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Immunohistochemical Assessment of the Compensatory Responses in Rat Olfactory Bulbs after 6-Hydroxydopamine-Induced Lesion of the Substantia Nigra

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We assessed changes of olfactory bulbs in rata with 6-hydroxydopamine destruction of the substantia nigra. The expression of marker proteins of immature and differentiated neurons and glia (vimentin, PSA-NCAM, tyrosine hydroxylase, and S100) was analyzed by immunohistochemical and morphometric methods. The number of periglomerular dopamine neurons and astroglia in the olfactory bulbs increased on the side of toxin injection and expression of PSA-NCAM and vimentin increased in the rostral migratory stream. Destruction of the substantia nigra shifted differentiation of neuronal progenitors towards the dopaminergic phenotype and increased their survival in the olfactory bulbs, which can be explained by increased expression of PSA-NCAM.

Key Words: *6-hydroxydopamine; PSA-NCAM; tyrosine hydroxylase; rostral migratory stream; olfactory bulbs*

Plastic changes in brain structures in response to neurodegeneration largely determine clinical picture of Parkinson's disease. Analysis of the compensatory reactions in CNS developing in response to lesion of the nigrostriatal system is necessary for the search of new therapeutic approaches, including cell transplantation. Depending on the time and severity of substantia nigra (SN) lesion, these changes at the cellular level include regulation of dopamine metabolism and neurotransmission, activation of axonal sprouting in survived neurons, and expression of dopamine synthesis enzymes by non-dopaminergic neurons [1,13]; in addition, the possibility of neurogenesis in the SN is discussed [3]. It is known that periglomerular neurons of the olfactory bulbs (OB) are the most plastic dopaminergic neuronal population; this population is continuously renewed due to migration of neuronal progenitor cells along rostral migratory stream (RMS). Their recovery in case of olfactory bulbs lesion occurs in a month, and functional maturation of new neurons takes up to 8 weeks [10]. Neurons of SN influence the olfactory system not only via direct afferents to OB, but also via paracrine dopaminergic regulation of neurogenesis in the subventricular zone [9]. Experimental lesion of SN leads to olfactory impairments, similar to those observed in patients with Parkinson's disease [1,9]; dysfunction of periglomerular dopamine neurons is considered to be a cause of hyposmia [2,9].

It is known, that dopamine modulates proliferation of neuronal progenitors. For instance, a decrease in the number of proliferating cells in the subventricular zone was observed in the models of SN destruction caused by MPTP and 6-OHDA toxins [8,14,15]. In other studies, no activation of neurogenesis after dopaminergic system lesion was found [3,11]. At the same time, some authors reported that destruction of SN in the experiment had no effect on migrating cells in RMS [6,14], but increases the number of TH^+ cells in the glomerular layer [8,14,15]; although studies of autopsy material from human brain showed ambiguous results [4]. Thus, the effect of nigrostriatal system

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lesion on neuronal differentiation and migration in OB remains a subject of discussion, and clarification of this issue is important, in particular, for understanding of the pathogenesis of non-motor symptoms of Parkinson's disease and further development of cell therapy of this pathology.

Our aim was to assess changes in RMS and in the renewing population of dopaminergic neurons of OB in rat brain after neurotoxic destruction of SN.

MATERIALS AND METHODS

Male Wistar rats (180-200 g) were anesthetized with ketamine (50 mg/kg) and benzodiazepine (5 mg/kg) and then, 6 μg of 6-hydroxydopamine (6-OHDA, Sigma; 3 μl toxin solution in 0.05% ascorbic acid) were injected into SN on the right side (coordinates: AP=4.2; V=1.9; L=7.0 by Paxinos and Watson atlas) using a dual stereotaxic apparatus (Stoelting); injection velocity 0.4 μl/min. Into the left SN, the same volume of vehicle without neurotoxin was injected. Motor activity were assessed in an open field that represented a square arena (0.6 m^2) divided into 25 equal squares. The total number of squares crossed by the animal was recorded over 3 min.

