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Neurons of the Paraventricular Hypothalamic Nucleus Under Normal and Modified Illumination Conditions: Immunohistochemical and Morphometric Parallels

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Using an immunohistochemical technique and densitometric analysis in experiments on rats, we examined the expression of an early-response protein, c-Fos, in the medial parvicellular subnucleus of the hypothalamic paraventricular nucleus (mpcPVN). During 7 days, the animals were kept under conditions of (i) normal photoperiod (group LD), (ii) continuous illumination (group LL), and (iii) continuous darkness (light deprivation, group DD). It was found that both normal photoperiodicity and artificial modifications of the latter result in significant changes of c-Fos immunopositivity in the nuclei of mpcPVN neurons (cells that synthesize corticotropin-releasing factor). Changes in the indices of concentration and amount of c-Fos in mpcPVN neurons were, from some aspects, similar to those observed under analogous experimental conditions in neurons of a leading pacemaker of the circadian periodicity, the suprachiasmatic nuclei (SChNs) of the hypothalamus [10], but, at the same time, demonstrated noticeable specificity from other aspects. In all experimental groups, the concentration and amount of c-Fos in mpcPVN neurons were greater during daytime than at night. Dramatic increases in the above parameters observed at daytime in rats subjected to light deprivation were the most significant effect of modifications of the photoperiod. Both continuous illumination and light deprivation resulted in some increase of the number (density) of c-Fos-immunopositive units in the mpcPVN. The nuclei of neurons of this structure demonstrated the phenomenon found earlier in SChN neuron; geometrical dimensions of the cell nuclei of these structures depended noticeably on both illumination conditions and periodicity. Probable mechanisms of the dependence of c-Fos expression in the hypothalamic nuclei on the photoperiod and its modifications, interdependence of such changes observed in the circadian pacemaker (SChN) and an important component of the system controlling the stress reactions (mpcPVN), and the relation of the above phenomena to the level of melatonin produced by the *epiphysis cerebri* (main neuroendocrine intermediator organizing the above-mentioned periodicity) are discussed.

Keywords: circadian rhythm, continuous illumination, light deprivation, hypothalamus, paraventricular nucleus, gene *c-fos*.

INTRODUCTION

In mammals, the suprachiasmatic nuclei (SChNs) of the hypothalamus serve as a pacemaker of the circadian periodicity, while the *epiphysis cerebri* (pineal gland) fulfills the role of the main neuroendocrinal intermediator in realization of this periodicity [1-7]. The SChNs receive, via the retino-hypothalamic tract, information on the level of illumination in the environment. Fluctuations of this level perceived by retinal photoreceptors is the main timing factor for an SChN "biological clock" [1, 3, 4, 7, 8].

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The paraventricular nuclei (PVNs) of the hypothalamus (i.e., crucial nuclear complex responsible for coordination of autonomic functions) play one of the main roles in neuroendocrinal regulation in general and in that realized under stress conditions in particular. Neuronal networks of these nuclei are significantly involved in the formation of responses of the organism to the action of stressing factors. These nuclei include a few neuronal populations, the subnuclei, which differ from each other in their structural/functional peculiarities and patterns of neural connections with different components of the nervous and neuroendocrine systems [3]. Multisynaptic pathways reaching the epiphysis via the PVNs regulate the synthesis of melatonin in the epiphysis at night and suppression of this process upon intensified illumination.

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Neurons of the medial parvicellular subnuclei of the PVNs (mpcPVNs) of the hypothalamus synthesize corticotropin-releasing factor, CTRF. Thus, these subnuclei play the role of one of the key structures in the mechanism of initiation and regulation of responses of the organism to the action of stressogenic factors. Various structural/functional subdivisions of the hypothalamus are closely interconnected with each other; thus, there are reasons to believe that not only SChNs but also mpcPVNs should be significantly involved in the processes of the neuroendocrine regulation in realization of the circadian periodicity.

