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PHYSIOLOGY

Myelinated Fibers of the Mouse Spinal Cord after a 30-day Space Flight

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Abstract—Myelinated fibers and myelin-forming cells in the spinal cord at the L3–L5 level were studied in C57BL/6N mice that had spent 30 days in space. Signs of destruction of myelin in different areas of white matter, reduction of the thickness of myelin sheath and axon diameter, decreased number of myelin-forming cells were detected in "flight" mice. The stay of mice in space during 30 days had a negative impact on the structure of myelinated fibers and caused reduced expression of the markers myelin-forming cells. These findings can complement the pathogenetic picture of the development of hypogravity motor syndrome.

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The development of life on Earth occurred under the influence of gravitational forces. Hypogravity motor syndrome, which is characterized by atony, atrophy, decreased muscle strength, and muscle fatigue [1] develops under gravity-free conditions in humans and animals. In the skeletal muscles that maintain posture, these pathological changes develop within the first day [1]. There is reason to believe that the cause of these disorders is the reaction of the central nervous system to the deficiency of afferentation. However, the effect of gravity-free conditions on the spinal cord as a whole and glial cells and fibers of the white matter are still poorly understood. Earlier, in a morphometric study of lumbar enlargement of the spinal cord, we found a decrease in its volume [4] using the hypogravity model [2]. In the same experimental model, we showed an increase in the level of expression of proteins (S100B, HSP25, and HSP70) that are responsible for protecting the neuronal response in response to hypogravity, as well as an increase in Olig2 protein expression, which regulates oligodendrocyte proliferation [4].

Molecular genetic analysis revealed a decrease in the expression of the *pmp2* and *pmp22* genes encoding myelin proteins, leading to defects in myelination [6], which entailed the destruction of the myelin sheath and a change in the rate of conduction of excitation along the axons [7]. In a study of the motor neurons of the lumbar enlargement of the spinal cord of mice that had spent 30 days in space, we found a decreased immunopositive expression of the HSP25, HSP70, and PSD95 proteins and synaptophysin, which play an important role in adaptive-compensatory processes [4]. However, the effect of gravity-free conditions on the fiber pathways of white matter and glial cells remains poorly understood.

In the present study we used the fluorescence microscopy and morphometry methods to obtain data demonstrating shifts in the state of myelinated fibers pathways and myelin-forming cells of mouse spinal cord cells after 30 days in space. These data allow us to conclude that the demyelination of the central nervous system is one of the factors in the development of hypogravity motor syndrome.

Experiments were conducted on male mice of the C57BL/6J strain (Puschino nursery of laboratory animals) with body weights of 25 ± 3 g. All procedures with the animals were carried out in accordance with the rules of the Ethics Committee of the Physiological Section of the Russian National Committee for Biological Ethics [8]. The "flight" group mice had spent 30 days in space on biosatellite BION-M1. The small number of animals in the "flight" group ($n = 3$) was due to the uniqueness of space projects. A control group of mice stayed on Earth for 30 days under the conditions simulating those on the biosatellite as much as possible. In some experiments, intact mice were used, which were kept under standard vivarium conditions. The animals were withdrawn from the

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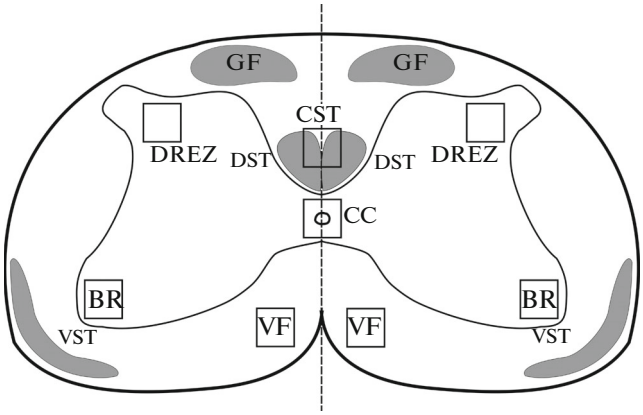

Fig. 1. The morphometry zones of myelinated fibers in the gracile fasciculus (GF), ventral spinocerebellar tract (VST), and dorsal spinocerebellar tract (DST) are shown in gray. The morphometry zones of the results of immunofluorescence detection of markers of myelin-forming cells are marked with squares: VF, ventral funiculus; VH, ventral horn; CST, corticospinal tract in the dorsal funiculus, DREZ, dorsal root entry zone; CC, the area of the central canal.

experiment by cervical dislocation, and lumbar spinal cord at the L3–L5 level was isolated.

