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Effects of Melatonin on the Behavioral Indices and Structural Characteristics of Cerebral and Spinal Neurons of Rats with Experimental Hemiparkinsonism

I. F. Labunets,[1] Yu. B. Chaikovskii,[1,2] S. I. Savos'ko,[2] G. M. Butenko,[1] V. F. Sagach,[3] and B. S. Kop'yak[3]

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In adult Wistar rats, a model of experimental hemiparkinsonism (EHP) was induced by stereotaxic microinjection of 8.0 μg 6-hydroxydopamine (6-HODA) into the left forebrain lateral ascending bundle. In such animals, we studied behavioral indices in the open field and elevated plus maze tests and also structural changes in the cerebral *substantia nigra* and spinal L5 ventral horn neurons. Six groups of animals were formed (in each group, $n = 8$): intact rats (I), animals that obtained microinjections of saline into the above lateral bundle (sham-injected, ShI), two groups with weak and intense motor EHP manifestations (frequency of circulatory movements in the apomorphine test less than one and six and more per 1 min, groups $HP_{<1}$ and $HP_{\geq 6}$, respectively), and two groups of rats with weak and intense EHP manifestations, which obtained daily i.p. injections of 10 mg/kg melatonin during 18 days (groups $HP_{<1}$+M and $HP_{\geq 6}$+M). In the open-field test, we observed in groups $HP_{<1}$ and $HP_{\geq 6}$ significant suppression of horizontal motor activity (very intense in the latter of the above-mentioned groups) and also significant weakening of vertical activity (number of rearings). Changes in the number of defecation acts were less expressed. In the elevated plus maze, manifestations of a significantly increased anxiety level were observed in rats of the above two groups. In the *substantia nigra* of animals with EHP, a considerable part of the neurons demonstrated clear signs of negative structural modifications; the number of neurons with the normal structure was considerably smaller than in the control. Analogous negative morphological changes were found in the lumbar segment of the spinal cord. Course injections of melatonin into rats with EHP noticeable weakened negative shifts in both open-field behavioral indices and level of anxiety (according to estimates in the elevated plus maze). Under the action of melatonin, morphological characteristics of neurons and gliocytes in the *substantia nigra* and spinal cord of rats with EHP demonstrated appreciable normalization. The mechanisms underlying functional and structural disorders in the CNS of animals after central microinjections of the 6-HODA neurotoxin and also neuroprotective effects of melatonin, which provide weakening of such disorders, are discussed.

Keywords: parkinsonism, 6-hydroxydopamine (6 HODA), melatonin, behavioral indices, *substantia nigra*, lumbar spinal cord, neurodegeneration.

INTRODUCTION

Parkinson's disease (PD) is a chronic progressive neurodegenerative disorder, in which massive loss of dopaminergic neurons in the *zona compacta* of the midbrain *substantia nigra* leads to an insufficiency of dopamine in the neostriatum and to the development of a classical complex of motor disorders ((hypokinesia, rigidity, and tremor) [1, 2]. Among possible reasons for degenerative modifications of nigral neurons in parkinsonism, oxidative stress, development of neuroinflammation, disorders in the biochemical pathways of degradation of proteins, glutamatergic excitotoxicity, etc., have been mentioned [3]. Thus, the pathogenesis of this disease is most likely multifactorial.

According to clinical observations in PD, non-motor symptoms (autonomic, sensory, and cognitive abnormalities, depression, sleep disturbance, etc.) are also typical of PD together with motor disorders. Non-motor symptoms can be manifested even earlier that motor dysfunctions; they are usually intensified

[1] Institute of Genetic and Regenerative Medicine, National Academy of Medical Sciences of Ukraine, Kyiv, Ukraine.
[2] Bogomolets National Medical University, Ministry of Public Health of Ukraine, Kyiv, Ukraine.
Correspondence should be addressed to
I. F. Labunets (e-mail: irina_labunets@ukr.net) and
Yu. B. Chaikovskii (e-mail: yuchaiko@i.ua).

as the disease progresses and can aggravate its clinical time course [2]. Such non-motor symptoms are considered to be related to the development of degenerative changes in different CNS parts (brainstem nuclei, thalamus, hippocampus, neocortex, olfactory bulb, etc.) [4]. In PD, changes in the spinal cord also deserve special interest; these changes can be involved in the formation of not only motor but also non-motor disorders related to this pathology.

Modern approaches in the therapy of PD involve application of complexes of the agents, which influence different pathogenetic links of this disease, namely the insufficiency of dopamine in the brain, disturbances of metabolism of this catecholamine, its imbalance with respect to other neuromediators, mitochondrial dysfunction, oxidative stress, etc. [2]. As is at present believed, introduction of neurotrophic factors, using approaches directed toward activation of endogenous synthesis of these factors, and also cellular therapy, in particular transplantation of dopaminergic neurons [6], can be promising in the treatment of this disease.

Among medicaments providing neuroprotective effects in parkinsonism, special attention is focused on the epiphyseal hormone melatonin. This agent is capable of simultaneously influencing a few pathogenetic factors, such as oxidative stress in the CNS, mitochondrial dysfunction, imbalance of neuromediators, and suppressed synthesis of neurotrophic factors; the properties of neural stem cells (NSCs) may also been modified under its action [7, 8]. It is clear that melatonin exerts a regulatory effect on the biorhythms of the organism, in particular on the sleep–wakefulness cycle; a disturbance of the latter is observed at the pre-clinical PD stage [2, 9]. As was reported, the biorhythms of the synthesis of this hormone are modified in this disease [10, 11].

The effects of melatonin on motor disorders under conditions of experimental parkinsonism may appear even more effective than those of the main remedy in PD, L-DOPA [12]. At the same time, information on the influence of this hormone on non-motor disorders in PD, which are, as a rule, resistant to therapy with levodopa preparations, is rather limited [2].