Modulation of the level and mode of illumination, i.e., the main external factor determining the circadian period, results in considerable changes of expression of an early-response gene, *c-fos*, in neuronal structures participating in the circadian regulation of functions. First of all, this is realized in SChN neurons. Correspondingly, the intensity of synthesis of the immunspecific c-Fos protein in these neurons undergoes changes, and this effect can be detected by the respective immunohistochemical technique [8, 9]. There are reasons to believe that changes in the c-Fos content are not a “passive” reaction of neurons of the above structures to changes in the illumination intensity; this protein can exert certain modulating effects on the activity of these neurons and, therefore, can play an active role in the processes of synchronization of such activity by external cyclic influences [5, 8].

In our earlier study [10], we found certain illumination level-related significant changes in the c-Fos content in SChN neurons of rats. These differences were found under conditions of both normal photoperiod and artificial modifications of the latter (constant illumination or constant darkness). Understandably, the possibility of corresponding changes in neurons of other hypothalamic nuclei has attracted substantial interest, the greatest with respect to the mpcPVN, the structure actively participating in the control of stress reactions. It should be taken into account that modifications of the photoperiod are, inevitably, rather powerful stressogenic factors. In this study, we examined the effects of normal photoperiodicity and experimental modifications of the latter in mpcPVN neurons of rats.

METHODS

Experiments were carried out on 36 adult mongrel male albino rats weighing 150 to 180 g. Animals were kept under standard vivarium conditions, at the controlled temperature and air humidity; free access to water and food was provided. Rats were divided into three groups; each of the latter was, in turn, divided into two subgroups including six animals. All stages of the experiments were carried out in accordance with the main requirements of the European Convention for the humane treatment of animals.

Intact animals of the 1st group were kept for 7 days under conditions of normal illumination periodicity (12/12 h light/darkness cycle, group LD). Illumination (50 lx in the cages) was provided from 8.00 until 20.00 with luminescent lamps. Rats of group 2 were kept for 7 days under conditions of continuous illumination of the analogous intensity (group LL, induction of the epiphyseal hypofunction). Animals of the 3rd group were kept during the same period in constant darkness (light deprivation, group DD, induction of the epiphyseal hyperfunction).

On the next day after termination of the 7-day-long conditioning period, animals were decapitated under Nembutal anesthesia (40 mg/kg i.p.). The brains were immediately dissected and immersed for 20 h in a 10% formalin solution in phosphate buffer (0.1 M, pH 7.2) at room temperature. After a standard procedure of dehydration and impregnation with chloroform and paraffin, tissue samples were embedded in paraffin.

The c-Fos protein was identified in slices of the hypothalamus using an indirect immunofluorescent technique. Fourteen-micrometer-thick slices were first deparaffinized in xylene, then rehydrated in six descending gradations (100-40%) of ethanol, and washed off three times in phosphate buffer (0.1 M, pH 7.2).

Rabbit antibodies (immunoglobulin, IG) against c-Fos (Sigma-Aldrich, USA) were used as primary ones. First, slices were incubated for 45 min in 0.3% solution of Triton X-100 (Sigma-Aldrich, USA) in 0.1 M phosphate buffer (pH 7.2) with 1% of goat serum added. Then, the above primary antibodies (1:1000) were put on sequential serial slices, and the latter were incubated for 24 h in a moist chamber at a low temperature (4°C). After the excess of primary antibodies was washed off in 0.1 M phosphate buffer, the slices were incubated for 60 min at 37°C with secondary antibodies (1:200). Goat gamma-globulin conjugated with fluorescein isothiocyanate (FITC, Sigma-Aldrich, USA), i.e., antibodies with respect to rabbit globulins, was used as secondary antibodies. After incubation, slices were washed off with 0.1 M phosphate buffer and immersed in a glycerol + phosphate buffer mixture (9:1) for further examination using luminescence microscopy.

The specificity of immunohistochemical reaction was controlled using all the above-mentioned procedures except the stage of incubation with primary antibodies against c-Fos [11].