For morphometry, the material was fixed with glutaraldehyde and osmium tetroxide, dehydrated. and embedded in Epon 812. Semifine transverse spinal cord sections were stained with toluidine blue. Images were obtained using an Olympus BX51WI microstructure microscope (Olympus, Japan) with an AxioCam MRm camera (Carl Zeiss, Germany), and the AxioVision Rel. 4.6.3 software (Carl Zeiss). Morphometry of digitized images was performed using AxioVision Rel 4.8. In a 100 × 100 μm square within the fasciculus gracilis, the ventral spinocerebellar and dorsal spinocerebellar tracts (Fig. 1), 50 profiles of myelinated fibers were examined by measuring their diameter, thickness of the myelin sheath, and the diameter of the axis cylinder. The ratio of the diameter of the axon to the diameter of the myelin fiber (myelin index, MI) was calculated for the myelinated fibers of each sample.

In free-floating spinal cord cross-sections with a thickness of 20 μm prepared using a Microm HM 560 cryostat (Thermo Scientific, United States), glial cells were detected using antibodies against the myelin oligodendrocyte-specific protein OSP (Santa Cruz Biotechnology, United States; dilution, 1 : 100), the myelin protein zero (P0, Abcam, United Kingdom; 1 : 100), oligodendrocyte transcription factor 2 (Olig2, Santa Cruz Biotechnology; 1 : 200) and Krox24 (R & D Systems, United States; 1 : 30). Donkey immunoglobulins conjugated to Alexa 488 fluorochrome (Invitrogen, United States; 1 : 200) and Alexa 647 (Invitrogen; 1 : 200) were used as secondary antibodies. For the imaging of cell nuclei, sections were additionally stained with a propidium iodide solution (PI, 10 μg/mL in phosphate buffered saline, Sigma-Aldrich, United States). Analysis of cross-sections of the lumbar enlargement at the L4–L5 level of the spinal cord was performed using a LSM 510 META confocal scanning microscope (Carl Zeiss). For immunohistochemical analysis of glia, the following areas were selected (Fig. 1): the ventral horn (VH), the corticospinal tract in the dorsal funiculus (CST), ventral funiculus (VF), central canal (CC), and dorsal root entry zone (DREZ). In all areas, immunopositive cells were counted in a square area 0.05 mm^2 in size in each of the six sections at 0.5-μm intervals. Digital images of spinal cord sections were analyzed using the ImageJ 1.47j software. During the counting, the number of cells belonging of immunopositive structures to specific cell was determined by the location of their nuclei detected using PI.

The fluorescence intensity value of the marker on the digital image of the section was used as an index of the OSP protein expression levels in cells.

Statistical processing of the results was performed by means of the Origin 8.0 software package using the Wilcoxon–Mann–Whitney U test. Differences were considered statistically significant at $p \le 0.05$.

The morphometric comparative study of white matter fibers in the spinal cord of the studied conductive tracts did not reveal significant differences between the diameter of myelin fibers, the diameter of the axon, and the thickness of the myelin sheath and MI between the control and the intact animal groups.

In myelinated fibers of white matter of the spinal cord in the ventral spinocerebellar tract of the experimental group of mice, a significant decrease in the average thickness of the myelin sheath by 15% and in the average diameter of the axon by 16% was detected, compared with the control group of animals kept on Earth under the conditions simulating the environment on the biosatellite (table).

Similar changes were found in the myelinated fibers of the dorsal spinocerebellar tract. The average thickness of the myelin sheath in the mice of the experimental group decreased by 13%, and the average diameter of the axon decreased by 8% compared with the control mice. These differences were not significant.

The average thickness of the myelin sheath in fasciculus gracilis area of experimental group decreased nonsignificantly by 5%. We also detected a small and nonsignificant increase in the average diameter of myelinated fibers by 6% as compared with the control animals.

In the dorsal spinocerebellar tract and fasciculus gracilis, no significant differences were found between the experimental, control, and intact groups in the diameters of myelinated fibers and the axis cylinder or thickness of the myelin sheath.

