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Age-Related Changes in Biogenic Amine Content and Oxidative Stress Profile in Rat Hypothalamus with Hyperhomocysteinemia

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Abstract—The article presents a detailed analysis of correlations between the content of a variety of biogenic amines in the hypothalamic structures responsible for the gonadotropin-releasing hormone synthesis and secretion (the medial preoptic area and median eminence), and such independent factors as total L-homocysteine plasma level elevation induced by L-methionine loading and aging. Both the nature and pattern of changes in oxidative stress profile were evaluated. It was shown that aging, when compared to hyperhomocysteinemia, is a determining factor influencing the biogenic amine content in the studied hypothalamic structures. Unlike the antioxidant defense system profile, no considerable changes in macromolecule oxidative modification were found, which is evidence of a balanced activity of pro- and antioxidant systems in the hypothalamus.

Keywords: L-homocysteine, aging, hypothalamus, biogenic amines, oxidative stress

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INTRODUCTION

Changes in the level of different biogenic amines are considered to play a key role in the mechanisms determining age-related changes in the neuroendocrine system that lead to loss of reproductive function. Decline of this function is accompanied by a gradual decrease in the level of biogenic amines [7, 39]. However, this change is nonspecific and similar effects can be observed under exposure to several neurotoxic xenobiotics, such as toluene and 1,2-dimethylhydrazine [1, 7].

Homocystein (HC) also belongs to compounds with neurotoxic properties [2, 54]. Many researchers have noted that HC serum levels increase with age in humans [22, 42], along with an increase in the intensity of free radical processes [24, 35, 53]. Thus, hyperhomocysteinemia (HHC) relates to the development of neurodegenerative and other age-associated diseases. However, the HC level does not rise with age in laboratory rats, in contrast to human beings [34]. This allows the use of an experimental HHC model to analyze the influence of age and HC as independent factors on the change in the biogenic amine content in the hypothalamus in female rats, which was the goal of the present study.

MATERIALS AND METHODS

The study was performed on 60 Wistar female rats of various ages. The animals were kept in a vivarium with artificial ventilation and a controlled light regime (daytime of 6:00 a.m. to 6:00 p.m.; nighttime of 6:00 p.m. to 6:00 a.m.). The rats received a standard diet and water. In the experiment, the animals were divided into two age groups: young pubescent, female rats with regular cycles aged 6–7 months (n = 41) and 22–24 month-aged rats, the estrous cycle had completely terminated and they were in the persistent diestrus stage (n = 19). Both age groups of animals were divided into two subgroups: the first (control) group included females that received ordinary water orally via a catheter daily for 30 days; the second (experimental) group included animals that received methionine at a concentration of 0.6 mg/kg body weight orally via a catheter daily for 30 days. This study was performed in accordance with the principles of the Helsinki Declaration on humane treatment of animals described in the European Community Directive number 86/609EU.

The estrous cycle stages were determined by vaginal smears. The isolated hypothalamuses were frozen and stored at –80°C. Quantitative analysis of noradrenaline (NA), dopamine (DA), and a serotonin metabolite (5-hydroxyindoleacetic acid (5-HIAA)) was performed with HPLC separately for the medial preoptic area (MPA) of the hypothalamus and the median eminence (ME) with arcuate nuclei [38].

The HC in blood serum was determined by IEA with Axis-Sheld Systems (Great Britain). The level of ROS production in the hypothalamus was evaluated from content of metabolites, which are formed by oxidative modification of macromolecules; the content of

nitrotyrosine—the nitration product of L-tyrosine amino acid formed with active participation of NO metabolites such as $OONO^-$ and $NO_2^{\bullet}$, and by 8-hydroxy-2'-deoxyguanosine (8-OH-dG), since guanine is the most easily oxidized base among the four DNA bases. To determine the antioxidant status in the hypothalamus, the ascorbic acid content and SOD activity were measured in female rats. In this study, we used test systems for enzyme immunoassay and colorimetric assays: DNA Damage (8-hydroxy-2'-deoxyguanosine) Enzo Life Sciences (United States); Nitrotyrosine—Hycult Biotech (Netherlands); Vitamin C (ascorbate)—Immunodiagnostik (Germany); and Superoxide Dismutase—Cayman Chemical Company (United States). The content of the test substances was calculated in ng/mg protein, which was determined by the Vera method.

Statistical analysis was performed with the Statistica 10.0 software package. The difference significance in the study groups was determined with the nonparametric Mann–Whitney U-test. The data are presented as medians (25–75‰). Differences were considered significant at $p < 0.05$. The Pearson's correlation coefficient was used to determine the relationship between the parameters based on the strength of an association. For a comprehensive and compact description of the objects under study, multivariate factor analysis was applied, which is the best way to describe the real behavior of the study population characteristics and to evaluate the reliability and accuracy of the conclusions drawn on the basis of the obtained data. We calculated factor loadings (a), which are interpreted as the correlations between the studied parameters and individual factors (hypothetical, not directly measurable, hidden signs associated with measurable indicators in some way). The higher is the load in absolute value, the greater is the association of variable with the factor and the greater this variable is determined by the action of the respective factors. The number of the factors to retain was determined based on the Scree plot [3, 32]. The principal component analysis was the method to determine the factors to retain; the data were normalized for the analysis.