Borders of the mpcPVN of the hypothalamus were estimated according to the atlas of cerebral structures of the rat [12]. Identification of c-Fos in neurons of this subnucleus and estimation of the content of this protein were performed using a computerized system for digital image analysis, VIDAS-386 (Kontron Elektronik, Germany) in the UV part of the spectrum. To obtain fluorescent images, a high-emission filter with the ranges of excitation and emission 370-390 and 420-450 nm, respectively, and a specialized wide-aperture objective were used. Images were entered into the above system of digital image analysis with the help of an 8-bit CCD camera, COHU-4922 (COHU Inc., USA). In such a way, we avoided the negative effect of image burn-in related to gradual destruction of FITC molecules under prolonged action of UV irradiation. The entered immunofluorescent image was digitized according to a densitometric scale (256 gradations of gray). The image was analyzed in an automated mode using VIDAS-2.5 applied software (Kontron Elektronik, Germany). Sites of the preparations where the fluorescence intensity significantly exceeded the background values (corresponding to the so-called nonspecific fluorescence) were automatically identified; these regions were localized within the borders of cross-sections of the nuclei of the neurons under study. The areas of these regions containing the immunopositive material and the total areas of cross-sections of the nuclei of mpcPVN neurons (S_i and S_n, μm^2) were measured; values of the normalized area of the immunopositive material ($S_i/S_n \cdot 100\%$) were calculated. Taking into account the intensity of fluorescence in the immunopositive areas and intensity of background fluorescence (D_i and D_0), we calculated parameters characterizing the concentration of c-Fos and content of this protein in the nuclei of immunopositive cells, $K_i = |\lg (D_i/D_0)|$ and $C_i = K_i \cdot S_i$ (arbitrary units, a.u.), respectively. These values are the relative parameters; this is why they are called below indices of the concentration and content of c-Fos in the nuclei of immunopositive cells.

The obtained numerical data were treated using VIDAS-2.5 applied and statistical software (Kontron Elektronik, Germany) and Excel-2003 (Microsoft Corp., USA). The mean, s.d., and s.e.m. values were calculated for all samplings; the distributions of the latter were preliminarily tested for fitting the Gaussian (normal) law. The samplings of immunopositive mpcPVN cells of different experimental groups, where the above parameters were calculated, included 122 to 168 units.

In addition, we calculated the density of localization of c-Fos-immunopositive neurons within the borders of examined sections of the mpcPVN. For this purpose, we preliminarily measured the numbers of cells with c-Fos-positive nuclei within several (4 to 7 for each animal) randomly selected fields of vision and calculated the mean numbers of such neurons per 1 mm^2 of the slice. The significance of differences between values in the experimental and control groups of animals was estimated by the Student's *t*-test; differences with $P < 0.05$ were considered to be significant.

RESULTS

Mean values of the total area of sections of c-Fos-immunopositive cell nuclei of mpcPVN neurons (S_n) in the groups of experimental animals kept under different illumination conditions varied from 32.33 ± ± 0.56 to 36.81 ± 0.51 μm^2. These values were noticeably greater than analogous values for the nuclei of SChN neurons measured under corresponding experimental conditions (25.2 to 30.0 μm^2) [10]. If cross-sections of the nuclei of neurons of these structures are approximated by circles, the mean diameters of the cell nuclei in the SChN and mpcPVN should correspond to about 5.7 to 6.2 and 6.4 to 6.9 μm, respectively. The respective differences are probably related to the fact that the dimensions of cells in the latter subnucleus are somewhat greater that those in the SChN.

The geometrical dimensions of c-Fos-positive nuclei in mpcPVN neurons demonstrated certain dependences on the time of day and experimental modifications of the photoperiod. In all three experimental groups (LD, LL, and DD), the mean S_n values were somewhat greater at 14.00 than those at 02.00; in the two former groups (LD and LL), these differences (8.9 and 7.4%, respectively) were statistically significant (Fig. 1A). In group DD, the "day" and "night" S_n values differed from each other insignificantly. The averaged S_n value (irrespectively of the time of day) in this group was somewhat smaller than the respective indices in the two former groups.

The mean values of areas covered with the c-Fos-immunopositive material were nearly identical in the group kept with the normal photoperiodicity (LD). In groups LL and DD , the respective values differed

