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Slc6a4, Tph2, Htr1b, Htr2a genes expression in the mouse spinal cord after microgravity exposure simulation on earth

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Abstract

Aim. To determine the level of gene expression of the serotonergic neurotransmission system (*Slc6a4*, *Tph2*, *Htr1b*, *Htr2a*) in the cervical and lumbar enlargement of the spinal cord for mice after 30-day microgravity exposure simulation by using the antiorthostatic unloading model by Morey-Holton et al. and a subsequent 7-day recovery period.

Methods. The experimental animals were divided into three groups: "Unloading" group with mice undergoes hindlimb-unloading procedure for 30 days (n=5); "Recovery" group with mice undergoes hindlimb-unloading procedure for 30 days, followed by readaptation within 7 days (n=5); "Control" group with mice kept at standard vivarium conditions (n=5). The expression level of genes encoding synaptic proteins in the central nervous system was estimated by a real-time polymerase chain reaction.

Results. There were no statistically significant differences between the studied groups regarding the *Tph2*, *Htr1b*, and *Htr2a* expressions in the cervical and lumbar enlargement of the spinal cord. Compared to the "Control" group, a statistically significant increase (6.3 times) in the level of *Slc6a4* expression in the lumbar spinal cord was revealed after microgravity exposure simulation ("Unloading" group), followed by a 3-fold decrease during the readaptation period ("Recovery" group).

Conclusion. The expression level of the *Slc6a4* gene, which encodes carrier protein involved in the function of serotonergic synapses, may indicate the potential involvement of this neurotransmitter system in the pathogenesis of movement disorders after microgravity exposure simulation on earth.

Keywords: serotonin (5-hydroxytryptamine), antiorthostatic unloading, spinal cord, real-time polymerase chain reaction (RT-PCR), *Slc6a4, Tph2, Htr1b, Htr2a*.

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Background: The effect of weightlessness on humans and vertebrates leads to molecular, cellular, and tissue changes that affect all body systems [1]. These changes are especially pronounced in the musculoskeletal system and manifest after the return of astronauts to Earth. However, the mechanisms underlying the development of motor disorders in cosmonauts remain largely uninvestigated to date. Clinical surveillance and analysis of the results of numerous studies indicate similarities between morphological and functional changes that develop under zero gravity and spinal cord and peripheral nerve injuries in patients with limited mobility [2,3]. This may be an effective method for better understanding underlying pathogenetic mechanisms.

Under the Bion M1 project, we have identified significant molecular changes in the spinal cord and sciatic nerve of mice after a 30-day orbital flight, which may play a vital role in the development of motor disorders [4]. Notably, significant changes in gene expression of the serotonergic neurotransmitter system were revealed. After the space flight and the subsequent 7-day recovery period, the expression level of the *Slc6a4* and *Htr2b* genes increased, and the expression level of the *Htr2a, Htr2c, Htr1a, Htr5a, Htr5b,* and *Htr7* genes decreased.

In previous studies, we examined the expression of genes encoding synaptic proteins in excitatory (i.e., cholinergic, glutamatergic) and inhibitory (i.e., glycinergic, GABA-ergic) neu-

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rotransmitter systems in the cervical and lumbar spinal cord of mice after 30 days of antiorthostatic suspension and 7 days of subsequent recovery. We found that the level of gene expression responsible for the state of these neurotransmitter systems in the spinal cord did not change when modeling the effects of hypogravity on Earth [5]. However, the serotonergic system modulates the functional activity of many neurotransmitter systems, including such as glutamatergic and GABA-ergic systems [6].

Serotonin (5-HT) is a monoamine neurotransmitter synthesized in various populations of neurons in the central nervous system (CNS) and is involved in modulating the activity of the spinal cord neural networks involved in locomotion. The CNS serotonergic nuclei have different projection zones and properties. In addition, the distribution of the various 5-HT receptor subtypes is not uniform. There are currently seven families of 5-HT receptors (5-$HT_{1–7}$), including at least 14 different receptor subtypes. Some of these receptors have been identified in the spinal cord [7].

