Effect of hindlimb unloading on stereological parameters of the motor cortex and hippocampus in male rats

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Hindlimb unloading (HU) can cause motion and cognition dysfunction, although its cellular and molecular mechanisms are not well understood. The aim of the present study was to determine the stereological parameters of the brain areas involved in motion (motor cortex) and spatial learning – memory (hippocampus) under an HU condition. Sixteen adult male rats, kept under a 12 : 12 h light–dark cycle, were divided into two groups of freely moving ($n = 8$) and HU ($n = 8$) rats. The volume of motor cortex and hippocampus, the numerical cell density of neurons in layers I, II–III, V, and VI of the motor cortex, the entire motor cortex as well as the primary motor cortex, and the numerical density of the CA1, CA3, and dentate gyrus subregions of the hippocampus were estimated. No significant differences were observed in the evaluated parameters. Our results thus indicated that motor cortical and hippocampal atrophy and cell loss may not necessarily be involved in the motion and spatial learning memory impairment in the rat. *NeuroReport* **27:1202–1205**

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Introduction

Hindlimb unloading (HU) is a ground-based model to mimic spaceflight microgravity in animals [1]. This model reproduces the absence of weight support on hindlimbs. Under such conditions, motor performance could change significantly and unloaded animals in comparison with space flight animals show similar atrophic changes in their bones and muscles [2]. As movement is mediated by muscular actions, which in turn are controlled by the nervous system, impaired movement as a result of HU could not only be because of changes in the contractile properties of the muscles but also long-term plastic changes in the neurons or networks of neurons involved in locomotor activity [3]. On the basis of the central effects of HU condition, Langlet *et al.* [4] reported that HU significantly decreased the hindlimb representation (−61%) on the motor cortex of the rat, suggesting that motor cortical maps might be more reactive to disuse or overuse than the somatosensory ones. Furthermore, perceptions of spatial orientation [5] and spatial learning and memory changes [6] under microgravity, and the hippocampus is critical in learning spatial orientation [7] and spatial cognition [8].

Since stereological parameters of relevant brain regions are altered by a variety of disorders [9,10] and treatments [11–14]. Thus, we hypothesized that the HU condition might exert its unfavorable effects on the motor cortex and hippocampus by alteration of volume and/or the numerical density of these structures. Therefore, in the present study, we estimated the stereological parameters of the motor cortex and hippocampus in freely moving rats and under HU.

Methods

The experiment was conducted in the Laboratory Animal Center and in compliance with the recommendations of the Animal Care Committee of the Aja University of Medical Sciences, Tehran, Iran. Sixteen randomly selected adult male Sprague–Dawley rats (200–300 g body weight) were housed in individual cages under controlled temperature (22°C), humidity (~40%), and lighting (12 : 12 h light–dark cycle) conditions, with free access to food and water. The rats were divided into two groups ($n = 8$/group) of freely moving [control (CTL) group] and HU (HU group) rats. HU was applied according to Morey-Holton and Globus [1]. Briefly, the rats were unloaded by the tail at about a 30° head-down angle to avoid contact of the hindlimbs with the ground and were allowed to walk freely on their forelimbs.

Perfusion and tissue preparation

After a period of 14 days of HU, the rats were anesthetized and perfused transcardially with 0.9% saline solution, followed by 10% buffered formalin. The brains were immediately removed, weighed, and postfixed in the

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same fixative solution overnight and immersed in 30% sucrose in PBS for 48 h and then stored at −80°C until further processing. Frozen brains were sectioned (30 μm) serially and coronally using a cryostat (SLEE Medical GmbH, Mainz, Germany). The sections were transferred to a 12-well plate containing a cryoprotectant solution at 90 μm intervals (every third section) and stored at −20°C until staining. All sections from the 10th well were mounted on a slide and stained with cresyl violet. Stained sections were compared with 'The Rat Brain in Stereotaxic Coordinates' atlas [15] to identify the motor cortex (both the primary and the secondary motor cortex; anterior–posterior 5.16 to −3.24 mm from the Bregma) and with the digital rat hippocampus atlas [16] to identify the hippocampus (anterior–posterior −1.72 to −6.94 mm from the Bregma).