Immunomorphological analysis was performed in 8 weeks after 6-OHDA injection (5 rats), intact rats (n=5) of the same age served as the control group. The animals were decapitated by guillotine, the brains were removed and fixed for 24 h in 4% formalin, embedded in OCT (Tissue Tek) medium, frozen, and sliced into 10-μ thick horizontal sections. For antigen retrieval, the sections in a citrate buffer (pH 6.0) were placed in a microwave oven for 10 min. Further immunohistochemical staining was performed according to the recommendations of the antibody manufacturers. We used rabbit polyclonal antibodies to GFAP (Dako) and tyrosine hydroxylase (TH) and mouse monoclonal antibodies to polysialylated neural cell adhesion molecule (PSA-NCAM, Novus antibodies), vimentin (Dako), and S100 protein (Dako). Appropriate secondary antibodies (Sigma) labeled with fluorochromes CF488 and CF555 were used for visualization of the reaction. Sections were examined under a Nikon Eclipse microscope equipped with a Nikon DS-Qi camera.

Fluorescence intensity (with background correction) was measured or the number of cells was estimated using a ×40 objective. At least 10 sections and 7-10 fields of view per section for each brain within the studied areas were analyzed. Measurements were performed along RMS in OB for vimentin and PSA-NCAM and in the glomerular layer and striatum for TH. $S100^+$ glial cells were counted in the granular layer of OB. The mean intensity of fluorescence was estimated in the studied areas in arbitrary units (255 gray levels) and expressed as percentage of its maximum value.

The data were averaged for each animal and median values for the group were calculated. Non-parametric Kruskal—Wallis ANOVA and Mann—Whitney tests were used for statistical analysis in Statistica 6.0 software.

