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ORIGINAL ARTICLE

Disturbances of diurnal rhythms of biogenic amines contents in hypothalamic nuclei as an evidence of neurotropic effects of enterotropic carcinogen 1,2-dimethylhydrazine

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Abstract

OBJECTIVES: Our data on the contents of norepinephrine (NE), dopamine (DA) and the metabolite of serotonin 5-hydroxyindoleacetic acid (5-HIAA) measured in the suprachiasmatic nuclei (SCN), preoptic area (PA) and median eminence (ME) of hypothalamus of rats after single subcutaneous injection of 1,2-dimethylhydrazine (DMH) as well as the effect of this carcinogen on formation of reactive oxygen species (ROS) in the PA are presented in this paper. **RESULTS**: Diurnal changes of DA in all studied brain structures and of NE in the PA have been observed in the control group. Their morning levels were higher than evening ones. Rhythms of 5-HIAA in the SCN and diurnal changes of ROS formation have been shown to have contrary changes in control. Both the morning (11 a.m.) and evening (11 p.m.) subcutaneous administration of DMH at the dose of 21 mg/kg of body weight resulted in changes of all rhythms observed in control. In some cases a phase shift was found, in others the rhythms of neurotransmitters and ROS formation disappeared entirely. **CONCLUSION**: The data obtained confirm the idea of dopaminergic and serotoninergic systems taking part in mechanisms of a response of the hypothalamic nuclei to non-photic stimuli. It is suggested that the effect of DMH on the content and diurnal rhythms of neurotransmitters in the hypothalamic structures under study is due to its affecting activities of the enzymes of biogenic amines synthesis, synaptic transmission, melatonin synthesis and secretion rhythms. The change in ROS formation that is caused by administration of DMH is likely to be due to a disturbance of diurnal rhythms of neurotransmitters that are one of the sources of formation of free radicals in the brain.

Abbreviations

5-HIAA – 5-hydroxyindoleacetic acid
5-HT – 5-hydroxytryptamine
DA – dopamine
DMH – 1,2-dimethylhydrazine
ME – median eminence
N-AcHT – N-acetyl-5-hydroxytryptamine
NE – norepinephrine
PA – preoptic area
ROS – reactive oxygen species
SCN – suprachiasmatic nuclei

Introduction

Various physiological and biochemical processes in mammals undergo circadian changes controlled by the suprachiasmatic nuclei (SCN) of hypothalamus. The clock in the SCN oscillates with a near 24-hr period, and it is entrained (synchronized) by diurnal changes of lighting environment [1]. One of the organism functions regulated by the SCN is a circadian rhythm of melatonin synthesis and secretion in the pineal gland with elevated level of this hormone in the dark [2]. On the other hand, melatonin modulates endogenous rhythms in the hypothalamus acting via its receptors in the SCN [3]. It is suggested that melatonin takes part in synchronization of circadian rhythms in the SCN, when the regular entrainment by the solar day/night cycle is impossible, particularly in the prenatal and early postnatal period of development [4]. The role of the SCN in the regulation of gonadotropins secretion diurnal rhythms and in a preovulatory surge in their blood content has been established [5]. The monosynaptic pathway that connects neurons of the SCN with gonadoliberinergic neurons of the preopric area (PA) of hypothalamus has been found [6]. A multisynaptic pathway that connects the SCN and median eminence (ME) of hypothalamus secreting gonadoliberin into the portal blood system of the pituitary gland via dopaminergic neurons has also been proposed [7]. Diurnal changes of various indices can be observed in the PA and ME as well as in the SCN. In particular, diurnal changes in gonadoliberin and neurotransmitters controlling its secretion (norepinephrine – NE, dopamine – DA, and 5-hydroxytryptamine – 5-HT) in the PA as well as rhythms of DA and 5-HT content in the ME of female rats have been found by us earlier [8, 9].

Recently the connection between biological rhythms and carcinogenesis has been studied extensively, and disturbances of the rhythms at late stages of cancer development were investigated in most studies. A dependence of an effect of different carcinogens on administration time, which is connected with diurnal changes in the activity of various defense systems of an organism, has been established [10]. Numerous data on such variations in animals and humans during carcinogenesis have been collected. Amplitude damping, phase shifts and/or period change, including appearance of ultradian rhythms (with a period less than 20 hr), have been shown [11]. However, data about the influence of carcinogens on circadian changes in the SCN (circadian rhythms main pacemaker), PA and ME, which are connected with the SCN, are not available.