We studied changes in the behavioral indices and their possible relation to structural modifications in the *substantia nigra* of the brain and in the lumbar part of the spinal cord under conditions of a model of experimental hemiparkinsonism (EHP) in rats. We also tried to estimate the efficiency of course introductions of melatonin with respect to the corresponding pathological shifts.

METHODS

Experiments were carried out on male rats Wistar (3 to 4 months old) weighing 240 to 250 g; the animals were obtained from a nursery farm at the Bogomolets Institute of Physiology. Rats were kept under standard vivarium conditions with a natural illumination mode and free access to food and water.

To create the state of EHP in rats of the experimental groups (of a general group of rats with hemiparkinsonism, HP), animals were stereotaxically injected into the left forebrain lateral ascending bundle with 8.0 μg 6-hydroxydopamine (6-HODA; Sigma, USA) in 4.0 μl of the solvent (saline with 0.1% ascorbic acid added as a stabilizer that inhibits neurotoxin oxidation) [13, 14]. All corresponding manipulations were performed under barbiturate anesthesia (50 mg/kg Nembutal; Sigma, USA, i.p.). Rats with sham introductions (ShI) obtained stereotaxic injections into the analogous cerebral locus of 4.0 μl of the above-mentioned solvent with no neurotoxin. One more group was formed from intact animals (group I).

One week after stereotaxic microinjection of 6-HODA, rats of the general HP group were subjected to test i.p. injections of an agonist of dopamine receptors, apomorphine (0.5 mg/kg; Sigma, USA). Then, the intensity of circulatory movements (number of body rotations per 1 min) to the side contralateral to the hemisphere where the neurotoxin was injected was estimated. As was demonstrated earlier, the intensity of such movements correlates with the level of unilateral degeneration of dopaminergic nigral neurons [13]. Among experimental rats, there were individuals with different, low and high, intensities of circulatory movements induced by apomorphine (less than one rotation vs. six or more rotations per 1 min). Taking into account these finding, two animal groups ($HP_{<1}$ та $HP_{\geq 6,}$ respectively) were formed from the general HP group.

Beginning from the 5th day after the apomorphine test, parts of the rats of the above-mentioned general HP group were i.p. injected either with melatonin (Sigma, USA; 10 mg/kg, daily, for 18 days, injections at 18.00) or with pure solvent of this hormone using the analogous protocol. As a

solvent, we used saline with the addition of ascorbic acid [15, 16]. Finally, taking into account all the above-described procedures, six animal groups (n = 8 in each) were formed: group 1 (intact), group 2, ShI (control sham-injected), group 3, $HP_{<1}$ (rats after injection of 6-HODA with weak manifestations of HP according to the results of the apomorphine test), group 4, $HP_{\geq 6}$ (rats with high-intensity HP manifestations, i.e., a high frequency of circulatory movements in the above test), group 5, $HP_{<1}$+M (rats with limited HP manifestations injected with melatonin), and group 6, $HP_{\geq 6}$+M (rats with high-intensity HP manifestations, which also obtained course injections of melatonin). Animals of groups 3 and 4 ($HP_{<1}$ and $HP_{\geq 6}$) were also subjected to i.p. course injections but only with the melatonin solvent and no melatonin.

Behavioral phenomena in rats of all groups were estimated in the course of testing in the open field and elevated plus maze [17, 18]. In the open-field test, we used the standard technique; the observation period lasted 3 min. Within this period, the index of horizontal motor activity (HMA, number of crossed squares of the arena), index of vertical motor activity (VMA, number of rearings with support on the wall and without it), and also the number of acts of defecation within the observation period (index that correlates with the level of emotionality) were measured.

In the elevated plus maze test, we estimated the level of anxiety in experimental animals. The rats were placed on the central platform of the maze, and times (sec) spent in the open arms, closed arms, and on the central open platform of the maze were measured within the 5-min-long observation period. The numbers of rearings and looking downward from the maze arms were also calculated.

Structural modifications in the midbrain *substantia nigra* and the lumbar part of the spinal cord of rats of all groups were examined after cessation of course injections of melatonin or its solvent. Samples for morphological examination were taken after transcardial perfusion/fixation of the animals by 4% solution of paraformaldehyde in 0.1 M phosphate buffer (pH 7.4). Then, the midbrain block and spinal segment L5 were excised and additionally fixed in 10% neutral formalin. After standard dehydration in alcohols of rising concentrations, 8- to 10-μm-thick histological slices were prepared and stained with toluidine blue using the Nissl technique. The borders of *substantia nigra* were identified according to the stereotaxic atlas [19]. In 10 fields of view of histological preparations, proportions of visually undamaged neurons in the *substantia nigra* and spinal L5 lamina 9, by Rexed, were calculated. The cells with clear signs of cytolysis, hydropic dystrophy, karyolysis, and karyopyknosis were considered to be impaired. Photomicrographs of the examined parts of the brain and spinal cord were obtained using a microscope, Olympus BX 51 (Japan).

Morphometric examination of the *substantia nigra* and spinal cord slices included measurements of mean values of the areas (μm^2) of cross-sections of the cytoplasm and nuclei of the neurons; for such measurements, we used the images of only those units whose cross-section passed via the center of the nucleus. Then, according to these values, the nucleo-cytoplasmic index (NCI) was calculated. Morphometric analysis was performed using Carl Zeiss software (AxioVision SE64 Rel.4.9.1).

For statistical analysis of the numerical data, MS Excel was used. The significance of differences in intergroup comparisons was estimated using the Student's t- and Mann–Whitney U-tests [20].