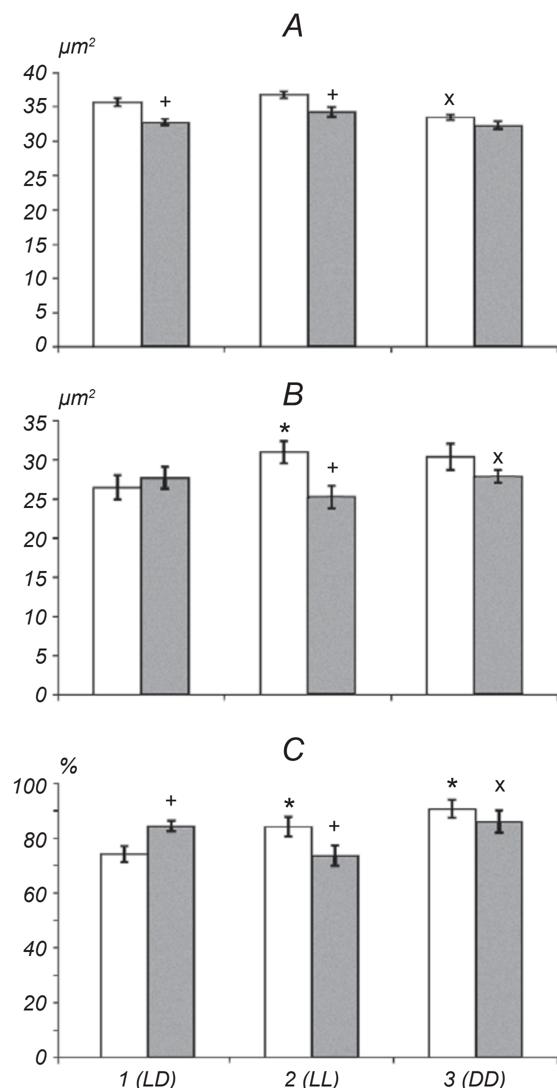

Fig. 1. Geometrical characteristics of neurons in the medial parvicellular part of the paraventricular nucleus (mpcPVN) of the hypothalamus. A) Areas of cross-sections of the nuclei, μm^2; B) areas where the intensity of fluorescence of the c-Fos-immunoreactive material significantly exceeded the background values, μm^2; C) normalized areas occupied by the c-Fos-immunoreactive material, % (total areas of cross-sections of the nuclei are taken as 100%). Means ± s.e.m. are shown. 1-3) Groups of animals kept under conditions of the standard (12/12 h) photoperiod (1, LD), continuous illumination (2, LL), and continuous darkness (3, DD). Open and hatched columns show the data for mpcPVN samples taken at 14.00 and 02.00. Asterisks show cases of significant differences ($P < 0.05$) from the control (LD) group, crosses show those for the "day" and "night" data in the same group, and diagonal crosses show cases of significant differences between groups 2 and 3.

noticeably from each other. The "day" values in the latter groups were greater than the nocturnal ones, and this difference exceeded the significance level in the continuous-illumination group (LL, Fig. 1B). The interrelations between the S_n and S_i values determined the existence of the corresponding differences between normalized (relative) areas occupied by the cFos-immunopositive material in the neuronal nuclei. This index in group LD was significantly higher at night, while opposite relations were observed in groups LL and DD (in the former group, this difference was significant, while in the latter one it was insignificant; Fig. 1C). In general, relative values of the c-Fos-positive areas (S_i) in the nuclei of mpcPVN neurons were noticeably greater than those in SChN neurons [10]. In the latter objects, this index varied from about 45 to 75%, while it could reach 86-91% in mpcPVN cells.

The index of c-Fos concentration, K_i, when measured in the nuclei of mpcPVN neurons during daytime and nighttime, demonstrated the most specific changes. In all three experimental groups, this index at 14.00 was significantly higher ($P < 0.05$) than that at 2.00. The respective differences were 55.5% in the group with the normal photoperiod (LD), 43.1% in the LL group, and 155.9% in the group kept in continuous darkness (DD). In other words, in the latter group the index of the "day" cFos concentration exceeded the "night" value by more than two and a half times. The "day" concentration index in the DD group was 47.3% greater than the respective ("day") value in the LD group. Therefore, most dramatic shifts in the optic density of the immunoreactive product reflecting the concentration of the early-response protein (c-Fos) were observed during daytime in animals subjected to light deprivation (Fig. 2A).