RESULTS AND DISCUSSION

The results of data analysis on all parameters studied are presented in Table 1. Results of the study showed that methionine intake, according to the used scheme of administration, leads to the development of HHC, a condition characterized by increased serum levels of HC, with a greater rise of HC serum levels in rats aged 22 to 24 months than in pubescent animals.

When analyzing the data of biogenic amine assay it was found that administration of methionine did not lead to significant changes of biogenic amine contents versus a control group of the same age. However, the biogenic amine content significantly decreased with age, both during physiological aging and during administration of methionine.

In our study, parameters that are markers of nucleic acids damage (8-hydroxy-2'-deoxyguanosine) and oxidative modification of proteins (nitrotyrosine) had no significant differences between the control subgroup and the subgroup with methionine loading, and there were no differences between young and old animals. It was shown that the ascorbic acid content was significantly lower in old animals in the control subgroup aged 22–24 months as compared with rats aged 6–7 months. It showed an observed tendency to decrease in rats subjected to forced oral administration of methionine. It was found that the SOD activity in young animals of the control subgroups was significantly higher than in the experimental subgroups after methionine loading. However, the intensity of SOD activity decreased by 22–24 months in rats without HHC, and the significant difference between the control and experimental animals disappears, retaining only the nature of a tendency.

With assessment of the relationship between indicators examined by the Pearson's rank correlation, it was shown that only the ascorbic acid content in the hypothalamus correlates in young animals with HC serum levels ($r = 0.75$; $p < 0.01$). Meanwhile, significant correlations between the studied parameters in the hypothalamus and HC serum level were absent in old animals. However, both low (for example, between NA and 5-HIAA in ME) and relatively high (between NA in MPA and NA in ME) significant correlations between the different neurotransmitters and a weak but significant correlation of these parameters with the content of ascorbic acid in the hypothalamus were observed. No significant relationships between the biogenic amine content and indicators of the oxidative modification of macromolecules and SOD or between individual indicators of oxidative stress (OS), were detected (Table 2).

For combined information about the structure of relationships between the studied parameters and the choice of the most significant of them, factor analysis was carried out to describe the influence of different conditions in the experiments (figure). It was found in evaluation of the load that the first factor was most closely associated with the age of the animal ($a = -0.93$), as well as NA in ME ($a = 0.92$), 5-HIAA in MPA ($a = 0.91$), NA in MPA ($a = 0.90$), and DA in ME ($a = 0.81$). Moreover, this factor was significantly more weakly associated with a change in such parameters as 5-HIAA in ME ($a = 0.72$), DA in MPA ($a = 0.67$), and ascorbic acid in the hypothalamus ($a = 0.66$). Changes in the serum HC level were insignificantly determined by the action of the retained factor ($a = -0.52$). Change in the 8-OH-dG is associated with the second factor ($a = -0.76$), which also insignificantly influences the change of such indicators as SOD ($a = 0.54$),

Table 1. The studied parameters in homogenates of hypothalamus and its structures in experimental groups, medians (25–75‰)