Stereological studies

Volume of the motor cortex and hippocampus was estimated by the point-counting method using Cavalieri's principle [17]. Briefly, a grid of points was laid over the image of the section on the monitor of a computer and the points falling on both areas were counted. The reference volume (V_{ref}) of the motor cortex and the hippocampus was determined by applying the following formula: $V_{ref} = \Sigma P \times a(p) \times d$, where the ΣP is the sum of the number of points (P) counted, $a(p)$ is the area associated with each point, and d is the distance between two serial sections. A total of 300–400 points per brain were counted on each area. We used the ImageJ software (free download software from NIH website; National Institutes of Health, Bethesda, Maryland, USA) and Stereology Tools macros for generated grids.

The numerical density (N_v) of neurons in layers I, II/III, V, and VI of the motor cortex, entire motor cortex (all cortical layers included), and primary motor cortex (M1 also known as a granular lateral area, all cortical layers included) as well as the numerical density of neurons in CA1, CA3, and dentate gyrus (DG) regions of the hippocampus were estimated using the optical dissector technique. Here, the $N_v = \Sigma Q / (\Sigma P \times a(f) \times h)$ formula was used, where the ΣQ is the sum of the cells counted from each dissector frame, ΣP is the sum of the number of dissector frames counted, $a(f)$ is the known area associated with each dissector frame, and h is the known distance between two dissector planes as described previously [17]. An electronic microcator with a digital readout (MT12; Heidnehain, Traunreut, Germany) was used to measure the movements in the Z-direction with a 0.5-μm precision eclipse microscope (E200; Nikon, Tokyo, Japan) with a high-numerical-aperture (NA = 1.25) × 60 oil-immersion objective at Histomorphometry and Stereology Research Center, Shiraz University of Medical Sciences, Shiraz, Iran. The known distance between the two dissector planes (h) was 15 μm and the counting frame area was 30 × 30 μm.

The total number of (N) neurons in the motor cortex was calculated by multiplying the numerical density of the motor cortex by the volume of the motor cortex ($N = V_{ref} \times N_v$).

Statistical analysis

Data on body and brain weight, estimated volume, numerical density, and total neurons showed a normal distribution as determined by the Kolmogorov–Smirnov statistic. The parametric t-test was used to compare the difference between the experimental groups, with the level of significance set at P value less than 0.05. The mean ± SEM are reported in the text.

Results

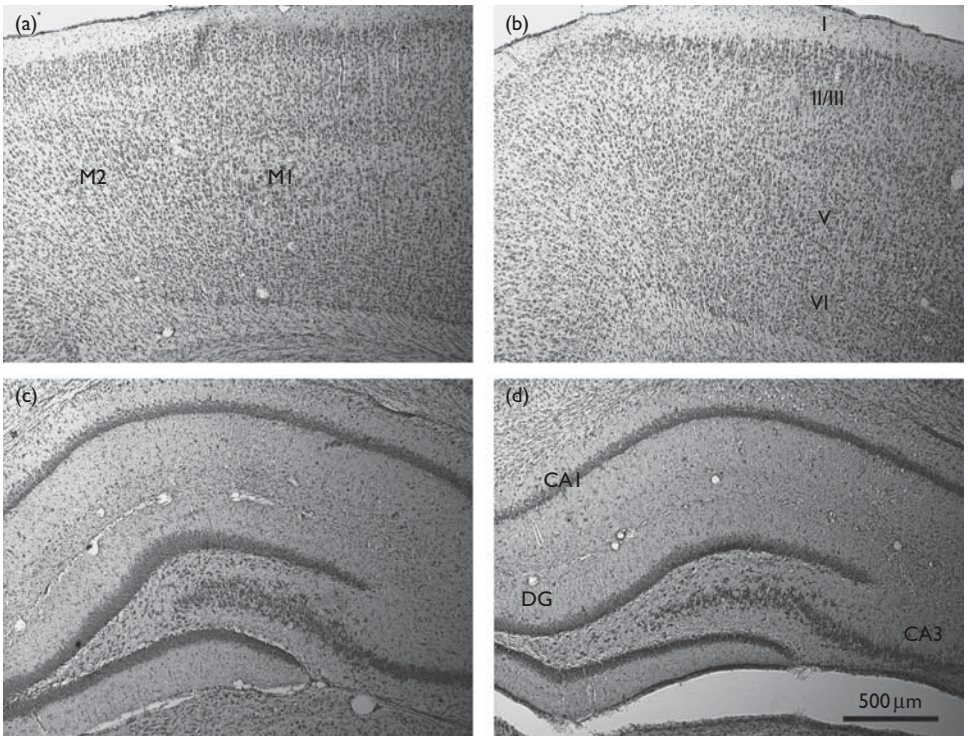
The representative micrographs, stained by cresyl violet in the motor cortex and the hippocampus of CTL and HU groups, are shown in Fig. 1. The motor cortex volume, numerical density of neurons in layers I, II–III, V, and VI of the motor cortex, the entire motor cortex, the primary motor cortex, and the total motor cortex neurons were not different ($P > 0.05$) between the freely moving and HU rats (Table 1). Similarly, no significant differences were recorded between the two experimental groups in terms of the hippocampal volume and the numerical density of CA1, CA3, and dentate gyrus subregions of the hippocampus (Table 2). The final body weight of the unloaded rats (227 ± 7 g) was lower ($P = 0.003$) than that of the control group (284 ± 13 g), but the brain weight was not affected by the treatment (1.34 ± 0.07 vs. 1.25 ± 0.07, respectively; $P > 0.05$).