It should be recognized that illumination mode-determined changes in both natural and normalized values of the area occupied by the immunopositive material were, in some cases, significant but, in general, relatively moderate (Fig. 1B, C). Thus, it is obvious that variations of the index of c-Fos content in the nuclei of mpcPVN neurons (C_i) nearly reproduced variations of the concentration indices for this protein. The content of c-Fos in the nuclei of these cells in all three experimental groups was at 14.00 much higher ($P < 0.05$) than that at night. The differences between the "day" and "night" values in groups LD, LL, and DD were equal to 48.8, 73.5, and 183.8%, respectively. The "daytime" index in the DD group exceeded the analogous value in the group with the normal illumination mode (LD) by nearly two times (by 82.5%, Fig. 2B). Absolute values of the indices of c-Fos content in the immunopositive nuclei of mpcPVN neurons measured in all experimental groups both at 14.00 and 02.00 were noticeably greater than those in SChN neurons [10]. This resulted from

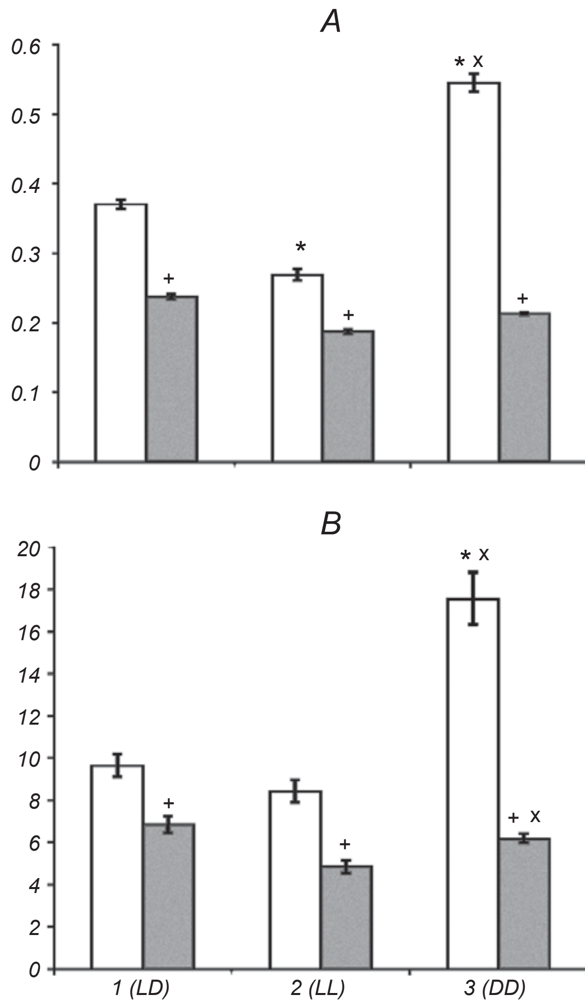

Fig. 2. Mean values of the index of concentration (A) and index of content (B) of cFos protein (arbitrary units) in the nuclei of mpcPVN neurons in animals of different experimental groups and during daytime and nighttime. Other designations are the same as in Fig. 1.