1. 5-HT_1 receptors are localized mainly in the neurons of the dorsal horns of the spinal cord.

2. 5-HT_2 receptors are more abundant in the neurons of the ventral horns, namely on motor neurons.

3. 5-HT_3 receptors appear to be associated exclusively with nociception.

4. There is evidence of 5-HT_7 receptors in the cinerea intermediate zone [8].

For example, under certain pathological conditions, as a result of impaired descending serotonergic projections, depletion of 5-HT and dysregulation of 5-HT transporters can occur in the spinal cord. This, in turn, may lead to varying degrees of motor dysfunction, including paralysis. The mechanism of these changes remains inadequately studied. Many works address the role of 5-HT and its receptors in the implementation of autonomic, motor, and sensory functions of the CNS [9, 10].

The genes *Slc6a4, Tph2, Htr1b,* and *Htr2a* play an important role in the serotonergic system's functioning. The *Slc6a4* gene encodes a serotonin transporter, a membrane protein responsible the reuptake of serotonin from the synaptic cleft [11]. The *Tph2* gene encodes an enzyme that controls the synthesis of serotonin [12]. The *Htr1b* and *Htr2a* genes encode specific postsynaptic 5-HT receptors (5-HT_{1B} and 5-HT_{2A}) [13]. The 5-HT_{1B} receptor exhibits predominantly antinociceptive activity and is thus associated with inhibition of sensitivity [7, 14]. Stimulation of 5-HT_2 receptors increases the activity of motor neurons and facilitates mono- and polysynaptic reflexes. Activation of 5-HT_{2A} receptors in spinal cord injury activates the potassium-chlorine cotransporter KCC2, restore endogenous inhibition, and reduce spasticity [7, 15].

Fig. 1. A mouse in a state of hind limb unloading (antiorthostatic suspension model by Morey–Holton et al.).

Aims. In this study, we aimed to describe the level of the serotonergic neurotransmitter system gene expression (*Slc6a4, Tph2, Htr1b,* and *Htr2a*) in the cervical and lumbar enlargement of the spinal cord in mice after 30 days of replicating the effects of hypogravity using the antiorthostatic suspension model (Morey–Holton et al.) and subsequent 7-day recovery.

Materials and methods. This study was performed on male BALB/c mice (animals aged 6–7 weeks). All experimental procedures with animals were conducted in compliance with the Physiological Section of the Russian National Committee for Biological Ethics [16]. The work was approved by the Biomedical Ethics Commission of the Institute of Biomedical Problems (Protocol No. 319 dated 04/04/2013).

The experimental animals were divided into three groups:

– The Suspension group included mice which were supported so that their hind limbs were unloaded for 30 days (n = 5);

– The Recovery group included mice which were supported so that their hind limbs were unloaded 30 days followed by a readaptation period 7 days (n = 5);

– The control group included mice kept under standard vivarium conditions (n = 5).

In compliance with the methods described by Morey–Holton et al., experimental mice were suspended by the tail to allow for the unloading of weight from their hind limbs in a specially equipped cage (Fig. 1) [17]. The animal could move around the cage using their forelimbs and had free access to food and water. Each animal was housed in a separate cage. The duration of the antiortho-

Table 1. Sequences and thermodynamic characteristics of primers used to determine the level of expression of targeted genes

Gene name (RefSeq ID)	Product length, bp	GC%	T_m
Slc6a4 (NM_010484.2)			
F: GCCATCAGCCCTCTGTTTCT	351	55.00	60.03
R: TGGCTTAGAGGGGAGGAGTC		60.00	60.03
Tph2 (NM_173391)			
F: TTCCAGTCGGTGAGTTGTGG	157	55.00	59.89
R: CTGTGATGCAAAGCGTGGAG		55.00	59.83
Htr1b (NM_010482)			
F: AGTCTGTGGCAGCGACTAAAG	311	52.38	60.34
R: TCTCAGCCCAAAGGAGAGGT		55.00	60.18
Htr2a (NM_172812)			
F: ATGCAGTCCATCAGCAACGA	252	50.00	60.04
R: AATGTACCGTGAGAAGGCGG		55.00	60.11

Note: bp — base pairs.

static suspension and recovery corresponded to those in the Bion M1 project [18].