RESULTS

Estimation of the Intensity of Changes in the Measured Indices Related to the Action of 6-HODA. As we found, the behavioral indices of rats in the tests used and morphometric parameters of neurons of the *substantia nigra* and spinal cord in groups 1 and 2 (i.e., in intact animals and in ShI rats subjected to microinjections of saline into the lateral ascending bundle) in most cases differed from each other insignificantly or even were practically identical. Thus, the procedure of stereotaxic microinjections *per se* did not induce significant consequences (changes in the measured indices). This allowed us to combine groups 1 and 2. All the measured values in these two control groups were averaged; the obtained data were taken as 100% in calculations of the normalized values in four groups HP and HP+M.

Behavioral Indices of Rats with EHP in the Open Field Test. The development of the EHP state significantly changed the behavioral indices demonstrated by rats in the above test [14] (Table 1). In rats with weak EHP manifestations (group 3, $HP_{<1}$), the intensity of locomotor activity (mean number of crossed squares of the arena) was, on average, 40% of that in the control groups

T a b l e 1. Behavioral Indices in Rats of the Experimental Groups in the Open Field Test

Experimental groups	Indices			
	HMA, number of crossed squares	VMA, rearings with support	VMA, rearings without support	number of acts of defecation
Intact (I)	78 ± 16	7.5 ± 0.8	3.4 ± 1.0	1.9 ± 0.6
Sham-injected (ShI)	72 ± 12	8.3 ± 1.1	4.3 ± 1.0	2.7 ± 0.6
Mean value for groups I and ShI	**75 (100%)**	**7.9 (100%)**	**3.85 (100%)**	**2.3 (100%)**
$HP_{<1}$	30 ± 11 ** (40 ± 15%)	6.3 ± 2.8 (80 ± 35%)	1.1 ± 0.3 ** (28 ± 8%)	1.4 ± 0.4 (61 ± 17%)
$HP_{\geq 6}$	4.0 ± 0.8 *** (5 ± 1%)	1.4 ± 0.6 ** (18 ± 7%)	0.4 ± 0.1 *** (10 ± 2%)	2.0 ± 0.6 (87 ± 26%)
$HP_{<1}$+M	29 ± 9 ** (39 ± 12%)	5.9 ± 1.1 * (75 ± 14%)	1.0 ± 0.2 ** (26 ± 5%)	1.8 ± 0.5 (78 ± 22%)
$HP_{\geq 6}$+M	10.6 ± 1.2 ***+ (14 ± 2%)	1.4 ± 0.4 *** (18 ± 5%)	0.6 ± 0.2 *** (16 ± 5%)	1.7 ± 0.9 (74 ± 40%)

Footnotes. HP) Hemiparkinsonism; one, two, and three asterisks show cases of significant differences in comparison with the control groups with $P < 0.05$, $P < 0.01$, and $P < 0.001$; crosses show the respective differences in comparisons of groups HP and HP+M. For detailed explanations, see Methods.

($P < 0.01$). In rats with intense manifestations of such pathology (group 4), suppression of locomotor activity was dramatic (20 times; $P < 0.001$). The number of rearings with support in group $HP_{<1}$ was somewhat smaller with respect to the control, but the difference was insignificant. In group 4 ($HP_{\geq 6}$), this index demonstrated more than a fourfold decrease ($P < 0.01$). Suppression of such manifestations of orientational activity as rearings without support, in groups $HP_{<1}$ and $HP_{\geq 6}$ was more intense; it was, respectively, about fourfold ($P < 0.01$) and by an order of magnitude ($P < 0.001$). The number of acts of defecation in the two above-mentioned groups within the observation periods was smaller than in the two control ones; however, the rather low frequency of such acts and the high variability of this index did not allow us to formulate concrete conclusions with respect to this behavioral component.

Course injections of melatonin did not change significantly the intensity of locomotion in group $HP_{<1}$+M, as compared with the respective index in group $HP_{<1}$. At the same time, this index in the group with intense manifestations of EHP ($HP_{\geq 6}$+M) exceeded nearly three times the analogous index in rats receiving no injection of melatonin (group $HP_{\geq 6}$). On the whole, injections of this hormone did not result in considerable systematic shifts in the number of rearings of both types and acts of defecations, compared with analogous indices in groups $HP_{<1}$ and $HP_{\geq 6}$.

