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MORPHOMETRIC STUDY OF HIPPOCAMPAL NEURONS IN CHRONIC IMMOBILIZATION STRESS

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Abstract:
Hippocampus ensures the implementation of the memory mechanisms, behavioral reactions, including avoidance of stress, aversive effects etc. The study was performed on the material of 20 male Wistar rats weighing 220-250 g, 10 of which were intact control group and 10 were experimental group, in which chronic immobilization stress was simulated. We determined the relative number of neurons in multiple fields of view on the total area of the pyramidal and polymorphic layers of CA1 and CA3 regions (further recalculated per 10,000 μm), larger and smaller diameters of neuron's bodies, their perimeters and areas with diameters of nuclei and nucleoli, nuclear-cytoplasmic ratio. It is found that under chronic immobilization stress in areas CA1 and CA3 of the hippocampus the morphologically similar neuronal lesions, decrease in their number, and change in nucleocytoplasmic ratio are observed.
Keywords: hippocampus, neurons, immobilization stress.

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МОРФОМЕТРИЧЕСКОЕ ИССЛЕДОВАНИЕ НЕЙРОНОВ ГИППОКАМПА ПРИ ХРОНИЧЕСКОМ ИММОБИЛИЗАЦИОННОМ СТРЕССЕ

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Аннотация. Гиппокамп обеспечивает реализацию механизмов памяти, поведенческих реакций, в том числе избегания стрессовых, аверсивных воздействий. Исследование выполнено на материале 20 крыс-самцов Вистар массой 220-250 г, 10 из которых составили интактную контрольную группу, 10 – экспериментальную, в которой моделировали хронический иммобилизационный стресс. Определяли относительное количество нейронов в нескольких полях зрения на полной площади пирамидного и полиморфного слоев областей CA1 и CA3 (с последующим пересчетом на 10000 мкм), больший и меньший диаметры перикарионов нейронов, их периметры, и площади, диаметры ядер и ядрышек, ядерно-цитоплазматическое соотношение. Установлено, что при хроническом иммобилизационном стрессе в областях CA1 и CA3 гиппокампа наблюдаются морфологически сходные повреждения нейронов, уменьшение их количества, изменение ядерно-цитоплазматического соотношения.
Ключевые слова: гиппокамп, нейроны, иммобилизационный стресс.

Hippocampus is one of the integrative brain structures that ensures the implementation of the memory mechanisms, behavioral reactions, including avoidance of stress, and aversive effects. It regulates the activity of the hypothalamic-pituitary-adrenal axis by both glucocorticoid-sensitive and glucocorticoid-insensitive ways. The hippocampus performs function of selecting and fixing the emotionally significant events [1, 2, 4, 15].

The studies carried out in the 80s of the last century shown the role of changes in hippocampal structures under stress. Five evidences of relation between stress and hippocampus were formulated: 1) the presence of mineral and glucocorticoid receptors in the hippocampus of animals and humans; 2) an inverted U-shaped curve of relationship between the levels of acute stress and memory; 3) stress-modulated inner excitability of the hippocampus and the activity-dependent synaptic plasticity associated with learning and memory; 4) relation of structural changes in CA3 region of the hippocampus to the time and intensity of chronic stress and increase in the level of stress hormones; and 5) reduction in the formation of nerve cells in ontogeny induced by stress and hormones. Initial studies were conducted on rats. The subsequent experiments on these and other animals, as well as human studies have given conflicting results. Some studies have shown that chronic stress or exposure to glucocorticoids induce the death of hippocampal neurons in rats [7, 12], shrews [9], and in primates [13], the other studies of this phenomenon have found this phenomenon neither in rats [3, 5, 14] nor in primates [10] or humans [11]. Therefore, the theory of the glucocorticoid cascade as the main mechanism of damage to hippocampal structure under stress, has not found any unconditional confirmation. Now it has been shown that glucocorticoids in stress play a role not so much the direct damaging factor as one that increases the sensitivity of hippocampal structures to other impacts [6].

One of the areas of current research is to find ways to correct impairments of integrative brain structures by using neurotropic, biologically active agents. In this context, we have investigated the effect of immobilization stress on the structural changes in the stress-sensitive CA1 and CA3 fields as a test model for further study of neurotropic drugs.