The animals were sacrificed by decapitation following inhalation anesthesia using isoflurane (Laboratorios Karizoo, S.A., Spain) and intraperitoneal administration of 3 mg/kg of zoletil 100 (Virbac Sante Animale, France). The spinal cord was isolated by hydraulic extrusion from the spinal canal, after which the areas of the cervical and lumbar enlargements were extracted. Spinal cord samples were taken simultaneously from control animals and the Suspension group. All samples were placed in liquid nitrogen immediately after isolation and further stored at −80°C.

The expression of genes that encode CNS synaptic proteins was studied using real-time polymerase chain reaction (RT-PCR). Total ribonucleic acid (RNA) was isolated from the spinal cord's lumbar and cervical enlargements using a commercial RNeasy Mini Kit (Qiagen) per the manufacturer's instructions. The synthesis of complementary deoxyribonucleic acid (cDNA) was performed using six-nucleotide random primers and RevertAid Reverse Transcriptase (Thermo Fisher Scientific). The concentration of the isolated RNA and the obtained cDNA was determined using a NanoDrop 2000 spectrophotometer (Thermo Scientific).

The quantitative analysis of the expression level was performed using a CFX96 thermal cycler (BioRad). The reaction mixture included qPCRmix-HS SYBR (Evrogen), primers specific to the target genes, and cDNA samples (Table 1). The design of primers specific to the regions of the target and reference genes was performed using the Primer3 4.1.0 software (Whitehead Institute for Biomedical Research) and the specificity of the primers obtained was tested using BLASTn (National Center for Biotechnology Information).

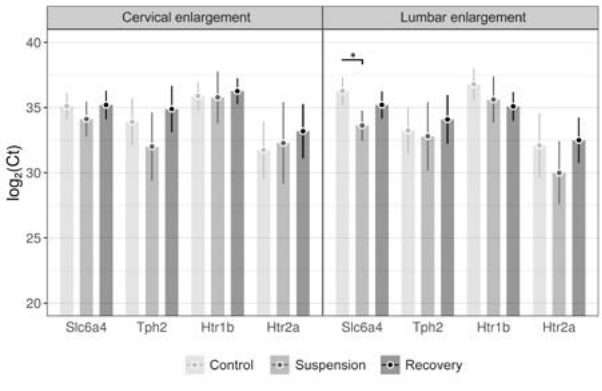

Fig. 2. Comparative analysis of gene expression of the serotonergic mediator system in the cervical and lumbar spinal cord of mice; *p = 0.0063.

The data obtained were analyzed using the environment for statistical computing R 3.6.3 (R Foundation for Statistical Computing) [19]. In order to analyze the relative expression of target genes, adjusted for the obtained values of the reference gene *Gapdh*, linear models with mixed effects (repeated observation covariance analysis, the unique identifier of the animal was included in the model as a random effect) were used, implemented in the lme4 1.1-21 package. The emmeans 1.4.5 package was used to assess the marginal Ct values, determine the boundaries of 95% confidence intervals for the marginal Ct values, and perform the Tukey procedure for testing hypotheses. Differences in the expression level between groups and between spinal cord regions within each group were considered statistically significant at $p < 0.05$.

Results and discussion. Analysis of the data obtained revealed combined changes in the nature of *Tph2* gene expression in the cervical and lumbar spinal cord (Fig. 2). There was an increase in the Suspension group by 3.7 and 1.4 times, respectively, compared with the control group, and a subsequent decrease by 2 and 2.5 times registered in the Recovery group compared to the Suspension group.