T a b l e 2. Behavioral Indices in Rats in the Elevated Plus Maze Test

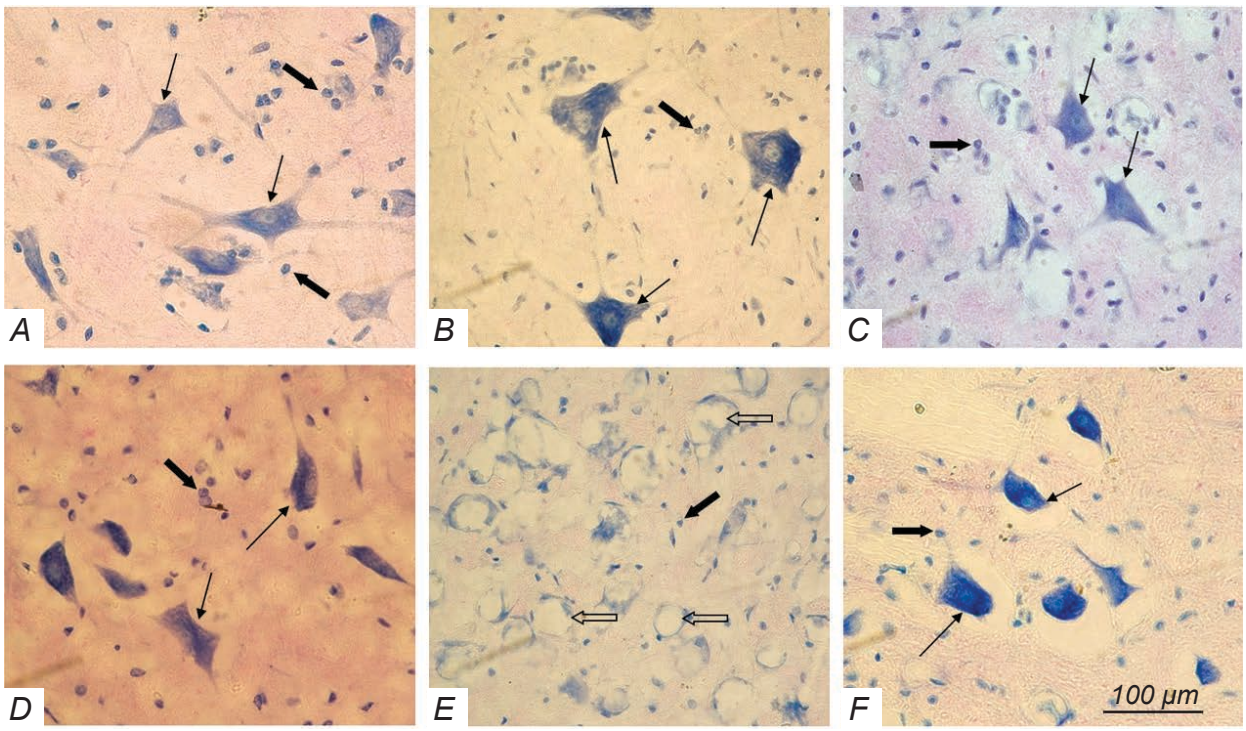
Experimental groups	Indices			
	time spent in open arms, sec	time spent in closed arms, sec	time spent in the center, sec	number of looking downward
Intact	159 ± 38	155 ± 32	43 ± 3	14 ± 1
Sham-injected	121 ± 19	174 ± 19	45 ± 4	16 ± 3
Mean value for groups I and ShI	**140 (100%)**	**164.5 (100%)**	**44 (100%)**	**15 (100%)**
$HP_{<1}$	22 ± 12 ** (16 ± 8%)	247 ± 47 * (150 ± 28%)	30 ± 11* (68 ± 25%)	4.3 ± 1.0 ** (29 ± 7%)
$HP_{\geq 6}$	25 ± 8 ** (18 ± 6%)	232 ± 15 * (141 ± 9%)	12 ± 3 ** (27 ± 7%)	3 ± 1 ** (20 ± 7%)
$HP_{<1}$+M	18 ± 4 *** (13 ± 3%)	272 ± 12 *+ (165 ± 7%)	22 ± 4 ** (50 ± 9%)	3.6 ± 0.9 ** (24 ± 6%)
$HP_{\geq 6}$+M	152 ± 36 ++ (108 ± 26%)	132 ± 32 + (80 ± 19%)	21 ± 5 **+ (48 ± 11%)	2.5 ± 0.5 ** (17 ± 3%)

Footnotes. Designations are the same as in Table 1.

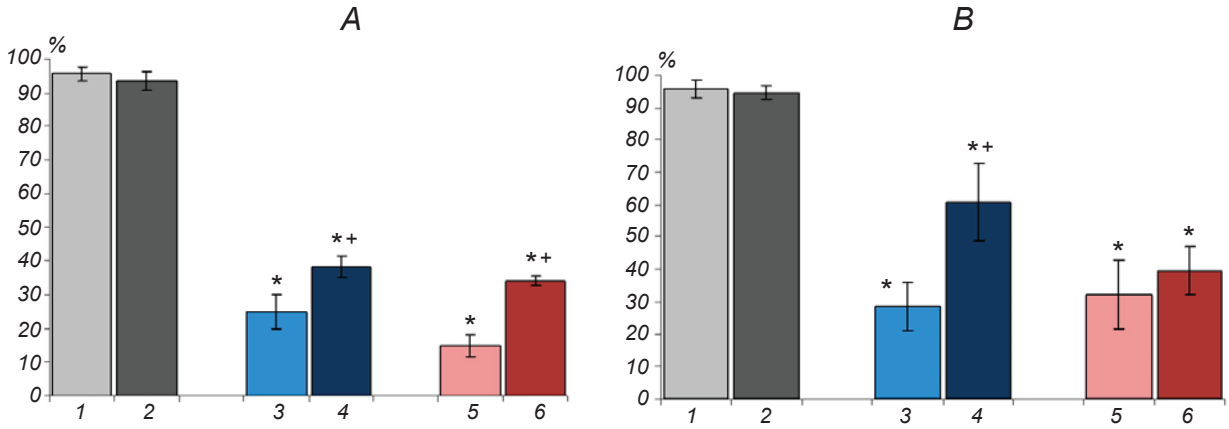
Indices of Behavior of Rats with EHP in the Elevated Plus Maze Test. After microinjections of 6-HODA, the time spent in open arms of the maze in rats of both experimental groups ($HP_{<1}$ and $HP_{\geq 6}$) was smaller than that in control animals (the difference was fourfold or even greater; $P < 0.01$). At the same time, the intervals spent in closed arms were greater by 50–40 % ($P < 0.05$). In rats with EHP, the time spent in the center was also significantly smaller: in group $HP_{<1}$, such a difference was obvious but insignificant (because of a high variability of the index), while in group $HP_{\geq 6}$, the respective difference was about fourfold ($P < 0.01$). In these two groups, the number of looking downwards was also much smaller than in the control ($P < 0.01$). Altogether, the observed shifts are indicative of a significantly increased level of anxiety in animals with EHP.

