm thickness were firstly deparrafined in xylene then rehydration was carried out in ethanol solutions of six descending concentrations (100-40%) and afterwards they were thrice irrigated every ten minutes in phosphate buffer (0.1M, pH 7.2).

Rabbits' antibodies (immunoglobulin - IGG) as initial antibodies, were used to c-Fos ("Sigma-Aldrich", USA). At first, sections were incubated during 45 minutes at 37?C in 0.3% solution Triton X-100 ("Sigma-Aldrich", USA) on 0.1 M phosphate buffer (pH 7.2) adding 1% of goat's serum. Then, primary to c-fos (1:1000) antibodies were brought on successive serial sections and incubated during 24 hours in a damp chamber under conditions of decreased temperature (4^0C). When excess of primary antibodies has been rinsed in 0.1 m phosphate buffer sections were incubated at 37^0C during 60 minutes with secondary antibodies in the solution 1:200.

Goat's gamma globulin, being the antibody to rabbit globulins, conjugated with fluoresceinisothiocyonate (FITC; "Sigma-Aldrich", USA) was used as secondary antibodies. After incubation sections were rinsed by phosphate buffer (0.1 M) and placed in a mixture of glycerol and phosphate buffer (9:1) for further investigation by means of luminescent microscopy.

Control of specificity binding of antibodies was conducted in the same way excluding the stage of incubation with initial to c-Fos antibodies.

Identification of c-Fos in the hypothalamic neurons and determination of the content of this protein were realized using computer system of digital analysis of VIDAS-386 image ("Kontron Elektronik", Germany) in ultraviolet spectrum. Filter of high emission with ranges of excitement and emission 370-390 and 420-450 nm correspondingly and specialized lens with wide aperture were used to obtain fluorescent image. Images with the help of eight-bet CCD-camera COHU-4922 ("COHU Inc", USA) were introduced into computer system of the analysis of VIDAS-386 images. In spite of all this the effect of preparation "burning", connected with gradual deterioration of FITC molecules under the influence of prolong ultraviolet irradiation, was made to be impossible. Introduced immune fluorescent image was quantized according to densitometric scale with 256 gradations of grey color. Image analysis was conducted in automatic mode with the help of software package application VIDAS-2.5 ("Kontron Elektronik", Germany). Parts of preparations in which fluorescence intensity probably exceeded background values (peculiar to the so-called non-specific fluorescence) were identified as to the program. The areas of such parts and the whole area of nuclei sections of PVN neurons which contained immune positive material (Si and Sя correspondingly, mcm^2) were measured. Indices, characterizing c-Fos concentration and contents of this protein in nuclei of immune positive cells, $Ki=|1g(Di/D0)|$ and Ci=KiSi (conventional units -c.u.) accordingly were calculated taking into consideration fluorescence intensity in immune positive areas and fluorescence intensity of the background (Di and D0). Since these indices are relative, and not the absolute values, we shall call them later on as indices of c-Fos concentration and content in immune positive cells.

Topographic belonging of immune positive neurons to separate structures of hypothalamus was mapped in accordance with stereotaxic atlas of rat brain.

The experimental data obtained were processed using package of applied and statistical programs VIDAS-2.5 ("Kontron Elektronik", Germany) and EXCEL-2003 ("Microsoft Corp.", USA). The value of arithmetical mean, average quadratic deviation and error of the average was calculated to select all indices. Selections of immune positive cells PVN consisted of 1200-130 units in which Si and Sя were measured and values of Ki and Ci were calculated in various groups of experimental animals.

Besides, we have calculated localization density of c-Fos-immune positive neurons within the limits of the investigated sections of this nucleus under study.

For that, the quantity of such cells was previously determined in several (four - seven for every animal), chosen by chance, fields of vision and calculated the average quantity of similar neurons per 1 mm2 of the section area. Reliability of differences of the values in the group of animals under study and control ones was determined according to (t) criterion Стьюдента. Values for which $P<0.05$ were considered to be reliable.