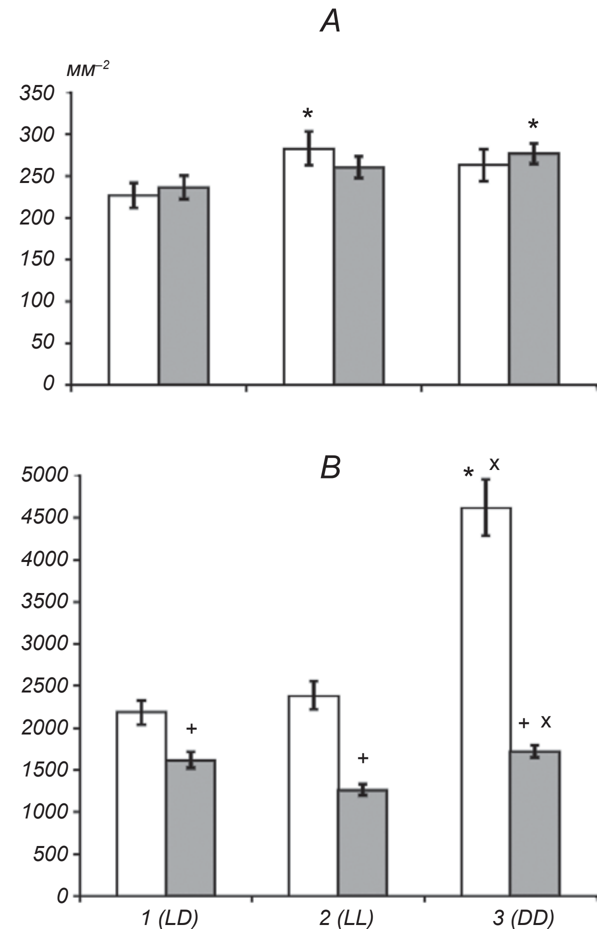

Fig. 3. Mean values of the density of localization of c-Fos-immunopositive mpcPVN neurons (A, mm^{-2}) and index of the integral density of c-Fos protein within the borders of sections of the above nucleus (B, arbitrary units). Other designations are the same as in Figs. 1 and 2.

the somewhat greater dimensions of the nuclei of mpcPVN neurons and greater areas occupied by the immunopositive product (at comparable values of the concentration index for the above product).

Mean values of the density of localization of c-Fos-positive mpcPVN neurons in the control group, when measured at 14.00 and 02.00, were close to each other (227 ± 15 and 236 ± 14 cells per 1 mm^2). Rats subjected to the action of continuous illumination and light deprivation demonstrated somewhat greater values of the respective indices (in group LL, 283 ± 20 and 260 ± 13 mm^{-2}, and in group DD, 263 ± 19 and 277 ± ± 12 mm^{-2}, respectively, Fig. 3A). Differences between the "day" and "night" values, however, did not reach the significance level. As was mentioned, these values were measured within randomized segments of the slices; this, probably, was the reason for the relatively high dispersions of this index. Nonetheless, it should be mentioned that the mean values of the density of c-Fos-positive cells in group LL at 14.00 and in group DD at 02.00 significantly exceeded the corresponding values in the control group.

Interrelations between the estimates of the total amount of the c-Fos-positive material calculated for different conditions of illumination and day periods were rather similar to those measured in the nuclei of separate mpcPVN neurons. In all three experimental groups, the value of this index at 14.00 significantly exceeded the respective values at 2.00; in groups LL and DD, the differences were more than twofold and threefold greater, respectively. The greatest total amount of c-Fos protein within the mpcPVN borders was observed during daytime in rats subjected to light deprivation. The "day" index of the integral content

of this protein in the mentioned group exceeded that observed also during daytime in the control group by more than two times.

DISCUSSION

Our experiments demonstrated that expression of the product of the early-response gene *c-fos*, i.e., c-Fos protein, in cells of the parvicellular subnucleus of the hypothalamic PVN undergoes considerable variations under conditions of both the normal photoperiod and experimental modifications of the latter. Changes in the c-Fos amount in the hypothalamic nucleus, which plays an important role in the formation of reactions to stressogenic factors (initiating changes in the level of corticoids), on the one hand, are rather similar to those observed in the circadian pacemaker (SChN) [10] and, on the other hand, demonstrate certain specificities. Such a pattern of c-Fos immunoreactivity in mpcPVN neurons is probably determined by the existence of rather intense (and illumination-modulated) effects on this nucleus coming from the SChN. The latter nucleus is the hypothalamic structure most directly involved in the organization of the circadian rhythmicity. As was mentioned, the PVN is one of the key structures in the hypothalamo-hypophyseal-adrenal system (HHAS), i.e., in the most important functional complex of structures responsible for general stress-reactivity of the organism.

Under conditions of the normal illumination mode (LD, 12/12 h), both absolute and relative areas occupied by the Fos-immunopositive product in the nuclei of mpcPVN neurons are somewhat greater during nighttime than during daytime. The "day" indices of concentration and amount of c-Fos in these neurons in the normal photoperiod, however, exceed considerably the respective "night" values. Such interrelations nearly repeat the respective dependences in SChN neurons [10]. At the same time, circadian variations of the c-Fos amount in mpcPVN neurons differ noticeably from those in suprachiasmatic neurons. In the mpcPVN, a rather typical effect observed in the SChN, namely smoothing of the circadian rhythmics of the c-Fos concentration and amount under conditions of continuous illumination (group LL), is nearly absent. The concentration and amount of this protein in the nuclei of mpcPVN neurons, when measured at 14.00, significantly exceeded the corresponding "night" indices. This was observed in all experimental groups and demonstrated no dependence of experimental modifications of the photoperiod (Fig. 2). The same can be said with respect to the integral estimate of c-Fos content within the borders of this hypothalamic subnucleus (Fig. 3B). Therefore, this peculiarity is sufficiently stable, and it is not influenced by changes in the photoperiod.