Course injections of melatonin did not result in significant changes in the time spent in open arms in rats of group $HP_{<1}$+M, as compared with group $HP_{<1}$. In group $HP_{\geq 6}$+M, this index was, however, significantly greater ($P < 0.01$) than in group $HP_{\geq 6}$ that obtained no melatonin; the respective index even somewhat exceeded the control values. Alterations of the time spent in closed arms naturally underwent opposite changes. The time spent in the center of the maze in rats of group $HP_{\geq 6}$+M was significantly greater ($P < 0.05$) than the analogous index in animals that were not injected with melatonin ($HP_{\geq 6}$). The numbers of looking downward in rats subjected to course injections of melatonin did not differ significantly from the corresponding values in rats with EHP but not injected with this hormone.

Effect of the Development of the EHP State on Morphological Characteristics of Nigral Cells. Neurons of the *substantia nigra* of rats of the control intact group demonstrated practically no signs of pathological modifications. The cells were characterized by the presence of centrally localized and structurally unchanged nuclei with one or several nucleoli, without manifestations of ectopia. Signs of intracellular and pericellular swelling in neurons of this cerebral structure in group I were usually absent. Pathological changes in microvessels of the *substantia nigra* were not observed;



F i g. 1. Photomicrographs of neurons of the *substantia nigra*. A) From rats of the intact group I; B) from animals after intracerebroventricular microinjections of saline (sham-injected animals, ShI); C) from rats with weak motor manifestations of experimental hemiparkinsonism, EHP (group $HP_{<1}$); D) from rats of group $HP_{<1}$ subjected to course injections of melatonin ($HP_{<1}$+M); E) from rats with intense manifestations of EHP ($HP_{\geq 6}$), and F) from rats of group $HP_{\geq 6}$ subjected to injections of melatonin ($HP_{\geq 6}$+M). Thin arrows show neurons with well-expressed dendrites, thick open arrows show neurons with signs of necrosis, and thick filled arrows show gliocytes. Staining by the Nissl technique.



F i g. 2. Diagrams of the density of structurally undamaged neurons in the *substantia nigra* (A) and gray matter of the spinal segment L5 (B) in rats of different experimental groups (1–6). Designations of groups are similar to those in Fig. 1. Asterisks indicate the cases of significant difference ($P < 0.05$) of the density from that in control groups; crosses indicate the difference with $P < 0.05$ in comparison between groups of rats with experimental hemiparkinsonism after course injections of melatonin and those, which received no melatonin injections.

diapedesis (passage of blood cells through the walls of the capillaries) was absent. Manifestations of slight perivascular swelling around separate blood vessels were observed in single cases. In rats of control group ShI, structural signs of nigral neurons demonstrated no significant differences from those in animals of group I. Only in rather rare cases were insignificant decreases in tinctorial properties of the cytoplasm observed in single neurons (Fig. 1A, B).

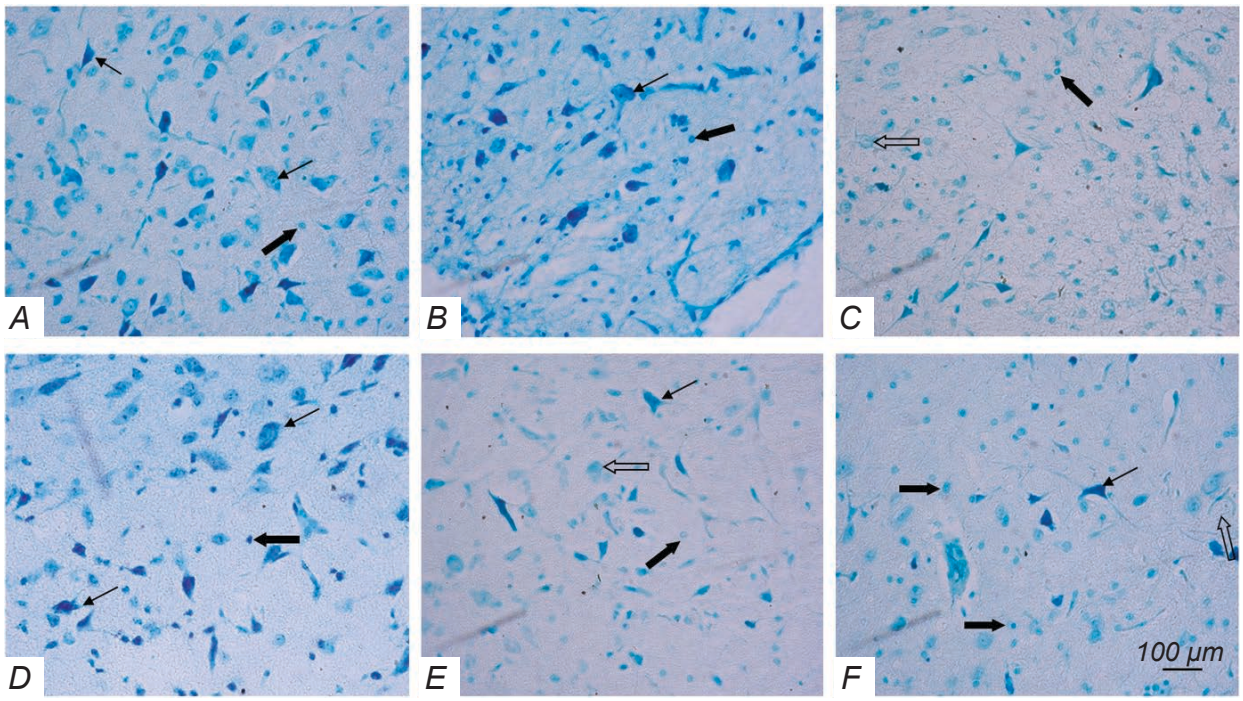
In rats with EHP (groups $HP_{<1}$ and $HP_{\geq 6}$), significant structural modifications were observed in a great number of nigral neurons. Among such changes, pyknosis of neurons and their nuclei and clearly pronounced dystrophic modifications of the dendrites in many cells should be mentioned (Fig. 1C, E). The hyperchromic nuclei in a considerable part of the neurons were distinguished by an ectopic localization of the nucleoli. Hyperchromatism of the nuclei was also observed in a significant proportion of glial cells. As a rule, the number of nigral neurons with clear negative structural changes was greater in group $HP_{>6}$ than in group $HP_{<1}$.