Materials and methods

The study was performed on the material of 20 male Wistar rats weighing 220-250 g, 10 of which were intact control group and 10 were experimental group, in which chronic immobilization stress was simulated. The animals were kept in cages per 10 animals under standard vivarium conditions with free access to food and water at 12-hour light regime and controlled temperature (22±2°C). The animals were stressed in the period from 9.00 a.m. to 2 p.m. The stress model was created by fixing the rats in the supine position in individual boxes for 2 hours every day for 5 days. At the end of the stress simulation the animals were removed from the experiment by exsanguination under ether anesthesia by drawing blood from the right ventricle. The study was conducted in compliance with the principles of the Helsinki Declaration on the humane treatment of animals, and in accordance with the decision of the regional ethics committee. Brains were removed completely from skull cavity and cut into four sections in the frontal plane. The material was fixed in 10% formalin solution by immersion method. To prepare the hippocampal preparation the corresponding frontal fragment was embedded in paraffin by standard procedure and stepped and serial sections of 4.0 μm thick were cut. Samples for histological examination and micropreparations were prepared by using a set of certified Leica equipment

(Germany). Sections for standard histological examination were stained with hematoxylin and eosin in the machine for histological sections and smears staining (Autostaniner XL ST5010; Leica, Germany), and with gallocyanin-chrome alum by Einarson's method, and thionine by Nissl's method. The main part of the morphological study was performed after the creation of an electronic image galley with the use of a semi-automatic scanner for micropreparations Mirax Desk (Carl Zeiss Microimaging GMbH, Germany). The morphometry was conducted with the use of Leica microscope software and the scanned image viewing program PannoramicViewer 1.15. We determined the relative number of neurons in multiple fields of view on the total area of the pyramidal and polymorphic layers of CA1 and CA3 regions (further recalculated per 10,000 μm), larger and smaller diameters of neuron's bodies, their perimeters and areas with diameters of nuclei and nucleoli, nuclear-cytoplasmic ratio. Each animal's linear and planimetric indices were measured at least 30 times. Quantitative data were recorded in MS Excel spreadsheets, which, as well as Statistica 6.0 were used for statistical processing. The reliability of the results was determined by Student's t test (t) and χ^2 with a confidence level of $p<0.05$.

Results and discussions. CA1 and CA3 regions in intact animals are clearly defined both topographically and by the typical structure of neuronal layers, especially pyramidal one. The obtained morphological patterns corresponded with the descriptions of cytoarchitectonic features of rat hippocampus described in the literature [8]. CA1 region is formed from middle densely spaced neurons with the average minimum size of 10.57+0.17 μm, maximum 16.03+0.28 μm, and with the average perikaryonic area of 139.24+4.47 μm^2. CA3 region consists of large, widely spaced neurons, which corresponding parameters are on average 12.10+0.20 μm, 20.43+0.29 μm, and 190.65+4.41 μm^2. The differences in the spatial density and number of neurons in these regions are distinguished both visually and upon morphometry. Their amount in the CA1 region varies from 33.3 to 67.0 per 10,000 μm^2 (average 51.2+4.5), in the CA3 region from 13.3 to 20.0 μm^2 (average 17.2+3.2), with significant differences ($p<0.05$). The diameters of the CA1 neuron cores were on average 36+0.13 μm, while the same of CA3 region were proportionally larger (8.85+0.17 μm), and did not differ significantly. Nuclear-cytoplasmic ratio (NCR) was also similar: 0.32+0.04 and 0.33+0.06. As the functional activity of neurons, we assessed the number of cells containing two nucleolus in the nucleus, which was 30.0% in the CA1 region and 22.0% in CA3 region.

During immobilization stress, we revealed some qualitative and quantitative changes in the neurons of the studied regions of rat hippocampus. Qualitative signs of damage are a typical picture of disorganization of neuronal layers, chromatolysis phenomena, polymorphic changes in nuclei such as swelling and pyknomorphic changes. Apical dendrites of CA3 large pyramidal neurons, clearly defined directly in the perikaryons are normal, but deformed and have «amputated» appearance in the stressed animals. The presence of the stress-caused damages in the hippocampus structures is confirmed by shifts in the morphometric parameters of the neurons. The maximum size of perikaryons decreased, in the CA3 region to a greater extent (up to 17.68+0.19 μm). Perikaryonic area of CA3 neurons decreased significantly ($p<0.05$) to 164.6+2.38 μm^2. The cell bodies lose their contour sharpness, and deformed. Given the fact that the largest size of the pyramidal neurons coincides with the orientation axis of the apical dendrite. Its reduction together with a reduced area of perikaryons may correspond to the described in the literature phenomenon of the stress-induced dendrite retraction [6]. The diameters of the CA1 neuronal nuclei insignificantly increased up to 7.83+ 0.09 μm, while the same of CA3 region decreased up to 8.16+0.10 μm, NCR at the same time did not changed in comparison with the indices in the intact animals. The most significant changes, reflecting the destruction and loss of the cellular composition of the hippocampus CA1 and CA3 regions, were determined when calculating the specific number of cells. Under chronic immobilization stress, it significantly decreased in the CA1 region up to 33.3+4.1 ($p<0.05$), in the CA3 region up to 14.7+2.7 per 10,000 μm^2, not differing from intact control. In the presence of synchronous changes in nuclei and perikaryons, reflected in unidirectional changes in their morphometric parameters, the indicator of the impaired functional activity of neurons is a revealed decrease in the number of binucleolar cells: a decrease in CA1 was up to 5.4% ($p<0.05$ by χ^2), in CA3 – 3.8% ($p <0.05$ by χ^2). This type of change from a functional point of view is consistent with the changes in neuronal processes that affect perikaryonic planimetric indices, the shifts in the density of synaptic contacts, and the decreased activity of synaptic transmission with the impaired interneuronal integration [15].

