DOI 10.1007/s10517-017-3609-1

Changes in the Neurochemical Composition of Motor Neurons of the Spinal Cord in Mice under Conditions of Space Flight

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Translated from *Byulleten' Eksperimental'noi Biologii i Meditsiny*, Vol. 162, No. 9, pp. 318-321, September, 2016
Original article submitted August 27, 2015

Expression of choline acetyltransferase, 200-kDa neurofilament protein, 28-kDa calbindin, neuronal NO synthase, caspase 3, and Ki-67 in the motor neurons of spinal cord segments T3-T5 in male C57Bl/6 mice after 30-day space flight in the Bion-M1 biosatellite was studied by immunohistochemical methods. Under conditions space flight, the size of motoneurons increased, the number of neurons containing choline acetyltransferase and neurofilaments, decreased, and the number of calbindin-positive neurons increased; motoneurons, expressing neuronal NO synthase and caspase 3 appeared, while Ki-67 was not detected. Fragmentation of neurons with the formation structures similar to apoptotic (residual) bodies was observed in individual caspase 3-positive motoneurons.

Key Words: *motoneurons; spinal cord; immunohistochemistry; microgravity*

Microgravity-induced deficit of extero- and proprioceptive influx that was revealed in animals after space flights and proved by morphological and cytochemical evidence [3] results in partial or total sensory deprivation of the motor function and impaired trophic of somatic musculature. Functionally and topographically different motoneurons and muscles innervated by them respond differently to space flight factors [9,11]. Exposure to microgravity causes morphological and cytochemical changes in neurons [9,12]. Neuronal changes can reflect adaptation of the spinal cord neural networks to the new gravity level [12]. The direction of changes in motor neurons after space flight has not been studied.

Here we studied immunohistochemical and morphometric characteristics of motoneurons of the thoracic part of spinal cord in mice after 30-day space flight in the Bion-M1 biosatellite.

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MATERIALS AND METHODS

Localization and morphofunctional characteristics of the spinal cord motoneurons was studied in male C57Bl/6 mice. The animals of the space flight group (n=3; weight 28.90±1.73 g) were exposed to microgravity during the 30-day space flight of the Bion-M1 biosatellite. Controls (n=3) were kept under vivarium conditions. Animals were sacrificed by cervical dislocation. The material was taken 12 h after landing of the satellite [1].

Spinal cord segments T3-T5 were fixed in 4% paraformaldehyde in PBS (pH 7.4). For cryoprotection, 30% sucrose solution was used. Serial cross-sections of the spinal cord (14-μ) were sliced on a cryostat.

Neurons immunoreactive for choline acetyltransferase (ChAT), 200-kDa neurofilament protein (NF), 28-kDa calbindin (CAB), neuronal NO synthase (nNOS), caspase 3, and Ki-67 were detected by immunohistochemical methods [13]. Primary antibodies (Abcam) used in the study are listed in Table 1; donkey secondary antibodies (Jackson ImmunoResearch) were conjugated with FITC or indocarbocyanide (1:100).

In case of NF detection, sections were preincubated with Fab-fragment of unconjugated donkey anti-mouse antibody for 1 h (dilution of 10 mg/ml, Jackson ImmunoResearch) prior to primary antibody application to prevent nonspecific staining.

Nissl staining of the total neuronal population was carried out using red fluorescent dyes: NeuroTrace 530/615 red fluorescent Nissl stains (1:200; Molecular Probes). Immunoreactive cells in the population of motoneurons were revealed by fluorescence. Thereafter, sections were washed in PBS and embedded in the VECTASHIELD immunofluorescence medium (Vector Laboratories). To distract nonspecific binding, some sections were incubated in the absence of primary or secondary antibodies. The preparations were analyzed under an Olympus BX43 microscope (Olympus Corporation), fitted with a set of fluorescent filters. The images were obtained using a cooled digital TSC-5.0ICE camcorder (Tucsen). Section area of motoneurons was determined using ImageJ software. In the same sections immunoreactive motoneurons were counted in lamina IX of the spinal cord gray matter in each section (absolute content). The proportion of immunoreactive motor neurons was calculated as the ratio of their number to the total number of Nissl —positive motor neurons taken as 100%. Motor neurons, cut through the nucleus with visible nucleoli and with fluorescence exceeding background glow, were included in the analysis. Structural changes in the spinal cord motor neurons of mice were assessed using thionin-stained sections.

The data were statistically processed (arithmetic mean±standard error) using Statistica 10.0 (StatSoft, Inc.) with ANOVA. Differences were considered significant at $p<0.05$.

RESULTS

In the gray matter of the spinal cord of control mice there were revealed motor neurons, immunopositive for ChAT, NF and CAB. All motor neurons were cholinergic and contained ChAT. Most cholinergic motor neurons also contained NF or CAB: NF was detected in 90.4% of ChAT-positive motoneurons, and CAB — in 1.4% of ChAT-positive motoneurons. A small number of CAB-positive motoneurons in the mouse spinal cord was noted in our previous studies [5,6]. At the same time, no motoneurons, immunopositive for nNOS, caspase 3 or Ki-67, were found in lamina IX. Mean section area of motor neurons varied depending on their neurochemical identity. Thus, NF-positive motor neurons had the largest average dimensions, CAB-positive motoneurons were the smallest; ChAT-positive motoneurons occupied an intermediate position.