Mean values of the cross-sectional area of the cytoplasm in nigral neurons of rats of groups $HP_{<1}$ and $HP_{\geq 6}$ were significantly smaller than analogous indices in control groups (in both cases, $P < 0.05$; decrements were 26–21%). Mean values of cross-sectional areas of nuclei in nigral neurons of rats of both groups with EHP, however, were changed insignificantly ($P > 0.05$). The above-noted shifts led to significantly higher values of the NCI

T a b l e 3. Morphometric Indices of Neurons of the *Substantia Nigra*

Experimental groups	Cross-sectional area of the cytoplasm, μm²	Cross-sectional area of the nucleus, μm²	Nucleo-cytoplasmic index
Intact	620.6 ± 52.4	201.7 ± 17.8	0.33 ± 0.02
Sham-injected	604.1 ± 50.3	182.7 ± 10.5	0.31 ± 0.02
Mean value for groups I and ShI	**612.3 (100%)**	**199.2 (100%)**	**0.32 (100%)**
$HP_{<1}$	452.7 ± 27.8 * (74 ± 4%)	212.3 ± 12.6 (106 ± 6%)	0.47 ± 0.04 * (147 ± 12%)
$HP_{\geq 6}$	484.7 ± 25.7 * (79 ± 4%)	198.2 ± 17.9 (99 ± 9%)	0.42 ± 0.04 * (131 ± 12%)
$HP_{<1}$+M	605.6 ± 39.5 $^{+}$ (99 ± 6%)	207.0 ± 24.0 (104 ± 12%)	0.35 ± 0.04^{+} (109 ± 12%)
$HP_{\geq 6}$+M	805.9 ± 35.0 *$^{+}$ (132 ± 6%)	234.2 ± 20.6 * (117 ± 10%)	0.29 ± 0.02^{+} (91 ± 6%)

Footnote. Designations are the same as in Tables 1 and 2.



F i g. 3. Photomicrographs of neurons of the spinal L5 segment of rats of the experimental groups. Designations are the same as in Fig. 1.

in neurons of the examined central structure in groups HP, as compared with the control ($P < 0.05$; Table 3). These changes were, perhaps, manifestation of certain negative metabolic abnormalities in neurons of the *substantia nigra* related to the EHP state.

The relative numbers of nigral neurons with clearly pronounced signs of pathological changes in rats with EHP injected with melatonin (groups $HP_{<1}$+M and $HP_{\geq 6}$+M) were considerably smaller than those in EHP animals with no melatonin injections. In the above two HP+M groups, however, appreciable proportions of neurons in the *substantia nigra* were characterized by hyperchromic staining. In these cells, nevertheless, degenerative changes in the nuclei and neuronal processes were observed noticeably more rarely than in groups $HP_{<1}$ and $HP_{\geq 6}$. At the same time, the action of melatonin weakened signs of perivascular and pericellular swelling but did not completely eliminate such signs. Pyknosis of the nuclei was observed in an appreciable number of glial cells.

Course injections of melatonin into EHP rats provided certain normalization of the mean values of the cross-sectional area of the cytoplasm and also of NCI values, as compared with the respective indices in the $HP_{<1}$ and $HP_{\geq 6}$ groups ($P > 0.05$, Table 3).

Therefore, course injections of melatonin provided rather clearly pronounced normalization of the examined structural characteristics of nigral neurons. The corresponding normalized values, which were appreciably modified in groups HP, demonstrated a trend toward recovery to the initial values under the action of melatonin and even sometimes exceeded the latter.

Effect of the Development of the EHP State on Morphological Characteristics of Spinal Cells. In the gray matter of the spinal segment L5 of rats with considerable motor manifestations of EHP (group $HP_{\geq 6}$), neurons in the state of apoptosis (hyperchromic, deformed) were rather numerous. Cells with signs of necrosis were also found. In animals with less intense EHP manifestations (group $HP_{<1}$), changes presumably qualified as necrotic (hydropic dystrophy and swelling of cells and their nuclei) were usual (Fig. 3C). In both groups of rats with EHP, the number/density of neurons with no signs of negative morphological modifications was much smaller than in the control (Fig. 2B).

Analysis of morphometric characteristics of spinal neurons of rats of the experimental groups showed that pathological structural changes in the ventral horns of the lumbar spinal cord of rats with EHP can be even more intense than those in the *substantia*

Table 4. Morphometric Indices of Neurons of the Spinal L5 Segment

Experimental groups	Area of the cytoplasm on the cross-section of neuron, μm²	Area of the nucleus on the cross-section of neuron, μm²	Nucleo-cytoplasmic index
Intact	233.3 ± 21.7	101.2 ± 6.7	0.46 ± 0.02
Sham-injected	246.0 ± 16.6	141.2 ± 7.5 *	0.59 ± 0.02 *
Mean value for groups I and ShI	**239.6 (100%)**	**121.2 (100%)**	**0.52 (100%)**
$HP_{<1}$	163.1 ± 16.8 * (68 ± 7%)	102.9 ± 10.5 * (85 ± 9%)	0.64 ± 0.03 * (123 ± 6%)
$HP_{\geq 6}$	114.2 ± 6.5 ** (48 ± 3%)	60.2 ± 5,3 ** (50 ± 4%)	0.52 ± 0.02 (100 ± 4%)
$HP_{<1}$+M	215.0 ± 25.0 $^{+}$ (90 ± 10%)	120.7 ± 14.0 $^{+}$ (99 ± 11%)	0.56 ± 0.02 $^{+}$ (108 ± 4%)
$HP_{\geq 6}$+M	191.8 ± 28.2 $^{+}$ (80 ± 12%)	115.4 ± 15.8 $^{+}$ (95 ± 13%)	0.63 ± 0.03 $^{+}$ (121 ± 6%)