Thus, the chronic immobilization stress, simulated by the above method, leads to the development of signs of neuronal damage in the critical CA1 and CA3

regions of the hippocampus, manifesting themselves both qualitatively and quantitatively. The nature and extent of changes in the used model was similar in CA1 and CA3 hippocampal regions. We revealed the signs of the loss of neuronal composition in the studied regions, the differences from intact indicators in planimetric indices of perikaryons, as well as manifestations of reduced functional activity of neurons at the level of the nuclear transcription apparatus. The obtained results justify the methodological approach applied to the morphological study of the correction of damaged hippocampal structures under stress.

References

1. Umrikhin A.E. Hippocampal neurotransmitter mechanisms of stress behavior and avoidance reactions // Bulletin of new medical technologies (electronic edition). 2013. No. 1. P. 55.

2. Bartsch T., Döhring J., Rohr A., Jansen O., Deuschl G. CA1 neurons in the human hippocampus are critical for autobiographical memory, mental time travel, and autonoetic consciousness // PNAS. 2011. Vol. 108, N 42. P. 17562-1756.

3. Bodnoff S.R., Humphreys A.G., Lehman J.C., Diamond D.M., Rose G.M., Meaney M.J. Enduring effects of chronic corticosterone treatment on spatial learning, synaptic plasticity, and hippocampal neuropathology in young and mid-aged rats // J Neurosci. 1995. Vol. 15. P. 61-69.

4. Clark R.E., Broadbent N.J., Larry R. Squire Hippocampus and Remote Spatial Memory in Rats // Hippocampus. 2005. 15(2). P. 260 – 272.

5. Coburn-Litvak P.S., Tata D.A., Gorby H.E., McCloskey D.P., Richardson G., Anderson B.J. Chronic corticosterone affects brain weight, and mitochondrial, but not glial volume fraction in hippocampal area CA3 // Neuroscience. 2004. Vol. 124, N 2. P. 429-438.

6. Conrad C.D. Chronic Stress-Induced Hippocampal Vulnerability: The Glucocorticoid Vulnerability Hypothesis// Rev Neurosci. 2008. Vol. 19, N 6. P. 395-411.

7. Dachir S., Kadar T., Robinzon B., Levy A. Nimodipine's protection against corticosterone induced morphological changes in the hippocampus of young rats // Brain Res. 1997. Vol. 748, No. 1-2. P. 175-183.

8. El Falougy H., Kubikova E., Benuska J. The microscopical structure of the hippocampus in the rat // Bratisl. Lek Listy. 2008. Vol. 109, No. 3. P. 106-110.

9. Fuchs E., Flügge G., Ohl F., Lucassen P., VollmannHonsdorf G.K., Michaelis T. Psychosocial stress, glucocorticoids, and structural alterations in the tree shrew hippocampus // Physiol Behav. 2001. Vol. 73. P. 285-291.

10. Leverenz J.B., Wilkinson C.W., Wamble M., Corbin S., Grabber J.E., Raskind M.A., Peskind E.R. Effect of chronic high-dose exogenous cortisol on hippocampal neuronal number in aged nonhuman primates // J Neurosci. 1999. Vol. 19. P. 2356-2361.

11. Müller M.B., Lucassen P.J., Yassouridis A., Hoogendijk W.J.G., Holsboer F., Swaab D.F. Neither major depression nor glucocorticoid treatment affects the cellular integrity of the human hippocampus // Eur J Neurosci. 2001. Vol. 14. – P. 1603-1612.

12. Sapolsky R.M., Krey L.C., McEwen B.S. Prolonged glucocorticoid exposure reduces hippocampal neuron number: Implications for aging // J Neurosci. 1985. Vol. 5. P. 1222-1227.

13. Sapolsky R.M., Uno H., Rebert C.S., Finch C.E. Hippocampal damage associated with prolonged glucocorticoid exposure in primates // J Neurosci. 1990.Vol. 10. P. 2897-2902.

14. Sousa N., Madeira M.D., Paula-Barbosa M.M. Effects of corticosterone treatment and rehabilitation on the hippocampal formation of neonatal and adult rats. An unbiased stereological study // Brain Res. 1998. Vol. 794, N 2. P. 199-210.

15. The hippocampal book / Edited by: P. Andersen, Morris R., Amaral D., Bliss T., 'O Keefe J. Oxford University Press. 2007. 832 p.