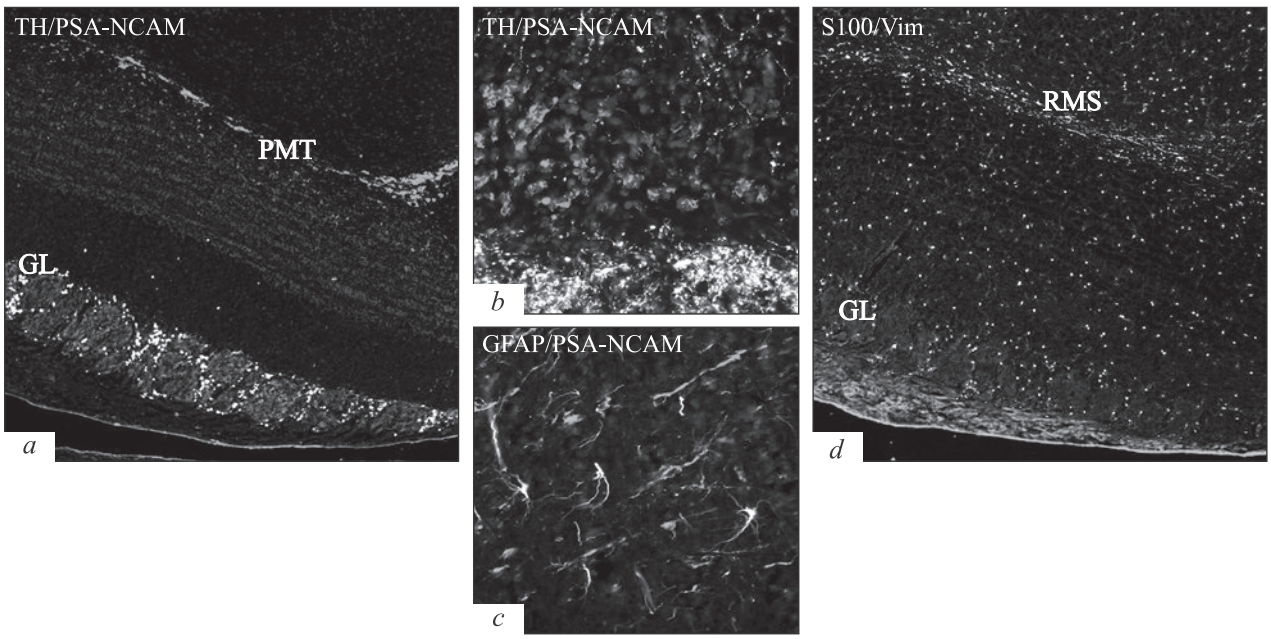

Fig. 1. RMS and OB structures in intact rats. *a*) OB; *b*) subventricular zone (fragment). Double staining for TH and PSA-NCAM. Nuclei were poststained with DAPI; *c*) granular layer of OB. Double staining for GFAP and PSA-NCAM (fragment); *d*) double staining for S100 protein and vimentin. GL: glomerular layer.

RESULTS

After SN destruction with 6-OHDA, horizontal motor activity of the animals in the open field test decreased, but in 6-8 weeks, this parameter showed a tendency to recovery. In previous studies we showed [13] that by week 8 after SN destruction, neurodegenerative changes in the striatum give way to the formation of new synaptic contacts between survived dopaminergic neurons. These observations are consistent with the observed tendency to recovery of motor activity. The terms of the onset of compensatory changes in the striatum were close to the period of neuronal renewal and functional maturation in OB [10]; therefore, we choose the term of 8 weeks after 6-OHDA injection.

In the control group, the highest density of TH^+ fibers was found located in the striatum and glomerular layer of OB (Fig. 1, *a*; Fig. 2, *a*), but solitary fibers were also detected in the subventricular zone (Fig. 1, *b*), in the anterior olfactory nucleus, and other structures of RMS. After 6-OHDA injection, the density of TH^+ fibers decreased. In 8 weeks after injection of the toxin, the density of TH^+ fibers in the striatum on the side of injection was significantly lower than in the contralateral hemisphere (Fig. 2, *a*, *b*). On the contrary, fluorescence intensity significantly increased in glomerular layer of OB on the side of SN lesion (Fig. 2, *a*, *b*). The mean density of periglomerular TH^+ neurons in OB on the side of lesion was 31±1.9 cells/0.1 mm^2, which significantly (by 35%) surpassed the corresponding parameter in the contralateral OB (23±1.5 cells/0.1 mm^2).

Intensive staining for vimentin and PSA-NCAM (Fig. 1) was typical of RMS. Vimentin was detected in ventricular ependimocytes, immature astrocytes of the subventricular zone, periventricular white matter, and along of migratory stream of neuronal progenitors in OB where longitudinal oriented vimentin$^+$ glial processes formed a dense network. High vimentin expression also was found in the olfactory nerve. The most intense PSA-NCAM$^+$ staining was found in the subventricular zone and along RMS, while radial migrating neurons in the granular layer of OB was less intensive stained.

The intensity of staining for PSA-NCAM in RMS structures on the side of 6-OHDA injection almost 2-fold higher than in the control (Fig. 3, *a*, *d*) and in the contralateral hemisphere; vimentin expression on the side of lesion was weakly, but significantly increased in comparison with the control and contralateral hemisphere (Fig. 3, *a*, *b*). The density of $S100^+$ astrocytes in the granular layer of ipsilateral OB was 23.0±0.7 cells/0.1 mm^2, while in the contralateral OB this parameter was significantly lower: 21.0±0.6 cells/0.1 mm^2. No co-localization of PSA-NCAM and glial markers GFAP (Fig. 1, *c*) and S100 was found in RMS of intact and 6-OHDA-treated rats. The increase of vimentin expression is probably associated with the