TABLE 1. Antigens, Revealed by Immunohistochemistry, and Primary Antibodies

Antigen	Antibodies, dilution
ChAT	Goat polyclonal, 1:300
NF	Mouse monoclonal, 1:300
nNOS	Goat polyclonal, 1:300
CAB	Rabbit polyclonal, 1:500
Caspase 3	Rabbit polyclonal, 1:200
Ki-67	Rabbit polyclonal, 1:100

In mice of the space flight group there were also motor neurons in lamina IX of the spinal cord gray matter, immunopositive for ChAT, NF and CAB. In contrast to the control group, in the space flight group motoneurons were immunopositive for enzymes nNOS and caspase 3. Ki-67-immunopositive motoneurons were not revealed. Decomposition of motor neurons into fragments, resembling apoptotic (residual) bodies, was observed in solitary caspase 3-positive motoneurons.

Comparison to the control group demonstrated significantly decreased number of cells, immunopositive for ChAT and NF (Table 2), and significantly increased number of CAB-positive neurons. In the spinal cord of mice of the space flight group 63.4% of motoneurons contained both ChAT and NF, 28.2% — both ChAT and CAB. All cholinergic motor neurons contained nNOS. Motor neurons, immunopositive for NF, CAB and caspase 3, were also immunipositive for nNOS. Colocalization of nNOS with CAB was detected in 30% of motoneurons, of nNOS with caspase 3 — in 57%, of CAB with NF — in 44% of motor neurons. Thus, after exposure to the space flight conditions the spinal cord motor neurons of mice differed from control in their functional properties.

Average cross-section area of immunoreactive motor neurons in animals of the space flight group was larger than that in the control group: sectional area of neurons, containing ChAT was by more than 1.8 times larger, NF — by 1.6 times, CAB — by 1.3 times. Section area of the spinal cord motor neurons of mice in the space flight group, calculated on the basis of Nissl staining, was 2 times larger than the corresponding area in control. Motor neurons in NF- and ChAT-immunopositive subpopulations had maximum dimensions, neurons, containing CAB, nNOS and caspase 3, were the smallest.

It is known that under conditions of prolonged microgravity enzymatic activity in the motor neurons of the spinal cord is reduced [4,12]; and there are also

TABLE 2. Content and Average Section Area (S) of Spinal Cord Motoneurons ($X \pm S_x$; n=3)

Marker	Control			Space flight		
	abs.	%	S, μ²	abs.	%	S, μ²
NeuroTrace red	7.30±0.17	100	256.20±14.35	7.10±0.33	100	536.80±10.13*
ChAT	7.30±0.17	100	256.20±14.35	5.00±0.29*	70.4	462.60±12.81*
NF	6.60±0.09	90.4	294.00±12.99	4.50±0.09*	63.4	478.2±65.7*
CAB	0.10±0.06	1.4	216.20±2.94	2.00±0.14*	28.2	281.80±6.59*
nNOS	N/d		N/d	6.50±0.15	91.5	358.00±10.76
Caspase 3	N/d		N/d	3.70±0.12	52	300.80±5.83
Ki-67	N/d		N/d	N/d		N/d

Note. N/d — not detected. *p<0.05 in comparison with control.

data on the decrease in ChAT expression by 54% in motoneurons of the spinal cord segments L4-L5 of rats, subjected to hindlimb support relief for 35 days [4]. Expression of nNOS may increase upon exposure of neurons to damaging factors, particularly after axonotomy [2].

It seems essential that these changes in the population of spinal cord motoneurons of mice after the space flight were directly or indirectly associated with calcium metabolism. The neuroprotection action of CAB consists in reduction of the intracellular calcium concentration [15]. Dysfunction of the calcium buffer system in neurons can lead to their degeneration [7,8].

The nNOS-produced NO may produce both protective and damaging effects. The destructive effect of NO is implemented through energy destabilization of cells and DNA deamination, irreversible activation of this process is mediated by pro-apoptotic enzymes — caspases [14]. After a long space flight against the background of significantly lower number of ChAT- and NF-positive motor neurons, the cross-section area of motor neurons was increased, which was associated with cell swelling due to excessive influx of calcium ions, also evidenced by elevated calbindin level.

Thus, reactive changes in the motor neurons of thoracic segments of the mouse spinal cord developing under conditions of prolonged space flight manifested in increased dimensions of cells throughout the population, decreased number of ChAT- and NF-positive subpopulations, increased number of CAB-positive subpopulations, and presence of motor neurons expressing nNOS and caspase 3. Morphological and immunohistochemical changes in motoneurons reflect adaptive mechanisms to the conditions of space flight, identification of effects of individual factors seems not yet possible.

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