No significant differences were revealed between groups in the expression of the *Htr1b* and *Htr2a* genes in the cervical spine. Changes in Htr2a gene expression, similar to the *Tph2* gene, were noted in the lumbar spine. It was found that levels increased by 4.3 times in the Suspension group compared to the control group. A 5.7-fold decrease in the Recovery group compared to the "Suspension" group was seen as well. However, it should be noted that these differences were not statistically significant in expression levels of the *Tph2* ($p = 0.1803$; $p = 0.6005$), *Htr1b* ($p = 0.5561$; $p = 0.639$), and *Htr2a* ($p = 0.2543$; $p = 0.4363$) genes in the cervical and lumbar enlargement of the spinal cord (the *p*-values presented correspond to the main effects in regression models).

Comparing the nature of Slc6a4 gene expression revealed a statistically significant ($p = 0.0063$) increase of 6.3 times baseline in the lumbar spinal cord of animals after exposure to simulated hypogravity (Suspension group). This was followed by a 3-fold decrease during the recovery period (Recovery group) when compared to mice in the control group.

We did not find any statistically significant differences in the level of expression of target genes in the cervical and lumbar enlargement of the spinal cord. Differences in the level of expression of the *Slc6a4* gene in the cervical enlargement were less pronounced than those registered in the lumbar spine, namely a two-fold increase in the expression level in animals of the Suspension group as compared to the control group.

CONCLUSIONS

1. Our data on the expression of the *Slc6a4* gene, which encodes the serotonin transporter protein, suggests the involvement of the serotonergic neurotransmitter system in the pathogenesis of motor disorders in replicated hypogravity on Earth.

2. The changes in gene expression in the serotoninergic neurotransmitter system reported here suggest that they may modulate the spinal cord neural networks function under antiorthostatic suspension conditions.

3. There are data confirm a central role for the serotonergic system in the pathogenesis of motor disorders in spinal cord injury [20–22]. Data on the expression of the *Slc6a4* gene indicate that the serotonergic neurotransmitter system plays a role in minimizing negative consequences resulting from hypogravity and prolonged hypodynamia in bedbound patients with limited mobility.

Author contributions. M.S.K. conducted the study, collected, and analyzed the results; A.N.L. conducted the study, collected, and analyzed the results; M.A.D. performed RT-PCR; A.A.I. was the work supervisor.

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Conflict of interest. The authors declare no conflict of interest.

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REFERENCES

1. Edgerton V.R., Roy R.R. Invited review: gravitational biology of the neuromotor systems: a perspective to the next era. *J. Appl. Physiol.* 2000; 89: 1224–1231. DOI: 10.1152/jappl.2000.89.3.1224.

2. Hides J., Lambrecht G., Ramdharry G. et al. Parallels between astronauts and terrestrial patients — Taking physiotherapy rehabilitation "To infinity and beyond". *Musculoskelet. Sci. Pract.* 2017; 27 (1): S32–S37. DOI: 10.1016/j.msksp.2016.12.008.

3. Scott J.M., Warburton D.E.R., Williams D. et al. Challenges, concerns and common problems: physiological consequences of spinal cord injury and microgravity. *Spinal Cord.* 2011; 49: 4–16. DOI: 10.1038/sc.2010.53.

4. Kuznetsov M.S., Lisukov A.N., Rizvanov A.A. et al. Bioinformatic study of transcriptome changes in the mice lumbar spinal cord after the 30-day spaceflight and subsequent 7-day readaptation on Earth: New insights into molecular mechanisms of the hypogravity motor syndrome. *Front. Pharmacol.* 2019; 10: 747. DOI: 10.3389/fphar.2019.00747.

5. Lisyukov A.N., Izmaylov A.A., Kuznetsov M.S. et al. Spinal cord neuroplasticity in tail-suspended mice. *Aviakosmicheskaya i ekologicheskaya meditsina*. 2019; 53 (6): 94–97. (In Russ.) DOI: 10.21687/0233-528X-2019-53-6-94-97.

6. Perrin F.E., Noristani H.N. Serotonergic mechanisms in spinal cord injury. *Exp. Neurol.* 2019; 318: 174–191. DOI: 10.1016/j.expneurol.2019.05.007.