Footnote. Designations are the same as in Tables 1–3.

nigra. In group $HP_{<1}$, the mean cross-sectional area of the cytoplasm in spinal neurons was 32% smaller than that in control groups ($P < 0.05$). In rats of group $HP_{\geq 6}$, the analogous decrement was noticeably greater (more than a twofold drop; $P < 0.01$). The mean cross-sectional areas of the nucleus in animals with EHP were also significantly smaller than those in the control ($P < 0.05$); in group $HP_{\geq 6}$, the decrement was practically the same as that typical of the cross-sectional area of the cytoplasm (two-fold decrease). A significant change (increase) in the mean NCI value ($P < 0.05$) was observed only in group $HP_{<1}$.

Therefore, injections of melatonin into rats with EHP provided a clear trend toward normalization of the values of cross-sectional area of the cytoplasm. The corresponding values in groups $HP_{<1}$ and $HP_{\geq 6}$ were somewhat smaller than in the control, but the respective differences were insignificant ($P > 0.05$). The recovery of mean values of cross-sectional areas of nuclei of spinal neurons under the action of melatonin was practically complete (Table 4).

DISCUSSION

Our study showed that induction of the EHP state in rats using the above-described approach was sufficiently effective. About half of animals subjected to stereotaxic microinjections of 6-HODA into the forebrain ascending lateral bundle demonstrated strong motor disorders. Such disorders were easily reproduced under conditions of the apomorphine test as circulatory movements of a rather high frequency (six or more body rotations per 1 min). In some experimental rats, however, the intensity of such motor phenomena was considerably lower (not more than one rotation per 1 min); at the same time, realization of such rotational movements was quite obvious. The main reason for such a difference was, most probably, an insufficient accuracy of stereotaxic microinjections of 6-HODA in some cases. After such injections, the neurotoxin spread due to diffusion and reached the zone of the lateral bundle, but its amount influencing the target cerebral structure was considerably smaller. This, naturally, resulted in a lower intensity of degeneration of nigral cells in animals of groups $HP_{<1}$ and $HP_{<1}$+M.

It is known that the development of motor disorders typical of PD is related to massive degeneration of dopaminergic neurons in the *substantia nigra*. It was convincingly demonstrated that injection of 6-HODA into the forebrain ascending lateral bundle leads to progressive death (mostly unilateral) of dopaminergic neurons in the above cerebral structure [14]. Considerable motor symptoms become apparent only after the loss of 60–70% of the total amount of nerve cells of this structure [1, 2]. Non-motor symptoms typical of parkinsonism can be found in the case of death of a smaller portion of nigral neurons, i.e., within the preclinical stage of this disease. This is why it was rather interesting to compare estimates of motor and non-motor disorders in experimental rats against the background of various levels of impairment of the nigral neurons.

As was found, in rats of group $HP_{<1}$, in which the portion of undamaged nigral neurons was nearly 55% [13], the HMA estimate (locomotor activity) was already somewhat smaller than that in control groups (intact and ShI animals). Indeed, in rats with $HP_{<1}$, the portion of damaged neurons during long-lasting observation decreased to 25% against

the background of a significant drop in HMA. In rats with $HP_{\geq 6}$, the portion of undamaged nigral neurons did not exceed 20% [13], which agreed with the considerably stronger decrease in HMA in such animals. The obtained data on the relation of morphological and functional changes in the CNS in EHP are quite comparable with the findings of other authors [1].

Under conditions of experimental parkinsonism, clear disorders of the ability of quadrupedal animals to maintain the vertical posture in rearings have been observed [18, 21]. In our experiments, we found that the number of rearing (usually interpreted as manifestations of orientational/research activity) performed with the support on the wall was noticeably smaller in rats with EHP (especially in group $HP_{\geq 6}$) than that in the control. The number of rearings without the support on the wall became considerably smaller even in rats of group $HP_{<1}$. Changes in orientational/research activity in rats even with comparatively weak manifestations of EHP, as well as an obvious trend toward suppression of locomotor activity in such animals, can indicate that the formation of motor behavioral disorders in rats with a moderate level of degeneration of nigral neurons is due to the involvement of negative modifications not only in the *substantia nigra* but also in other CNS structures. Our data and findings of other authors indicate that this occurs, even at early stages of nigral degeneration, in the spinal cord, i.e., in the CNS part responsible for direct control and final coordination of motor functions [5]. In motoneurons of the spinal cord, significant degenerative changes develop in a parallel manner with relatively weak destructive changes in the cay PD-related CNS structure, *substantia nigra*. Electrostimulation of spinal structures combined with injections of levodopa used in relatively low doses in PD therapy provided certain recovery of the impaired motor functions [4, 22, 23]. In this disease, degenerative structural changes in spinal neurons are considered to be related to accumulation of a toxic protein (alpha-synuclein), the development of reactive gliosis with noticeable activation of microglia, decrease in the activity of mitochondrial complex IV in neurons and gliocytes, disturbances in the metabolic glucose pathways, and increase in the expression and activity of certain proteins, such as calpain, caspase-3, and p53 [4, 24].

It was recently reported that the development of degenerative changes in neurons of the spinal cord in PD can forestall disorders in nigral neurons [4]. We found considerable changes in spinal motoneurons in rats with moderately manifested EHP. Such manifestations were rather significant; the intensity of pathological modifications in the spinal cord in groups $HP_{<1}$ was quite comparable with negative alterations in the *substantia nigra*. This is why we conclude that degenerative changes precisely in the spinal cord is an important factor responsible for disorders of motor activity at both preclinical and clinical PD stages.