Information about the important role of the pineal gland and its hormone melatonin in tumor development draws still more attention. Pinealectomy triggers carcinogenesis, while the melatonin administration inhibits cancer development. Recent studies showed that melatonin inhibits carcinogenesis caused by 1,2-dimethylhydrazine (DMH) which is an agent that selectively and effectively causes colon cancer in rats [12].

Taking into account the facts mentioned above it seems reasonable to investigate the effect of the DMH administration on diurnal rhythms in hypothalamus and to evaluate a dependence of carcinogen effect on administration time.

The aim of the present study was to investigate the influence of the morning and evening administration of DMH on biogenic amines (NE and DA) and the 5-HT metabolite 5-hydroxyindoleacetic acid (5-HIAA) content in the SCN, PA and ME of female rats. The effect of DMH on nocturnal levels of melatonin and its precursors in the pineal gland and on the intensity of free radical processes in the PA was also studied.

Material and methods

The experiments were performed on 80 Wistar female rats (180–220 g of body weight). The animals were kept in an animal facility with cyclic changes of lighting environment (day – from 8 a.m. till 8 p.m.; darkness – from 8 p.m. till 8 a.m.) and were given a standard lab chow and tap water *ad libitum*. All rats under study were divided into 3 groups. The first group contained intact animals (control). Two other groups included animals exposed to single administration of the carcinogen in the morning (10 to 11 a.m.) or evening (10 to 11 p.m.). 1,2-dimethylhydrazine hydrochloride (Sigma, USA) was administered subcutaneously at a single dose of 21 mg (calculated as free base) per kg of body weight. DMH was dissolved *ex tempora* in 0.9% sodium chloride solution.

The control animals were decapitated in the morning (11 a.m.), evening (5 p.m.) and at night (1 a.m.). The experimental animals were decapitated in 24 hr (11 a.m.), 30 hr (5 p.m.) and 38 hr (1 a.m.) after morning administration of DMH and in 18 hr (5 p.m.), 26 hr

(1 a.m.) and 36 hr (11 a.m.) after evening administration of the carcinogen. The PA, ME and SCN were isolated from the brain of the decapitated rats, frozen in liquid nitrogen and kept at –70°C till the day of analysis. After thawing the brain structures were homogenized in 0.5 ml of 0.1 N perchloric acid containing 0.05% of potassium metabisulfite and centrifuged (10,000 g, 15 min, +4°C). The supernatant was transferred to the microfiltration unit and filtrated through a nylon filter (0.2 μm pore size). The filtrate was analyzed on that same day by high-performance liquid chromatography with electrochemical detection [13].

Biogenic amines and 5-HIAA in the hypothalamic structures were separated using a reversed phase Separon SGX C_{18} column (150 x 4 mm, 5 μm). The mobile phase contained 6 mM citrate buffer, 2 mM Na_2EDTA, 1.1 mM sodium octyl sulfonate and 7% acetonitrile (vol/vol). The flow rate was 0.75 ml/min. The working electrode oxidation potential of +0.65 V vs. Ag/AgCl reference electrode was used.

Indoleamines in the pineal gland were separated on a reversed phase Separon SGX C_{18} column (150 x 3 mm, 5 μm). The mobile phase contained 50 mM KH_2PO_4, 0.13 mM Na_2EDTA, 0.7 mM sodium dodecyl sulfate and 25% acetonitrile (vol/vol). The flow rate was 0.8 ml/min. The working electrode oxidation potential of +0.95 V vs. Ag/AgCl reference electrode was used.

The content of the biogenic amines and their metabolites in the hypothalamic structures was expressed as ng per mg of protein estimated by Lowry method [14]. The content of indoleamines was expressed as ng per pineal gland. To estimate formation of reactive oxygen species (ROS), the intensity of H_2O_2-luminol-dependent chemiluminescence was measured and expressed as relative unit per mg of protein [15].

Statistically significant differences were revealed by ANOVA method and Student *t*-test. One-way ANOVA method was used for determination of diurnal rhythm significance when overall comparison of several independent groups was needed. If the presence of statistically significant difference between the data obtained at 3 time points during the 24 hr was confirmed by one-way ANOVA method, Student *t*-test was used to compare maximal and minimal values. The effects of DMH on the levels of biogenic amines and 5-HIAA at different times during the day were analyzed by two-way ANOVA method. All results are expressed as means ± SD. Differences were considered as significant when the level of significance (p) was less than 0.05.