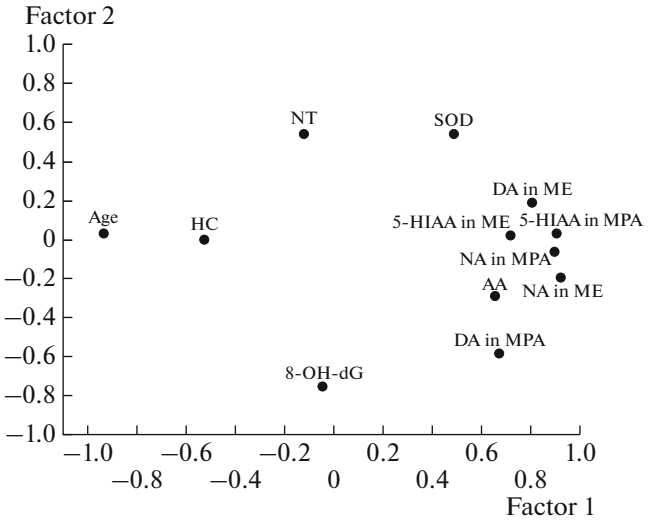
Parameter	Young rats of 6–7 months of age, $n = 41$		Old rats aged 22–24 months, $n = 19$	
	control subgroup	experimental subgroup	control subgroup	experimental subgroup
Homocysteine, μmol/L	6.2 (5.0–7.3)	10.0 (6.7–30.6)***	6.0 (5.4–6.7)	57.20 (14.3–118.8)***
NA in ME, ng/mg of protein	25.6 (28.6–40.6)	25.5 (29.3–41.0)	10.8 (10.1–12.8)**	11.1 (9. 6–15.8)**
5-HIAA in ME, ng/mg of protein	6.4 (4.4–7.2)	6.2 (4.9–7.8)	3.4 (3.0–5.1)*	3.4 (1.8–5.3)*
DA in ME, ng/mg of protein	30.3 (21.8–35.9)	26.8 (24.1–31.8)	13.4 (9.5–15.4)**	14.3 (8.9–16.4)**
NA in MPA, ng/mg of protein	28.1 (21.4–33.1)	29.3 (25.0–36.6)	6.2 (4.8–7.9)**	7.5 (5.1–9.0)**
5-HIAA in MPA, ng/mg of protein	4.0 (3.4–5.6)	4.1 (3.1–4.9)	1.7 (1.4–2.8)**	1.9 (1.7–2.1)**
DA in MPA, ng/mg of protein	11.5 (8.8–16.5)	13.6 (10.6–15.6)	5.3 (2.8–7.0)**	4.2 (3.3–5.1)**
8-OH-dG, ng/mg of protein	8.8 (7.8–9.1)	8.4 (8.2–9.1)	8.8 (8.7–9.1)	8.6 (8.3–9.1)
Nitrotyrosine, nmol/mg of protein	13.2 (12.8–14.1)	12.5 (11.6–13.5)	13.7 (13.6–13.9)	3.6 (12.5–15.1)
Ascorbic acid, ng/mg of protein	36.3 (34.6–40.4)	38.7 (36.6–39.5)	32.0 (26.8–35.0)*	1.6 (27.7–37.6)
SOD, U/mg of protein	0.98 (0.58–1.13)	0.36 (0.26–0.52)***	0.51 (0.40–0.68)*	0.30 (0.19–0.60)

* $p < 0.05$; ** $p < 0.001$—difference significance between young and old animals in the respective groups; *** $p < 0.001$—difference significance of the subgroup with methionine loading from control subgroup.

Table 2. Correlation coefficient matrix of the study parameters

Parameters	NA in ME	5-HIAA in ME	DA in ME	NA in MPA	5-HIAA in MPA	DA in MPA	SOD	Ascorbic acid	8-OH-dG	Nitro-tyrosine
NA in ME	1.00	0.65*	0.74*	0.88*	0.78*	0.74*	0.26	0.62*	0.10	–0.13
5-HIAA in ME	0.65*	1.00	0.73*	0.49*	0.66*	0.37	0.37	0.45	0.24	0.10
DA in ME	0.74*	0.73*	1.00	0.63*	0.75*	0.30	0.30	0.46*	–0.01	0.17
NA in MPA	0.88*	0.49*	0.63*	1.00	0.76*	0.64*	0.29	0.63*	–0.16	–0.20
5-HIAA in MPA	0.78*	0.66*	0.75*	0.76*	1.00	0.54*	0.50	0.49*	–0.04	–0.27
DA in MPA	0.74*	0.37	0.30	0.64*	0.54*	1.00	0.12	0.61*	0.38	–0.28
SOD	0.26	0.37	0.30	0.29	0.50*	0.12	1.00	0.24	–0.28	0.14
Ascorbic acid	0.62*	0.45	0.46*	0.63*	0.49*	0.61*	0.24	1.00	0.19	–0.08
8-OH-dG	0.10	0.24	–0.01	–0.16	–0.04	0.38	–0.28	0.19	1.00	–0.11
Nitrotyrosine	–0.13	0.10	0.17	–0.20	–0.27	–0.28	0.14	–0.08	–0.11	1.00

* $p < 0.01$—correlation significance.



Two-dimensional graph of factor loadings of the study parameters. DA in ME—dopamine in the median eminence with arcuate nuclei; 5-HIAA in ME—5-hydroxyindoleacetic acid in median eminence with arcuate nuclei; NA in ME—noradrenaline in median eminence with arcuate nuclei; DA in MPA—dopamine in the medial preoptic area; 5-HIAA in MPA—5-hydroxyindoleacetic acid in the medial preoptic area; NA in MPA—noradrenaline in the medial preoptic area; AA—ascorbic acid; HC—homocysteine; NT—nitrotyrosine; 8-OH-dG—8-hydroxy-2-deoxyguanosine.

nitrotyrosine in the hypothalamus ($a = 0.54$), and DA in MPA (−0.59).

Thus, it is shown that a change in, for example, DA in MPA, as well as of most other biogenic amines in hypothalamic structures, depends on and differs significantly in relation to the experimental conditions associated with the study of age-related changes in the hypothalamus. In addition, such a change also depends on another factor that is associated with neither the HHC under study nor the aging process and is characterized by a change of OS parameters.