Discussion

In the present study, we investigated the stereological parameters of the brain areas involved in motion (motor cortex) and spatial learning memory (hippocampus) under simulated microgravity. Under the HU condition, the hindlimbs do not make contact with the ground; therefore, the force imposed to the hindlimbs is suppressed. In this situation, the cutaneous receptors are not activated, the sensory input is reduced, and prolonged bed rest, immobilization, and even spaceflight in humans can be mimicked [18].

We estimated motor cortex volume, numerical density of neurons in layers I, II–III, V, and VI of the motor cortex, the entire motor cortex, the primary motor cortex, and total motor cortex neurons in hindlimb unloaded and freely moving rats. As the sections were stained with cresyl violet and examined under a light microscope, we omitted the layer IV when estimating the numerical density of this lamina because previous reports had shown that particular or targeted investigations may confirm the structural presence of the lamina IV in the rat motor cortex [19,20]. It was reported that HU as a non-traumatic situation considerably affected the cortical motor map as the hindlimb representation area was decreased by 61% [4]. In humans, immobilization of

Fig. 1



The representative micrographs (stained by cresyl violet) of the motor cortex and hippocampus in CTL and HU rats: (a) motor cortex in control; (b) motor cortex in HU; (c) hippocampus in control; (d) hippocampus in HU rat. CTL, control; DG, dentate gyrus; HU, hindlimb unloading; M1, primary motor cortex; M2, secondary motor cortex.

Table 1 Estimated motor cortex volume and numerical density of neurons in freely moving (control) and hindlimb unloading rats housed in a 12 : 12 h light–dark cycle

	CTL	HU	*P* value
Motor cortex volume (mm^3)	46.3 ± 1.3	43.4 ± 2.5	0.31
Layer I (10^3 cell/mm^3)	6.6 ± 0.3	6.6 ± 0.2	0.77
Layer II–III (10^3 cell/mm^3)	71.6 ± 3.6	74.4 ± 4.1	0.61
Layer V (10^3 cell/mm^3)	59.6 ± 3.4	52.9 ± 2.4	0.12
Layer VI (10^3 cell/mm^3)	36.0 ± 1.2	35.4 ± 1.4	0.75
Entire motor cortex (10^3 cell/mm^3)	66.3 ± 4.4	61.3 ± 2.2	0.21
Primary motor cortex (10^3 cell/mm^3)	65.1 ± 2.2	56.6 ± 3.94	0.089
Total motor cortex neurons (10^5)	30.8 ± 1.7	26.9 ± 2.5	0.21

Data are expressed as mean ± SEM.
CTL, control; HU, hindlimb unloading.

Table 2 Estimated hippocampus volume and estimated numerical neuron density in freely moving (control) and hindlimb unloading rats

	CTL	HU	*P* value
Hippocampal volume (mm^3)	83.6 ± 3.8	74.0 ± 3.5	0.09
CA1 (10^4 cell/mm^3)	83.8 ± 2.7	81.2 ± 3.1	0.56
CA3 (10^4 cell/mm^3)	52.3 ± 3.9	48.4 ± 3.5	0.49
Dentate gyrus (10^4 cell/mm^3)	152.0 ± 4.4	161.0 ± 4.0	0.13

Data are expressed as mean ± SEM.
CTL, control; HU, hindlimb unloading.

lower legs had no significant effect on the motor cortex area size of the tibial anterior muscle within the first days of immobilization, but there was a significant decrease in the motor cortex area size for the inactivated muscle after 4–6 weeks of immobilization [21].