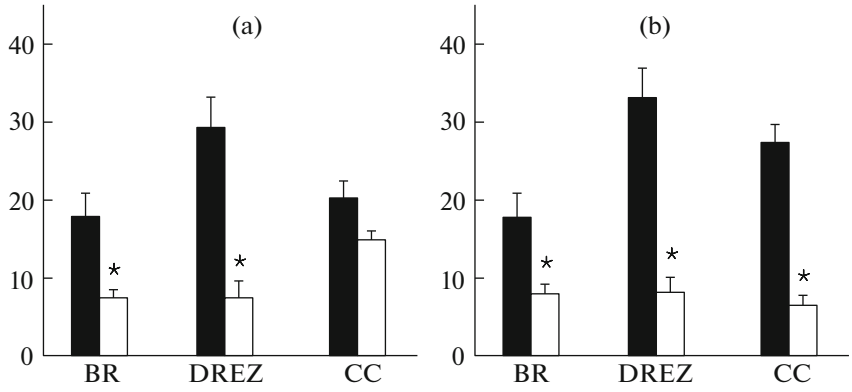

Fig. 2. Number of (a) $P0^+$ and (b) $Krox24^+$ cells (ordinate, the measurement in the area with $S = 0.05$ mm^2) in the lumbar enlargement of the spinal cord of mice. The abscissa axis shows counting zones. Dark bars, control (30 days on Earth under the conditions stimulating the conditions on biosatellite, $n = 8$); light bars, experiment (30 days in space flight, $n = 3$). * $p < 0.05$.

In fibers of the white matter of the experimental group of animals in all conduction tracts, we revealed signs of myelin destruction in the form of lower acuity of the contours of individual myelinated fibers and blur boundaries between the myelin sheath and axis cylinder.

The average MI value for the experimental and control groups in the ventral spinocerebellar tract and dorsal spinocerebellar tract were almost identical, while a nonsignificant increase by 9% was detected in the fasciculus gracilis in the experimental group.

Thus, the morphological analysis of myelinated fibers of conduction tracts showed a reduction of the thickness of the myelin sheath and morphological signs of the beginning of demyelination in all studied tracts, which can be regarded as evidence of the effect of gravity-free conditions of space flight on the myelinated fibers.

The study of glial cells by the immunofluorescence method revealed the presence of OSP^+ and $Olig2^+$ cells in all investigated areas in mice of the intact, control, and experimental groups. In all areas of the spinal cord, a decrease in the expression of oligodendrocyte proteins was observed in the experimental group of mice, for which significant amounts of shifts of immunopositive cells expressing the Olig2 protein, in CST and the fluorescence intensity in the white matter (VF) for the OSP were also detected. In the CST of mice of the experimental group, an eightfold reduction of the OSP protein fluorescence intensity was shown. In the CST of the experimental group of mice, the amount of $Olig2^+$ cells was 1.4 times lower than in the control mice. The decreased expression of SP and Olig2 in the "flight" group was shown. These findings, together with the lack of significant destructive changes, suggest shifts in the functional state of the myelinated fibers in the central nervous system.

Morphometry data of myelinated fibers in the ventral spinocerebellar tract

	Control				Experiment			
	D	*d*	*m*	*MI*	*D*	*d*	*m*	MI
N1	5.51	3.05	2.46	0.5535	4.62	2.49	2.13	0.5390
N2	5.64	3.17	2.47	0.5620	4.93	2.75	2.19	0.5578
N3	5.67	3.10	2.57	0.5467	4.61	2.47	2.14	0.5358
M	5.6	3.10	2.5	0.5535	4.68*	2.53*	2.14*	0.5406

D, the diameter of myelin fiber; *d*, the diameter of axon; *m*, thickness of the myelin sheath; MI, thickness of the myelin sheath; N1, N2, N3, number of the animal; *M*, the arithmetic mean value; * $p < 0.05$ in comparison with control.

Cells expressing the myelin protein P0 and transcription factor Krox24 were detected in the spinal cord of the experimental and control groups. The highest number of $P0^+$ and $Krox24^+$ cells was observed in the area of the CC and DREZ in mice of the "flight" group and in the control group. According to these markers, shifts were observed only in the gray matter. The number of $P0^+$ cells decreased in VH and DREZ by factors of 1.4 and 3.8, respectively (Fig. 2a). In the "flight" group, the number of $Krox24^+$ cells in the VH, DREZ and CC decreased by factors of 2.3, 4.2, and 4.3, respectively, compared with the control group of mice (Fig. 2b). The count of $P0^+/Krox24^+$ cells did not reveal significant changes in any of the areas of the spinal cord studied.

The decreased number of myelin-forming cells in the spinal cord suggests the role of this factor in the pathogenesis of hypogravity motor syndrome. It is known that the Krox24 transcription factor is one of the early-response proteins involved in the regulation of the cell cycle and differentiation of myelin-forming cells and is induced by neurons [9]. It is believed that the decrease in the expression of this protein may be a consequence of impaired relationship between neurons and glial cells, as evidenced by the phenotypic changes of macroglia cells identified in the

hind-limb-unloading model [4]. The decreased number of $Olig2^+$ cells in the corticospinal tract and decreased fluorescence intensity of the OSP protein in the ventral funiculus also indicate that destructive changes in the white matter oligodendrocytes develop during space flight.

Thus, the data suggest that space flight affects the state of the myelin sheath in the spinal cord, which can complement the pathogenetic picture of the development of hypogravity motor syndrome.

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