Results

Diurnal rhythms of the NE, DA and 5-HIAA content in the SCN, PA and ME

Diurnal changes of biogenic amines in all studied hypothalamic structures were found in the control group. Levels of biogenic amines were higher in the morning than in the evening and night (Figure 1). According to the result of the one-way ANOVA method, significant diurnal changes were found for

Table I. Statistical significance of diurnal changes of catecholamines and 5-HIAA content in the SCN, PA and ME of rats. DMH-M and DMH-E - morning and evening administrations of DMH, DMH-E/Control - the effect of evening administration of carcinogen, DMH-M/DMH-E - a comparison of morning and evening administrations of carcinogen.

		SCN		PA		ME	
		F-ratio	p-value	F-ratio	p-value	F-ratio	p-value
Diurnal rhythm (oneway ANOVA method)							
Control	NE			3.69	**< 0.05**		
	5-HIAA	3.73	**< 0.05**				
	DA	4.40	**< 0.05**	4.36	**< 0.05**	5.16	**< 0.02**
DMH - M	NE					7.03	**< 0.005**
	5-HIAA	7.24	**< 0.005**				
	DA	7.44	**< 0.005**				
DMH - E	NE	23.09	**< 0.00001**			4.12	**< 0.05**
	5-HIAA	11.74	**< 0.0005**	4.28	**< 0.05**		
	DA						
Effect of DMH (two-way ANOVA method)							
DMH - E/ Control	NE	7.97	**< 0.01**	8.93	**< 0.005**	11.34	**< 0.002**
	5-HIAA			3.90	**< 0.05**		
	DA	8.95	**< 0.005**				
DMH - M/ DMH -E	NE	10.34	**< 0.005**	18.78	**< 0.0005**	6.85	**< 0.05**
	5-HIAA	4.16	**< 0.05**				
	DA	9.55	**< 0.005**			5.52	**< 0.05**