Under EHP conditions, such a non-motor symptom of PD as an abnormally high level of anxiety was clearly manifested. As is generally known, the enhancement of anxiety accompanies the development of stress reactions with crucial involvement of the hypothalamo-hypophyseal-adrenal axis [17]. In PD, not only the current functioning of this axis is disturbed, but its responses to regulatory influences are also significantly modified [25, 26]. The abnormal increase in the anxiety level in rats with EHP was especially intense in the $HP_{\geq 6}$ group. Behavioral changes in rats with EHP are, perhaps, due to (in any case partly) the imbalance of dopamine and other cerebral neuromediators.

Our experiments showed that course injections of melatonin provide significant neuroprotective effects under conditions of the analyzed pathology. After melatonin injections, proportions of undamaged neurons in both *substantia nigra* and spinal cord were found to be appreciably greater than those in animals that did not obtain such treatment. Positive alterations of the morphometric parameters were indicative of activation of some compensatory mechanisms and synthetic reparative processes under the action of melatonin, which contribute to partial normalization of the corresponding morphological substrate and improved functioning of neurons under EHP conditions.

The significant neuroprotective effect of melatonin after various experimental impairments of the nervous system, in particular central morphofunctional disorders in PD, may be related to the multifactorial nature of its effects in the organism. Melatonin provides antioxidant influences on neurons of the brain and spinal cord, which are based on a rise in the activity of antioxidant enzymes and result in a decrease in the amount of free radicals [15, 27–29]. Melatonin affects the content and activity of a few immune factors (T lymphocytes, macrophages, and cytokines); such effects restrain the development of neuroinflammation in the

CNS in neurodegenerative diseases [30, 31]. We, in particular, observed a drop in the number of macrophages and their functional activity in the *substantia nigra* of rats with EHP after melatonin injections [32]. In addition, a considerably decreased level of thymulin in the blood of rats with such pathology was restored to nearly control values after melatonin injections [15, 16]. As is known, the above-mentioned highly active hormone of the thymus regulates the differentiation of T lymphocytes, influences the balance of their subpopulations and activity of macrophages, decreases the content of pro inflammatory cytokines (tumor necrosis factor α, interleukins IL-1β and IL-6) in the brain, and increases the level of anti-inflammatory cytokines (IL-10) [33, 34].

One more probable mechanism of realization of the neuroprotective effect of melatonin in PD/parkinsonism is activation of neurogenesis in the CNS. It was demonstrated that this hormone intensifies the synthesis of neurotrophic factors (BDNF and NGF) and also increases the proliferative and differentiative potential of NSCs in the main zones of adult neurogenesis (subventricular zone of the brain lateral ventricles, hippocampus, and olfactory bulb) [15, 35]. The fact found in our recent study looks intriguing; this is the melatonin-induced increase in the number of NSCs just in the olfactory bulb of animals with moderate manifestations of EHP symptoms (which corresponds to group $HP_{<1}$) [15]. It was reported that a disturbance in olfactory reception is one of the first non-motor symptoms of PD, and some structural changes in the olfactory bulb are observed at the preclinical stage of this disease [2]. Neural stem cells were found not only in the above-mentioned zones but also in the *substantia nigra*; it was demonstrated that NSCs in this structure are capable of differentiating into neurons under the action of certain external factors [36]. It cannot be ruled out that precisely melatonin can play the role of such an external factor; melatonin receptors do exist in the main zones of neurogenesis and also in the *substantia nigra* [7, 8]. Since melatonin is present not only in the circulatory bed but also in the cerebrospinal fluid, it can also realize its effects on NSCs in the spinal cord; indeed, such cells were reported to be present in some spinal structures [37].

Melatonin also provides a rather significant antistress effect via changes in the functional state of the hypophyseal-adrenal axis [8]. The functioning of this axis in PD was shown to be significantly modified; it cannot be ruled out that, under the action of melatonin, the circadian pattern of interaction between this hormone and dopamine of the brain is recovered [38]. In addition, it was shown that glucocorticoids significantly influence the neurogenesis process in the brain; these agents change the vitality of neurons, synaptic functions, and production of neurotrophic factors [39].

Therefore, the neuroprotective effects of melatonin under EHP conditions are due to certain normalization of the structural characteristics and functional state of nigral and segmental (spinal) neurons. This leads to a noticeable decrease in the intensity of the motor and non-motor disorders resulting from the development of the analyzed experimental pathology. Our data indicate that melatonin appears to be a promising neuroprotective agent in the complex pathogenetically valid pharmacological therapy of PD. The mechanisms underlying the effects of melatonin and schemes of its use need further advanced examination. The possibility of using the above agent upon manifestations of non-motor disorders at both preclinical and clinical stages of PD is of special interest.

All aspects of the experiments on animals corresponded to the regulations of the Helsinki Declaration (1975) revised and supplemented in 2000, principles of the European Convention for the Protection of Vertebrate Animals Used for Experimental and Other Scientific Purposes (86/609/EEC, Strasbourg, 1986), and directives of the National Scientific Ethics Committee. The performance of experiments was approved by the Bioethics Committees of the Institute of Genetic and Regenerative Medicine and Bogomolets National Medical University (Kyiv, Ukraine).

The authors of this study, I. F. Labunets, Yu. B. Chaikovskii, S. I. Savos'ko, G. M. Butenko, V. F. Sagach, and B. S. Kop'yak, confirm that, in the course of performance of the experiments, they had no conflict of interest pertinent to commercial or financial relations and relations with organizations or persons somehow or other related to the study, as well as to interaction within the research group.

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