7. Cope T.C. *Motor neurobiology of the spinal cord*. 1 ed. CRC Press. 2001; 360 p.

8. Gackière F., Vinay L. Serotonergic modulation of post-synaptic inhibition and locomotor alternating pattern in the spinal cord. *Front. Neural. Circuits.* 2014; 8: 102. DOI: 10.3389/fncir.2014.00102.

9. Ghosh M., Pearse D.D. The role of the serotonergic system in locomotor recovery after spinal cord injury. *Front. Neural. Circuits.* 2014; 8: 151. DOI: 10.1016/j.expneurol.2019.05.007.

10. Bardoni R. Serotonergic modulation of nociceptive circuits in spinal cord dorsal horn. *Curr. Neuropharmacol.* 2019; 17: 1133–1145. DOI: 10.2174/1570159X17666191001123900.

11. Murphy D.L., Moya P.R. Human serotonin transporter gene (SLC6A4) variants: their contributions to understanding pharmacogenomic and other functional G×G and G×E differences in health and disease. *Curr. Opin. Pharmacol.* 2011; 11: 3–10. DOI: 10.1016/j.coph.2011.02.008.

12. Pratelli M., Pasqualetti M. Serotonergic neurotransmission manipulation for the understanding of brain development and function: Learning from Tph2 genetic models. *Biochimie.* 2019; 161: 3–14. DOI: 10.1016/j.biochi.2018.11.016.

13. Palacios J.M. Serotonin receptors in brain revisited. *Brain Res.* 2016; 1645: 46–49. DOI: 10.1016/j.brainres.2015.12.042.

14. D'Amico J.M., Li Y., Bennett D.J. et al. Reduction of spinal sensory transmission by facilitation of 5-HT1B/D receptors in noninjured and spinal cord-injured humans. *J. Neurophysiol.* 2013; 109: 1485–1493. DOI: 10.1152/jn.00822.2012.

15. Gackière F., Vinay L. Serotonergic modulation of post-synaptic inhibition and locomotor alternating pattern in the spinal cord. *Front. Neural Circuits.* 2014; 8: 102. DOI: 10.3389/fncir.2014.00102.

16. Genin A.M., Ilyin E.A., Kaplansky A.S. Bioethic rules of research with humans and animals in aviation, space and marine medicine. *Aviakosmicheskaya i ekologicheskaya meditsina*. 2001; 35 (4):14–20. (In Russ.)

17. Morey-Holton E.R., Globus R.K. Hindlimb unloading rodent model: technical aspects. *J. Appl. Physiol.* 2002; 92: 1367–1377. DOI: 10.1152/japplphysiol.00969.2001.

18. Andreev-Andrievskiy A., Popova A., Boyle R. et al. Mice in Bion-M 1 space mission: Training and selection. *PLoS One.* 2014; 9 (8): e104830. DOI: 10.1371/journal.pone.0104830.

19. *R: A Language and Environment for Statistical Computing. Foundation for Statistical Computing.* Austria, Vienna. 2017. Available online www.r-project.org (access date: 14.02.2019).

20. Bos R., Sadlaoud K., Boulenguez P. et al. Activation of 5-HT2A receptors upregulates the function of the neuronal K-Cl cotransporter KCC2. *Proc. Natl. Acad. Sci. USA.* 2013; 2 (110 (1)): 348–353. DOI: 10.1073/pnas.1213680110.

21. Gerin C.G., Hill A., Hill S. et al. Serotonin release variations during recovery of motor function after a spinal cord injury in rats. *Synapse*. 2010; 64 (11): 855–861. DOI: 10.1002/syn.20802.

22. Hayashi Y., Jacob-Vadakot S., Dugan E.A. et al. 5-HT precursor loading, but not 5-HT receptor agonists, increases motor function after spinal cord contusion in adult rats. *Exp. Neurol.* 2010; 221 (1): 68–78. DOI: 10.1016/j.expneurol.2009.10.003.