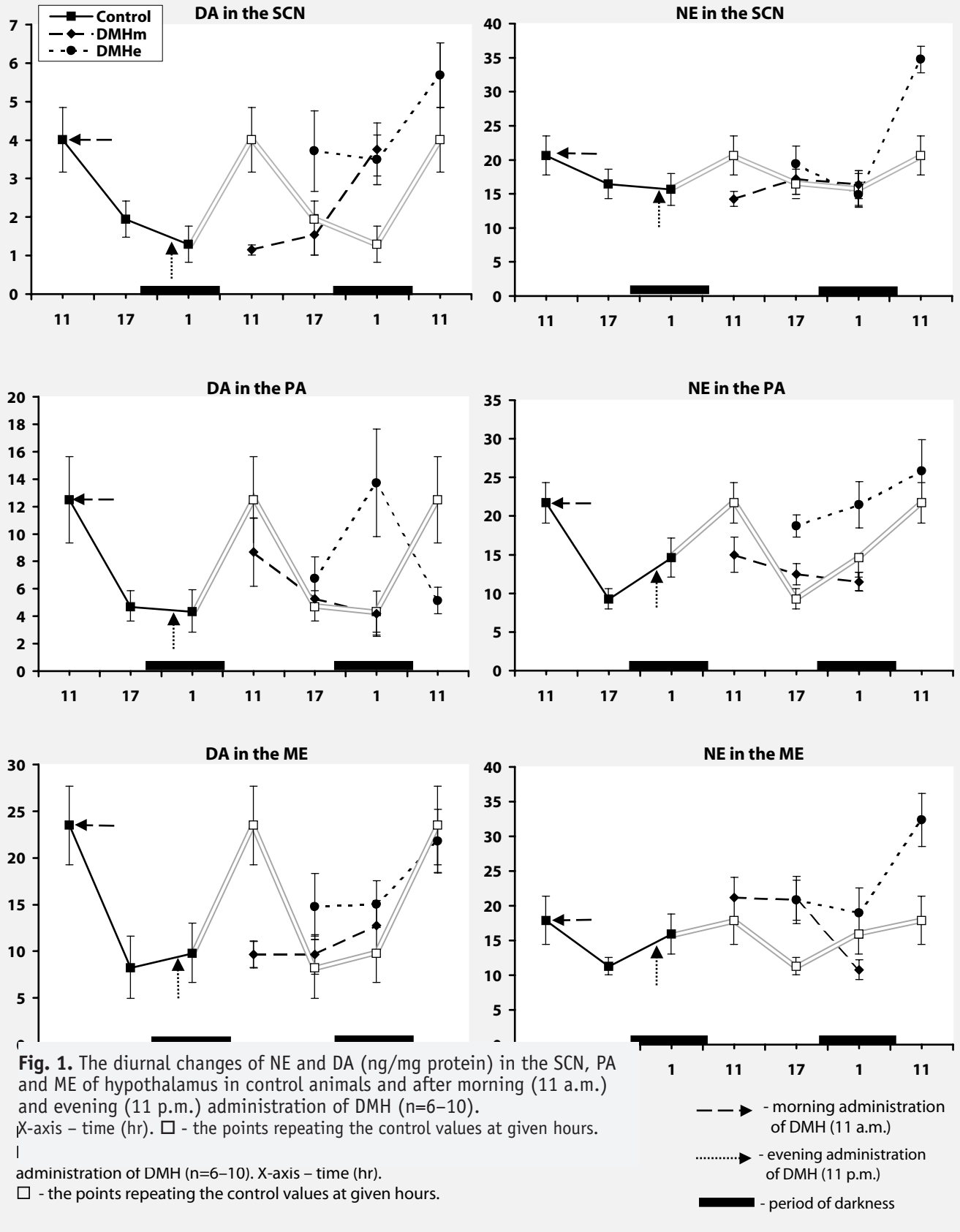

Fig. 1. The diurnal changes of NE and DA (ng/mg protein) in the SCN, PA and ME of hypothalamus in control animals and after morning (11 a.m.) and evening (11 p.m.) administration of DMH (n=6–10). X-axis – time (hr). ☐ - the points repeating the control values at given hours.

administration of DMH (n=6–10). X-axis – time (hr).
☐ - the points repeating the control values at given hours.