Discussion of the results

The results of the carried out experiments are

evidence that product expression of the activity of gene "previous response" c-fos - protein c-Fos - in neurons of sc PVN of rats, hold under conditions of normal photo periodicals (12.00C:12.00T), experiences rather distinct circadian fluctuations. At night the concentration index of this protein in the nuclei of the indicated neurons is almost one third less than corresponding value of the given parameter at night, and the difference between the average night and day values of the index c-Fos contents constituted 29,2% (table). However, standardized mean value of the area, occupied by immune positive material on nuclei cuts of neurons, is more at night. Such differences were evidently leveled in case of calculation of integral density index of immune positive product in the plane of tissue sections scPVN, but as a whole, the mentioned circadian variations of c-Fos expression under conditions of standard are completely evident.

Under conditions of light stress (Group LL) concentration index of c-Fos protein in nuclei of scPVN neurons at day and night is less than corresponding values under normal conditions of lighting (table). Besides, this index decreased at night in comparison with the patterns taken for the investigation in day time. Under such experimental conditions index of c-Fos contents experienced similar circadian fluctuations (table). It is necessary to note a tendency to general intensity reduction of the given protein expression under conditions of constant lighting. The value of the above mentioned index, taken as average for group LL as a whole (without taking into account a period of twenty-four hours), which is 20% less than corresponding value in standard, is evidence of it.

However, protein production of c-Fos "previous response" under conditions of light deprivation (group DD) experiences the most evident modifications. In this group during night period, indices characterizing the mentioned process, less differed from the control than in the day time. Particularly, index of c-Fos concentration and contents in nuclei of sc PVN neurons exceeded almost twice the corresponding values in group LD during this time period (table). It is clear, that it resulted in corresponding, almost two-times excess, of the total contents of c-Fos protein in the structure under study in comparison with the standard (table).

Melatonin level - hormone of the pineal gland

Table

Characteristics of c-Fos-immune positive neurons in medial small-cellular subnucleus of paraventricular nucleus of hypothalamus in rats under conditions of different light regimen (x±Sx)

Series of experimental animals	Area of the material immune reactive to c-Fos, mcm^2	Concentration of protein index of c-Fos in neurons, $O|_\phi$	Index of c-Fos protein contents in neuron, $O|_\phi$	Total content of c-Fos protein in the structure, $O|_\phi$/mm^2
Intact, LD 14.00 a.m.	25,98 ± 1,489	0,363 ± 0,0059	9,46 ± 0,529	2145 ± 127
Intact, LD 02.00 p.m.	27,12 ± 1,402	0,233 ± 0,0031 p_1<0,001	6,70 ± 0,394 p_1<0,01	1582 ± 89 p_1<0,01
Constant lighting, LL 14.00 a.m.	30,40 ± 1,364 p=0,050	0,264 ± 0,0078 p<0,001	8,28 ± 0,531	2341 ± 167
Constant lighting, LL 02.00 p.m.	24,72 ± 1,405 p_1<0,05	0,184 ± 0,0023 p<0,001 p_1<0,001	4,76 ± 0,303 p<0,01 p_1<0,001	1238 ± 58 p<0,01 p_1<0,001
Constant darkness, DD 14.00 a.m.	29,83 ± 1,681	0,535 ± 0,0122 p<0,001	17,25 ± 1,236 p<0,001	4537 ± 325 p<0,001
Constant darkness, DD 02.00 p.m.	27,26 ± 0,797	0,209 ± 0,0017 p<0,001 p_1<0,001	6,07 ± 0,214 p_1<0,001	1681 ± 64 p_1<0,001

Notes: p - reliable changes in regard to parameters of the animals which were under conditions of standard photoperiod of the same hour's interval; p1 – concerning parameters of animals of the previous hour's interval within the limits of series.