The effects of HU, simulated microgravity, or spaceflight on the learning and memory abilities have been explored in previous studies. For instance, the HU condition induced an impairment in learning and memory in rats evaluated by the spatial learning test using a radial arm maze [6]. In addition, the perception of spatial orientation and mental performance could be influenced during spaceflight [5,22]. In the present study, no significant differences were observed between HU and CTL groups on the basis of all evaluated parameters in contrast to previously reported induced neuronal apoptosis in the cortex and hippocampus [6] and suppressed neurogenesis in the hippocampus [23] by the HU condition.

When we analyzed primary motor cortex individually, a reduction in the numerical density of neurons in HU rats was much more than that in the entire motor cortex in comparison with the control group, although this was not statistically significant ($P = 0.089$). As M1 represents paw movements (and presumably also tail, trunk, neck, jaw, and tongue movements) and the secondary motor cortex (M2, also known as a granular medial area) represents entirely and largely exclusively whisker movements [24], this decrease in neuronal density seems logical. In

addition, on the basis of studies by Schneider *et al.* [25–27] the effects found in microgravity could be secondary effects associated with psychological parameters, especially stress, which could impact on the motor performance and working memory, rather than the primary physiological effects of microgravity.

In the current investigation, following 14 days of HU, the mean body weight of HU rats was significantly lower than that of the control group, which is quite usual under such conditions [28]. This bodyweight loss might be because of context stress, although it has been reported that corticosterones were not elevated significantly after 14 [6] 21 [28] days of unloading.

Our results thus showed that motor cortical and hippocampal atrophy and cell loss may not necessarily be involved in the motion and spatial learning memory impairment in rats. Determination of stereological parameters of other structures such as the striatum and the cerebellum, which are involved in posture, as well as the sensorimotor cortex, could be useful. Furthermore, changes in the motor cortex neural or synaptic morphology under HU conditions cannot be ignored [29].

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Conflicts of interest

There are no conflicts of interest.