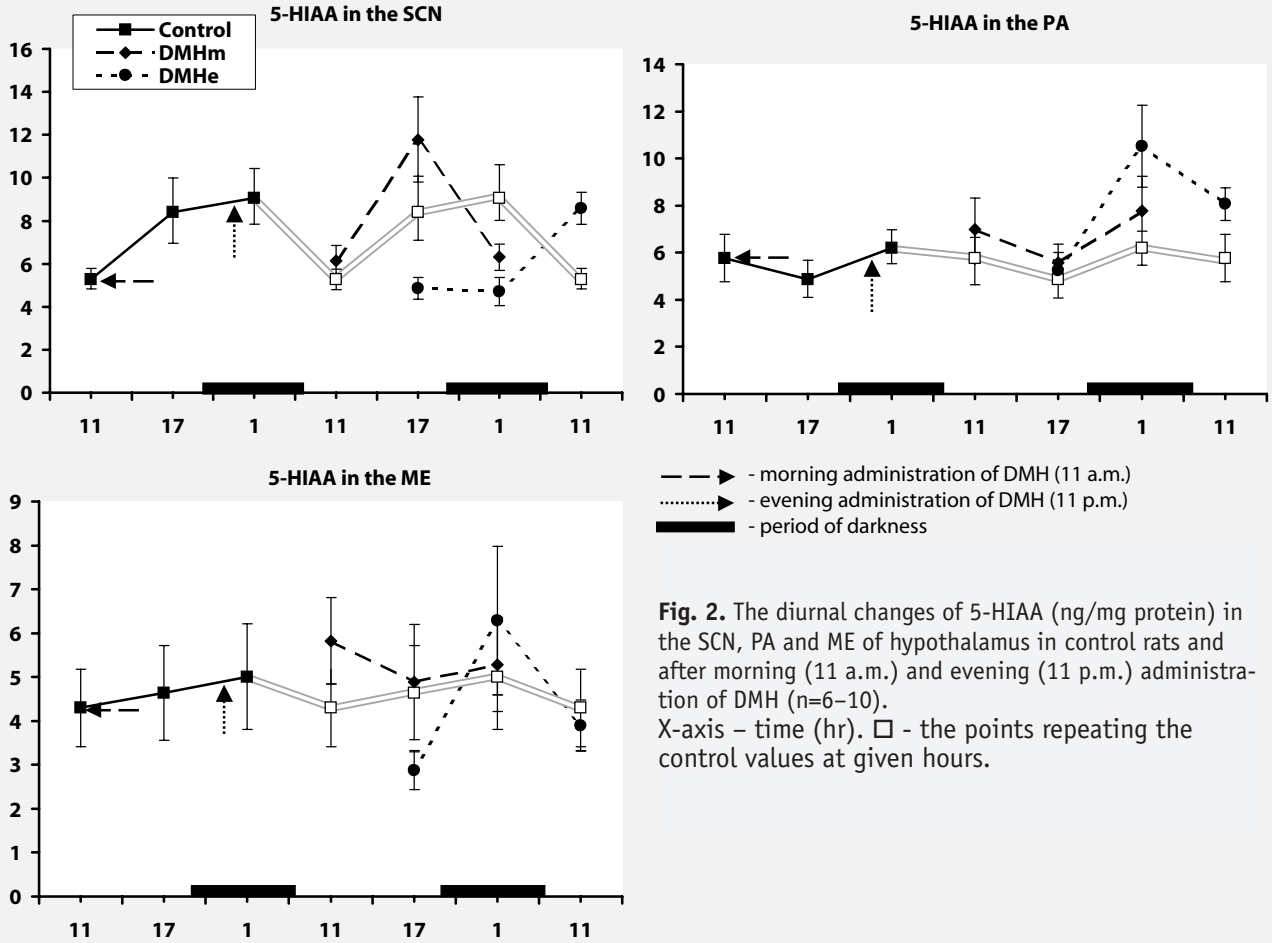

Fig. 2. The diurnal changes of 5-HIAA (ng/mg protein) in the SCN, PA and ME of hypothalamus in control rats and after morning (11 a.m.) and evening (11 p.m.) administration of DMH (n=6–10).
X-axis – time (hr). □ - the points repeating the control values at given hours.

NE in the PA and for DA in all studied brain regions (Table1). The significance was also confirmed by a comparison of maximal and minimal values by post-hoc Student *t*-test. The morning level of NE in the PA was significantly different from its evening level only ($p<0.001$, *t*-test). The morning level of DA in the PA and ME was significantly higher than its evening and night levels ($p<0.05$, *t*-test). In the SCN a significant decrease in the DA content in comparison with its morning level was observed at night hours only ($p<0.05$, *t*-test). The diurnal changes in the 5-HIAA content were observed in the SCN only, where the nocturnal level of this metabolite was significantly higher than its morning value ($p<0.05$, *t*-test) (Figure 2).

The effect of administration of DMH

a) Disturbances of diurnal rhythms

Morning administration of DMH eliminated daily changes in the contents of NE in the PA, of DA in the PA and ME ($p>0.05$, one-way ANOVA). Significant differences from the control values have been observed only in the morning DA level in the ME ($p<0.01$, t-test) (Figure 1). Evening administration of DMH disturbed diurnal rhythms of NE in the PA and of DA in the SCN and ME ($p>0.05$, one-way ANOVA). In the PA, evening admin istration of DMH caused a significant ($p<0.001$, t-test) increase in the evening NE level, when compared to control. In the SCN, evening administration of the carcinogen increased the nocturnal level of DA, when compared to control ($p<0.05$, t-test).

b) An appearance of new variations

According to results of the one-way ANOVA method, morning administration of DMH caused variations in the NE content in the ME that were not observed in the control (Table1). A significant decrease in this neurotransmitter level at night hours, when compared to its morning ($p<0.05$, *t*-test) and evening levels ($p<0.01$, *t*-test), has been observed. It should be noted that in this group the evening content of NE in the ME was significantly higher than the control value ($p<0.05$, *t*-test). Evening administration of DMH resulted in an appearance of significant variations in the NE content in the SCN and ME that were not observed in control (Table1). The morning level of this catecholamine in the above brain structures was