being the principal humoral mediator of arrangement of circadian rhythms, should be logically considered as the most significant factor determining the observed shifts of intensity of c-fos gene expression in scPVN neurons under conditions of standard and experimentally changed photo periodicity. In case of normal alternation of the periods of lighting and darkness c-Fos concentration and contents increase in the day-time, when melatonin level is minimal in the blood. That's why it might possible to think that enhancement of melatonin secretion and an increase of its level prevent intensification of gene c-fos expression and enhancement of synthesis of the corresponding c-Fos protein. However, under conditions of experimental induction of the pineal gland hypofunction (holding animals at a constant lighting, light stress) the effect, expected on the basis of such considerations, evident increase of concentration and quantity of immune positive product in scPVN neurons, is not observed. General reduction of intensity of the given protein expression is the principle phenomenon under such conditions .But induction of the pineal gland hyperfunction in case of light deprivation leads to the evident increase of c-Fos concentration and contents in the day-time - period, when in health , melatonin level in the blood is minimal. Under conditions, mentioned above, holding the animals in a constant darkness (which, as steady lighting, also is stressogenic factor) the level of this hormone in the day-time must be significantly more than corresponding value under conditions of standard photoperiodicity. It is not excluded that just strong divergences between the evident and "expected" (normal) melatonin levels in the animals of group DD in the day-time is one of the essential causes of strong c-fos gene expression in this period of twenty-four hours.

Conclusions

1.In medial small-cellular subnuclei of paraventricular nucleus of the rat hypothalamus the dynamics of expression of the product activity of gene "previous response" c-fos-protein c-Fos has a distinct circadian rhythmicity.

2.Protein production of c-Fos "previous response" under conditions of light deprivation experiences the most evident modifications.

Perspective of further investigations

Melatonin level presents itself an important factor, which influences on intensity of c-fos expression, but these values are not connected with simple dependence. Interrelations of the above mentioned indices are evidently rather complicated, and mechanisms of such interrelations require further investigations.

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ДИНАМІКА АКТИВНОСТІ ГЕНА C-FOS У ПАРАВЕНТРИКУЛЯРНИХ ЯДРАХ ГІПОТАЛАМУСА ЩУРІВ ЗА СВІТЛОВОГО СТРЕСУ

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Резюме. З'ясовано вплив стресу (світлової деривації та стимуляції) на стан гена ранньої функціональної активності c-fos у суб'ядрах паравентрикулярного ядра (ПВЯ) гіпоталамуса щурів у різні проміжки доби (вдень і вночі). Експресія продукту цього гена - білка c-Fos - у тварин, котрі утримувалися в нормальних умовах чергування освітлення та темряви, демонструвала досить чіткий циркадіанний характер. Водночас зміна тривалості циклу світло-темрява призводить до вираженого десинхронозу.

Ключові слова: ген c-fos, імуноспецифічний білок c-Fos, паравентрикулярне ядро гіпоталамуса, постійне освітлення, світлова депривація.

ДИНАМИКА АКТИВНОСТИ ГЕНА C-FOS В ПАРАВЕНТРИКУЛЯРНЫХ ЯДРАХ ГІПОТАЛАМУСА КРЫС ПРИ СВЕТОВОМ СТРЕССЕ

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Резюме.Исследовано влияние стресса (световой депри-

вации и стимуляции) на состояние гена ранней функциональной активности c-fos в нейронах субъдрах паравентрикулярного ядра (ПВЯ) гипоталамуса крыс в различные промежутки суток (днем и ночью). Экспрессия продукта этого гена - белка c-Fos - у животных, которых содержали в нормальных условиях чередования освещения и темноты демонстрировала довольно четкий циркадианный характер. В то же время, изменение длительности цикла свет-темнота приводит к выраженному десинхронозу.

Ключевые слова: ген c-fos, иммуноспецифический белок c-Fos, паравентрикулярное ядро гипоталамуса, постоянное освещение, световая депривация.

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