References

1 Morey-Holton ER, Globus RK. Hindlimb unloading rodent model: technical aspects. *J Appl Physiol (1985)* 2002; **92**:1367–1377.
2 Edgerton VR, Roy RR. Neuromuscular adaptations to actual and simulated spaceflight. In: Pollock DM, editor. *Comprehensive physiology*. John Wiley & Sons, Inc, USA; 2011. pp. 721–763.
3 Canu MH, Falempin M, Orsal D. Fictive motor activity in rat after 14 days of hindlimb unloading. *Exp Brain Res* 2001; **139**:30–38.
4 Langlet C, Bastide B, Canu MH. Hindlimb unloading affects cortical motor maps and decreases corticospinal excitability. *Exp Neurol* 2012; **237**:211–217.
5 Glasauer S, Mittelstaedt H. Perception of spatial orientation in microgravity. *Brain Res Brain Res Rev* 1998; **28**:185–193.
6 Sun XQ, Xu ZP, Zhang S, Cao XS, Liu TS. Simulated weightlessness aggravates hypergravity-induced impairment of learning and memory and neuronal apoptosis in rats. *Behav Brain Res* 2009; **199**:197–202.
7 Crusio WE, Schwegler H. Learning spatial orientation tasks in the radial-maze and structural variation in the hippocampus in inbred mice. *Behav Brain Funct* 2005; **1**:3.
8 Silvers JM, Tokunaga S, Berry RB, White AM, Matthews DB. Impairments in spatial learning and memory: ethanol, allopregnanolone, and the hippocampus. *Brain Res Brain Res Rev* 2003; **43**:275–284.
9 Morgan JT, Barger N, Amaral DG, Schumann CM. Stereological study of amygdala glial populations in adolescents and adults with autism spectrum disorder. *PLoS One* 2014; **9**:e110356.
10 Rubinow MJ, Mahajan G, May W, Overholser JC, Jurjus GJ, Dieter L, *et al.* Basolateral amygdala volume and cell numbers in major depressive disorder: a postmortem stereological study. *Brain Struct Funct* 2016; **221**:171–184.
11 Aslan H, Songur A, Tunc AT, Ozen OA, Bas O, Yagmurca M, *et al.* Effects of formaldehyde exposure on granule cell number and volume of dentate gyrus: a histopathological and stereological study. *Brain Res* 2006; **1122**:191–200.
12 Basta D, Tzschentke B, Ernst A. Noise-induced cell death in the mouse medial geniculate body and primary auditory cortex. *Neurosci Lett* 2005; **381**:199–204.
13 Namavar MR, Raminfard S, Jahromi ZV, Azari H. Effects of high-fat diet on the numerical density and number of neuronal cells and the volume of the mouse hypothalamus: a stereological study. *Anat Cell Biol* 2012; **45**:178–184.
14 Ragbetli MC, Aydinlioglu A, Koyun N, Ragbetli C, Bektas s, Ozdemir S. The effect of mobile phone on the number of Purkinje cells: a stereological study. *Int J Radiat Biol* 2010; **86**:548–554.
15 Paxinos G, Watson C. *The rat brain in stereotaxic coordinates*. New York, NY: Academic Press; 2007.
16 Kjonigsen LJ, Leergaard TB, Witter MP, Bjaalie JG. Digital atlas of anatomical subdivisions and boundaries of the rat hippocampal region. *Front Neuroinform* 2011; **5**:2.
17 Smith AM, Pappalardo D, Chen WJ. Estimation of neuronal numbers in rat hippocampus following neonatal amphetamine exposure: a stereology study. *Neurotoxicol Teratol* 2008; **30**:495–502.
18 Canu MH, Picquet F, Bastide B, Falempin M. Activity-dependent changes in the electrophysiological properties of regular spiking neurons in the sensorimotor cortex of the rat in vitro. *Behav Brain Res* 2010; **209**:289–294.
19 Alaverdashvili M, Hackett MJ, Pickering IJ, Paterson PG. Laminar-specific distribution of zinc: Evidence for presence of layer IV in forelimb motor cortex in the rat. *Neuroimage* 2014; **103**:502–510.
20 Skoglund TS, Pascher R, Berthold CH. The existence of a layer IV in the rat motor cortex. *Cereb Cortex* 1997; **7**:178–180.
21 Liepert J, Tegenthoff M, Malin JP. Changes of cortical motor area size during immobilization. *Electroencephalogr Clin Neurophysiol* 1995; **97**:382–386.
22 Manzey D, Lorenz B. Mental performance during short-term and long-term spaceflight. *Brain Res Rev* 1998; **28**:215–221.
23 Yasuhara T, Hara K, Maki M, Matsukawa N, Fujino H, Date I, *et al.* Lack of exercise, via hindlimb suspension, impedes endogenous neurogenesis. *Neuroscience* 2007; **149**:182–191.
24 Brecht M, Krauss A, Muhammad S, Sinai-Esfahani L, Bellanca S, Margrie TW. Organization of rat vibrissa motor cortex and adjacent areas according to cytoarchitectonics, microstimulation, and intracellular stimulation of identified cells. *J Comp Neurol* 2004; **479**:360–373.
25 Schneider S, Brummer V, Göbel S, Carnahan H, Dubrowski A, Struder HK. Parabolic flight experience is related to increased release of stress hormones. *Eur J Appl Physiol* 2007; **100**:301–308.
26 Schneider S, Brummer V, Carnahan H, Dubrowski A, Askew CD, Struder HK. What happens to the brain in weightlessness? a first approach by EEG tomography. *Neuroimage* 2008; **42**:1316–1323.
27 Schneider S, Brummer V, Mierau A, Carnahan H, Dubrowski A, Struder HK. Increased brain cortical activity during parabolic flights has no influence on a motor tracking task. *Exp Bain Res* 2008; **185**:571–579.
28 Knox M, Fluckey JD, Bennett P, Peterson CA, Dupont-Versteegden EE. Hindlimb unloading in adult rats using an alternative tail harness design. *Aviat Space Environ Med* 2004; **75**:692–696.
29 Trinel D, Picquet F, Bastide B, Canu MH. Dendritic spine remodeling induced by hindlimb unloading in adult rat sensorimotor cortex. *Behav Brain Res* 2013; **249**:1–7.