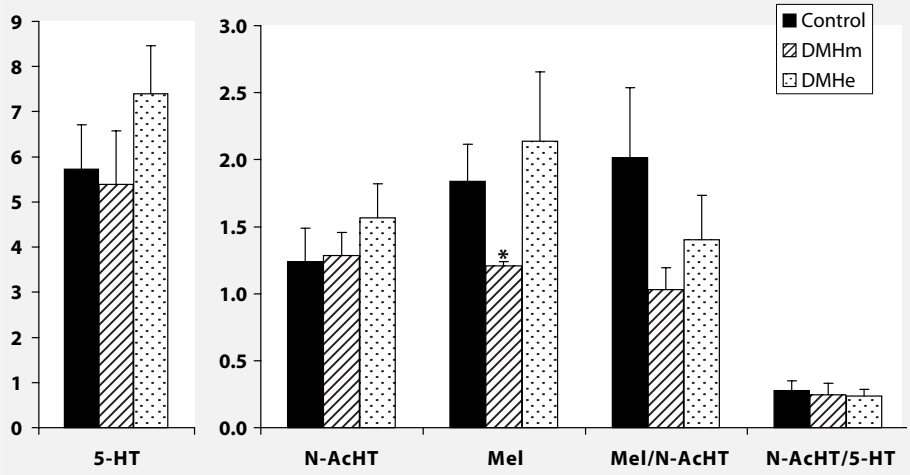

Fig. 3. The contents of melatonin (Mel) and its precursors 5-HT and N-AcHT (ng/pineal gland), and the N-AcHT/5-HT and melatonin/5-HT ratios in the pineal gland in control and after morning (11 a.m.) and evening (11 p.m.) administration of DMH (n=5–8).
X-axis – time (hr). * - significant differences (p<0,05) between experimental and control values.

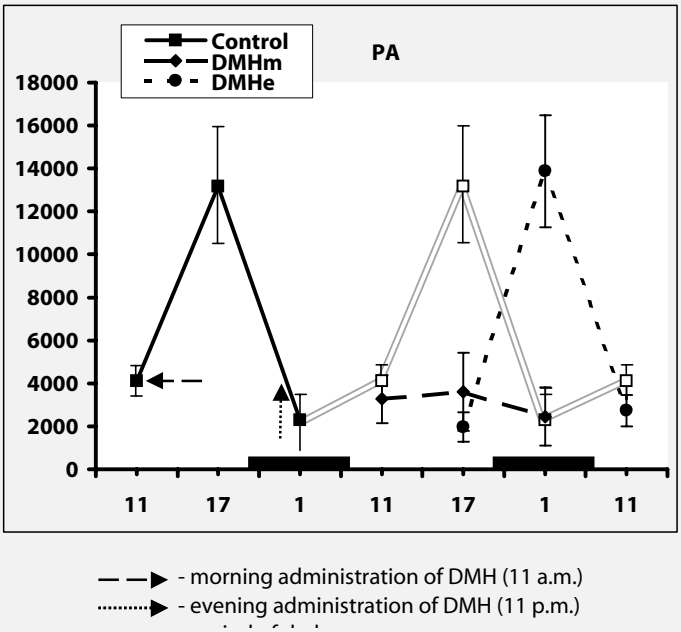

Fig. 4. Luminol-dependent chemiluminescence level (relative units/mg protein) in the PA of hypothalamus at various time in control and after morning (11 a.m.) and evening (11 p.m.) administration of DMH (n=5–6).
X-axis – time (hr). □ - the points repeating the control values at given hours.

significantly increased, when compared to its evening and night levels in the same group and to its morning level in the control group ($p<0.001$, *t*-test for SCN and $p<0.05$, *t*-test for ME). Also, the effect of evening administration of DMH was in a considerable increase in the evening NE level in the ME ($p<0.05$, *t*-test), when compared to the control values. Evening administration of DMH caused an occurrence of corresponding changes in the 5-HIAA level in the PA that were not observed in control (Table1), the nocturnal content of the metabolite being significantly higher than the evening one ($p<0.05$, *t*-test) (Figure 2). Besides, nocturnal level of 5-HIAA was increased, when compared to the control value at the same night hours ($p<0.05$, *t*-test).

c) A phase shift of diurnal rhythms

The diurnal rhythms of DA in the SCN after morning administration of DMH and of 5-HIAA in the same brain structure after evening administration

of the carcinogen were still significant (Table 1) but opposite to those in control. Thus, after morning administration of DMH the DA nocturnal content in the SCN exceeded considerably its morning level ($p<0.01$, *t*-test) (Figure 1). Besides, the DA morning level was significantly lower ($p<0.01$, *t*-test) than the control one, and its nocturnal level was significantly higher ($p<0.05$, *t*-test) than that in control. Evening administration of the carcinogen resulted in an increase in the 5-HIAA content in the SCN at morning hours, when compared to the control values for evening and night hours ($p<0.01$, *t*-test) (Figure 2). Meanwhile, the evening and night levels of this metabolite were significantly decreased ($p<0.05$, *t*-test), whereas the morning one was increased ($p<0.01$, *t*-test), when compared to control.

The analysis by two-way ANOVA method revealed no effect of morning administration of DMH on the DA content in the SCN and of evening administration of the carcinogen on the 5-HIAA level in the same brain structure ($p>0.05$, two-way ANOVA). A conclusion can be drawn that the above mentioned administrations of DMH cause no significant changes in diurnal rhythms of DA and 5-HIAA in the SCN, but only a phase shift can be observed.