At the same time, indices of the concentration and content of c-Fos in mpcPVN neurons demonstrated practically the same peculiarity as was found in the SChN. These indices increased sharply during daytime in animals subjected to light deprivation (group DD). Interpretation of these effects, which are nearly parallel in the mpcPVN and SChN, meets certain difficulties. As was mentioned [10], the level of melatonin can be considered the most important factor influencing expression of the *c-fos* gene in these nuclei under conditions of the normal and artificially modified photoperiodicity. This hormone is produced by the epiphysis, and its level inversely depends on the level of illumination. In the case of the usual light periodicity, the level of melatonin reaches maximal values at the night, while long-lasting exposure of the animals to the conditions of continuous illumination results in more than a 15-fold drop in the respective index [13]. Under conditions of experimental induction of the epiphyseal hypofunction (LL, illumination stress), the expected effect (significant rise in the concentration and amount of the immunopositive product in neurons of the mentioned nuclei) was, however, practically absent. At the same time, induction of the epiphyseal hyperfunction under conditions of light deprivation resulted in very considerable (in fact, several-fold) rises in the concentration and, correspondingly the amount of c-Fos during daytime. Such an effect appears somewhat unexpected if we take into consideration the fact that ancestors of the laboratory rats were nocturnal or, in any case, "twilight" animals. Thus, it could be expected that light deprivation should play the role of a less powerful stressogenic factor than conditions of continuous illumination. At the same time, we actually observed in our experiments the opposite situation. In any case, it can be stated that the level of melatonin demonstrates no simple interdependence on the intensity of expression of the *c-fos* gene in the hypothalamic nuclei (in both the SChN, i.e., the circadian pacemaker, and in the mpcPVN, i.e., the HHAS component). This aspect probably needs further examination.

Some general increase (by 16-17%) of the number (spatial density) of c-Fos immunopositive neurons within the mpcPVN borders with any modifications of illumination conditions (Fig. 3A) is probably related to the fact that both continuous illumination and light

deprivation act as rather considerable stressogenic factors.

In our experiments, we observed in the mpcPVN a phenomenon quite comparable to that found in SChN cells. The geometrical dimensions of the nuclei of these cells did not maintain stable values but demonstrated noticeable circadian variations. In all three experimental groups, the mean areas of cross-sections of the nuclei of mpcPVN neurons were at 14.00 noticeably greater that those at 02.00, and this difference exceeded the significance level in groups LD and LL (Fig. 1A). "Circadian differences" between the areas of nuclear cross-sections in these groups (8.9 and 7.4%) correspond to the differences between volumes of these nuclei equal to about 10% and even more. Therefore, the dependence of geometrical dimensions of the nuclei of mpcPVN neurons on the circadian rhythm was not so intense as in SChN cells [10] but, nonetheless, was quite noticeable. This finding confirms the conclusion that the geometrical dimensions of nerve cells and of their compartments cannot be considered absolutely constant parameters (as was usually supposed, explicitly or implicitly, *a priori*). They undergo considerable dynamic changes under the action of certain factors of rather moderate (physiological) intensity. This was earlier demonstrated with respect to the cells of other hypothalamic nuclei in mammals [14] and medullary reticular neurons (Mauthner cells) in fishes [15]. The specific mechanisms of this phenomenon deserve special studies.

The comparison of immunohistochemical and morphometric characteristics of neurons of two hypothalamic nuclei examined under conditions of the normal photoperiod and artificial modifications of the latter shows that cerebral structures involved in organization of the circadian rhythm and realization of the stress reactions closely interact, on the one hand, with each other and, on the other hand, with the important neuroendocrine intermediator structure, the *epiphysis cerebri*. These structures are probably interconnected by not only direct functional connections but also by feedbacks. Probably, this situation is the reason for the rather complicated pattern of changes (sometimes, unexpected) observed in the mentioned CNS structures upon experimental modifications of the illumination mode.

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