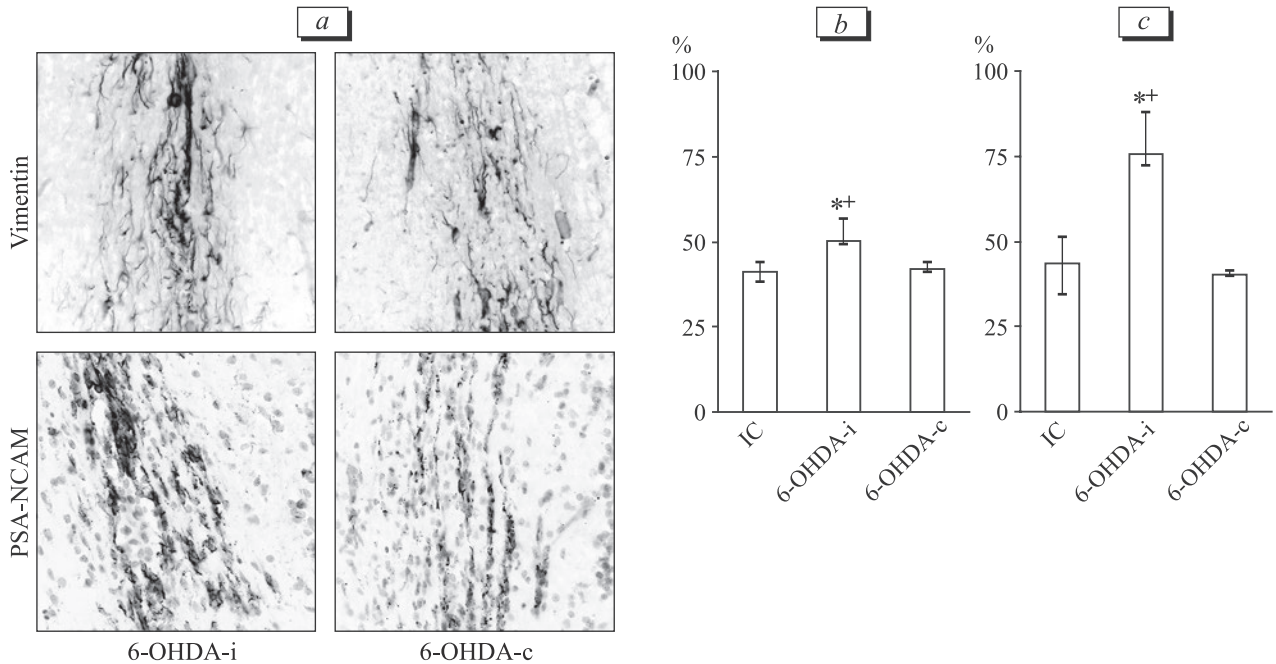

Fig. 2. Changes in the intensity of staining for vimentin and PSA-NCAM in RMS after unilateral injection of 6-OHDA into SN. *a*) Localization of vimentin and PSA-NCAM in RMS on the side of toxin injection and in the contralateral hemisphere. Immunofluorescence staining, negative image. *b*, *c*) Changes in the intensity of staining for vimentin (*b*) and PSA-NCAM (*c*). 6-OHDA-i: toxin injection, ipsilateral hemisphere; 6-OHDA-i: toxin injection, contralateral hemisphere; IC: intact control (right hemisphere). $p<0.05$ in comparison with *contralateral hemisphere, $^+$IC (Mann—Whitney test).