Also, the diurnal rhythm of DA in the PA was not changed significantly after evening administration of DMH. Meanwhile, in the PA a morning decrease and night increase in this catecholamine level were observed, when compared to control ($p<0.05$, *t*-test). The analysis by two-way ANOVA method revealed no significant differences in the diurnal DA content in the PA in control and after evening administration of the carcinogen ($p>0.05$). Therefore, it is possible that in this case we have had a phase shift of the diurnal rhythm.

A comparison of the effects of morning and evening administrations of the carcinogen

According to the results of two-way ANOVA method, the evening administration of the carcinogen significantly increased the total NE levels in all studied brain structures as well as the total DA content in the SCN and the total 5-HIAA content in the PA (Table 1). However, this approach failed to show a significance of the effect of morning administration of DMH on contents of these compounds in the brain structures studied. The statistical method used revealed a significant increase in the NE and DA contents in the SCN and PA and in the NE content in the ME as well as a significant decrease in the 5-HIAA content in the SCN after evening administration of the carcinogen, when compared to the data obtained for the groups with morning administration of the carcinogen (Table 1).

The content of melatonin and its precursors in the pineal gland after administration of DMH

The contents of melatonin and its precursors 5-HT and N-acetyl-5-hydroxytryptamine (N-AcHT) in the pineal gland at night as well as the ratios N-AcHT/5-HT and melatonin/N-AcHT in 26 hr after evening administration of DMH do not change significantly, when compared to control (Figure 3). However, the nocturnal melatonin level measured in 36 hr after morning administration of the carcinogen was significantly decreased, the ratio melatonin/N-AcHT also had a tendency towards a decrease, whereas the contents of 5-HT and N-AcHT and the ratio N-AcHT/5-HT in this group were not different from the control values.

Diurnal changes of ROS in the PA

In the PA, apart from diurnal rhythms of biogenic amines, we have found a diurnal rhythm of formation of ROS ($p<0.005$, $F=11.38$, one-way ANOVA). As can be seen from the data presented (Figure 4), the evening level of ROS formation in the PA in control exceeded significantly the corresponding values at the morning and night hours ($p<0.01$, *t*-test). Morning administration of DMH decreased significantly ($p<0.05$, *t*-test) the evening level of ROS formation down to the morning and night values. After evening administration of DMH diurnal rhythms of ROS formation were still significant ($p<0.0005$, $F=14.29$, one-way ANOVA). However, a peak of ROS formation shifted towards night hours. It should be noted that the diurnal rhythm of ROS formation in the PA and its alteration under the influence of DMH are opposite to those of DA in the same brain structure (Figure 1).

Discussion

We have found that the DMH administration causes significant changes of studied diurnal rhythms in SCN, PA and ME. This effect of DMH can be a consequence of either its direct influence on the hypothalamic structures or its indirect influence on nervous and endocrine mechanisms regulating levels of neurotransmitters and their diurnal rhythms in studied brain regions. In previous experiments it was shown that DMH possesses an antigonadotropic effect that can be due to the influence of the carcinogen on gonads directly or due to the effect on the hypothalamo-pituitary mechanism of reproduction regulation [16, 17]. It has been established that the level of specific radioactivity in testicles and the pituitary gland in 3 hr after administration of [^{3}H]-DMH to male rats exceeded significantly that in the liver, the organ that metabolizes the carcinogen. This fact allows us to suggest a direct effect of the carcinogen on the

gonads. The [^{3}H]-labelling of the hypothalamus was significantly higher than that of the brain stem and cortex, which indicates a selective effect of DMH on this brain structure [16]. The data about the effect of DMH on the biogenic amines contents in the whole hypothalamus are of special interest [17]. The NE level in the hypothalamus in 30 min after administration of the carcinogen to male rats has been shown to be decreased by 37% and remain low during 24 hr. The DA level was also decreased significantly (by 68%) but only in 24 hr after administration of DMH. It has been suggested that DMH selectively inhibits the activity of the catecholamines biosynthesis enzymes, 3,4-dihydroxyphenylalanine (DOPA) decarboxylase and dopamine β-hydroxilase, thus preventing formation of DA from DOPA and then the synthesis of NE from DA. In our experiments in one day after morning administration of DMH we also observed a decrease in levels of DA in the SCN and ME. Besides, the level of the neurotransmitters measured in 26 hr after evening administration of the carcinogen was either the same as the control value or higher than that (DA in the PA and SCN). Such a difference in the effects of morning and evening administration of DMH cannot be accounted for by inhibition of the biosynthesis of DA and NE in the hypothalamic structures. Rather, the influence of the carcinogen on diurnal rhythms of the synthesis of these neurotransmitters or availability of these rhythms of sensitivity of the neurotransmitter systems in the studied hypothalamic structures to the influence of DMH can be supposed.

This has also been confirmed by the difference in the effects of morning and evening administrations of the carcinogen that was revealed by the two-way ANOVA method. Whereas morning administration of DMH caused no significant changes in the mean diurnal contents of the compounds monitored, changing only the mode of diurnal dynamics of their levels, evening administration of the carcinogen in a number of cases resulted in an increase in their total contents. Neurons of the SCN have been shown to have diurnal rhythms of sensitivity to various stimuli, particularly to administration of melatonin. Maximal sensitivity was found at a late subjective day and early subjective night [3]. Therefore, the observed increase in total level of neurotransmitters after evening administration of DMH and the lack of this effect after morning administration of the carcinogen are likely to be connected with the increased sensitivity of the neurons producing these neurotransmitters to the carcinogen effect at the evening hours.

DMH has been shown to inhibit the excitatory signal transduction in sympathetic ganglia of rats at 30 min after its administration [18]. The authors believe that the effect of DMH can be either related to a direct inhibition of cholinergic transduction or due to the excitatory effect of the carcinogen (like catecholamines) on α-adrenoreceptors. DMH can be suggested to influence the signal transduction within the CNS, and particularly within hypothalamus as a main target for this agent in the brain. When the SCN are deinnerved and contacts between neurons inside the nuclei are broken, the endogenous circadian rhythms are partially kept, but their phase relationships are disturbed [4]. Therefore, the changes in the neurotransmitters and 5-HIAA contents in the SCN caused by DMH could be induced by the disturbance of the signal transduction in afferent fibers innervating these hypothalamic nuclei as well as between their neurons. The changes in the neurotransmitters levels observed in the PA and ME could be a consequence of the disturbances found in the SCN, which the former structures are connected with, or due to the direct effect of DMH on these structures.

It has been found that, besides the light, various non-photic stimuli can modulate circadian changes in the SCN [1]. Non-photic stimuli can synchronize, phase shift or change the period of circadian rhythms [19]. The phase shifts of the circadian rhythms of neuronal activity in the SCN and motor activity are caused also by the administration of some pharmacological agents, such as the benzodiazepine triazolam or 5-HT agonists, to animals [1]. The serotoninergic system is suggested to take part in the mechanisms of the response of the SCN to non-photic stimuli [20]. The dopaminergic system is likely also to be involved in these processes as it is shown by the data about 12 hr phase shift in the daily rhythms of the 5-HT and 5-HIAA contents in the SCN after administration of the DA agonist bromocriptine [21]. According to our data, the possibility of the mechanisms providing the response of the SCN to non-photic stimuli being involved in the phase shift in the diurnal rhythms of DA and 5-HIAA in these nuclei cannot be ruled out.

An activation of sympathetic neurons is known to be the main stimulus increasing the synthesis and secretion of the pineal hormone melatonin at night hours. The decrease in the melatonin content in the pineal gland after morning administration of DMH could be a consequence of a disturbance of the sympathetic innervation of the pineal gland as well as of a decrease in the activity of enzymes of the synthesis of this pineal hormone under the influence of this carcinogen.

The diurnal changes in ROS formation in the PA observed in the control rats are probably related to diurnal changes in neurotransmitters in this hypothalamic structure that is rich in catecholaminergic synapses. This suggestion is strengthened by the data

that biogenic amines, DA in particular, undergoing autooxidation or enzymatic oxidation by monoamine oxidase (MAO), can be a source of ROS formation (mainly H_2O_2) in the brain [22]. The activity of MAO was shown to undergo diurnal changes in various brain structures including hypothalamus [23] Since the diurnal changes in biogenic amines and ROS formation in the PA have been shown in our experiments to be in a counterphase, it can be suggested that the increase in free radical activity in the evening is due to metabolism of DA and NE being enhanced by MAO in the light period, which results in a simultaneous decrease in neurotransmitters and increase in formation of free radical products of their oxidation.

The data obtained indicate that DMH causes the different changes of diurnal rhythms of the biogenic amines contents in various hypothalamic structures of rat depending on the time of cancerogen administration (morning or evening hours). This fact allows supposition that the neurotropic effects of DMH are due to diurnal changes in the sensitivity of the organism to the influence of xenobiotics.

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