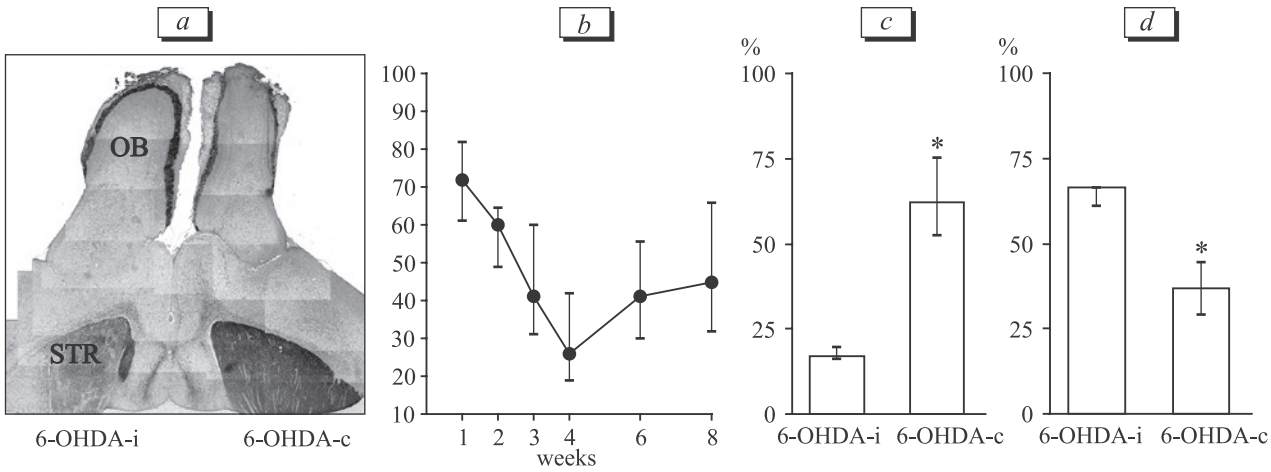

Fig. 3. Changes in locomotor activity of animals and TH expression in the striatum and OB after unilateral injection of 6-OHDA into SN. *a*) Localization TH on a horizontal section of OB. Immunofluorescent staining, negative image. *b*) Changes in locomotor activity in the open-field test (number of crossed squares) after 6-OHDA injection. *c*, *d*) Changes in the intensity of TH staining in the striatum (*c*) and glomerular layer of OB (*d*). 6-OHDA-i: toxin injection, ipsilateral hemisphere; 6-OHDA-i: toxin injection, contralateral hemisphere. CTR: striatum. **p*<0.05 in comparison with the ipsilateral hemisphere (Mann—Whitney test).

revealed reactive gliosis in OB and can attest to an increase in the number of astrocytes in OB due to not only their *in situ* proliferation, but also migration from subventricular zone.

Our study demonstrated an increase in the number of periglomerular TH^+ cells and density of their processes on the side of 6-OHDA injection, which is consistent with published data for of Parkinson's disease models caused by injection of MPTP and 6-OHDA [5,14,15]. Although injection of 6-OHDA into SN suppressed cell proliferation in the subventricular area [8,14], the number of neuronal progenitors expressing specific proteins doublecortin and nuclear antigen of proliferating cells (PCNA) in RMS remained constant [6,14], which attested to better survival and preserved migration of immature neurons in OB under conditions of SN destruction. The number of TH^+ neurons in OB increased probably due to a shift of neuronal progenitors differentiation towards the dopaminergic phenotype [14,15]. It can be assumed that more intensive staining for PSA-NCAM in RMS is related to enhanced expression of this marker by migrating cells, but not to an increase in their number. It is known that polysialylation of NCAM is essential for survival of immature neurons as it suppresses their apoptosis [7], which can lead to an increase in the number of newly formed neurons. It should be emphasized that despite the obvious compensatory nature of this process, the increase in the number of periglomerular dopamine neurons in OB can led to their more pronounced inhibitory effect in glomeruli and contribute to the development of hyposmia in Parkinson's disease [12]. In addition, according to neurochemical studies on the MPTP model, newly formed periglomerular TH^+ neurons are characterized by impaired dopamine metabolism [12]. Thus, functional interpretation of the changes observed in OB after SN destruction is ambiguous.

In general, comparison of our results and published data showed that the reactions of cell populations in OB after SN destruction strongly depend on the period after damage and that the changes revealed by us reflect the early period of compensation.

In the model of Parkinson's disease caused by unilateral injection of 6-OHDA into SN, disruption of innervation of the dopaminergic subventricular zone on the affected side led to an increase in the number of periglomerular dopaminergic neurons in OB, which is associated with a shift in the direction of differentiation of neuronal progenitors towards the dopaminergic phenotype and improvement of their survival in OB. The latter can be determined by enhanced PSA-NCAM expression on the side of injury.

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