Age-related changes, particularly the decreased absorption of B vitamins in the gastrointestinal tract, as well as several other causes that ultimately can cause disruption of the normal functioning of the methionine cycle (especially with genetic predisposition), lead to an increase in serum HC level and development of HHC [54, 56]. This condition further contributes to the development of pathological processes in the body and causes the appearance of different diseases at elder and senile age. It is known that HC is an amino acid, the toxic effects of which are manifested as follows: oxidation, hypomethylation, protein homocysteinization [51], metalloproteinase activation, and telomere damage [43]. All of these pathological processes are also associated with the aging process. HHC is known to have an adverse effect on the regulation of vascular tone, lipid metabolism, and the coagulation cascade. Elevated HC levels are observed in the diagnosis of many geriatric pathologies, including broken bones caused by osteoporosis [31], coronary atherosclerosis, stroke, Alzheimer's disease [46], senile dementia, and neurological and psychiatric dysfunction [40, 41, 50]. There is evidence that the HC level increases in blood in women after menopause [21]; however, other authors demonstrated that the age rather than menopausal status was the determining factor in the increase of HC plasma levels in pre- and postmenopausal women of different ages [13].

HC is known to easily penetrate the blood-brain barrier [9, 14, 41], where ionotropic and metabotropic glutamate receptors are involved in the implementation of its neurotoxic effects [2, 5, 60].

The modern literature contains quite a large number of works describing mechanisms of HC action that cause neuronal death—OS induction, DNA damage, and activation of pro-apoptotic factors [8, 14, 30, 35, 44, 57]. Studies involving cerebellar cell culture showed that HC activates NMDA-receptors, thereby increasing the ionized calcium level and increased ROS production in neurons [10]. This leads to the development of OS, which is considered one of the causes of the toxic effect of HC [51]. It was noted that the incubation of neurons with SOD and catalase antioxidant enzymes reduces the negative effect of HC [29].

One of the most significant manifestations of the toxic effect of HC is oxidative damage to DNA [24, 35]. It was found that it is caused by increased activity of nucleases under the influence of ROS, primarily of hydroxyl radicals [35], as well as superoxide and hydrogen peroxide [29] formed by autooxidation of HC [16]. It was shown that the accumulation of homocysteic acid triggers apoptosis in neurons [2, 5]. Furthermore, in the presence of adenosine, HC is effectively converted to adenosine homocysteine, thus lowering the methylation level of molecules, which is necessary for the metabolism of nucleic acids, amines, or other neurotransmitters [33, 37, 59].

It is also known that the function of NO-synthase is disturbed under HHC. This results in the accumulation of a powerful oxidant, peroxynitrite [17, 58], which also leads to DNA damage [15]. Methionine added to the diet of pregnant rats, by leading to the development of HHC, causes oxidative stress in the brain of the offspring, SOD deficiency, and increased susceptibility of neurons to death [6]. We have previously shown that the administration of methionine to pregnant females results in the development of prenatal HHC and an increase of the 8-OH-dG level in the brain of newborn rats, a marker of nucleic acid damage, and a decrease in SOD activity [45].

In this study, which was performed on female rats of different ages, the 8-OH-dG content in the hypothalamus did not change with the methionine loading. Oxidative DNA damage correlated with the rate of glutathione oxidation, which, for example, increases progressively with age in the hippocampus, cortex but

not in the hypothalamus [28]. HC inhibits the activity and expression of several antioxidant enzymes, namely SOD, heme oxygenase-1, and glutathione peroxidase [48, 55]. In young animals methionine administration caused a significant decrease in SOD activity, which may be due to its inactivation with peroxynitrite [23], as well as a decrease in the total amount of the enzyme for consumption on the utilization of superoxide radicals, which are generated during HHC.

The enzymatic activity of antioxidants, especially of SOD [4], is known to decrease with age, which is confirmed by the data obtained in our study involving 22–24 month old animals; however, methionine only slightly reduced the activity of the enzyme. Meanwhile, one cannot suggest that the effect of HHC in old animals is accompanied by a lower accumulation of ROS. Perhaps, these results are explained by the fact that the role of the other components of the antioxidant defense strengthens with age with a sufficiently reduced activity of SOD. It was noted that old animals show an increase in the number of glutathione peroxidase in mitochondria [27], i.e., the effectiveness of antioxidant defense increases.

Furthermore, according to our data, the total HC concentration in blood plasma cannot serve as a clear criterion for evaluation of oxidative stress in the hypothalamus of animals subjected to methionine loading. Despite the good permeability of methionine through the blood-brain barrier, data on the HC content in the hypothalamus could be more informative, since HC exerts most of its toxic effects directly in the vessels, affecting endothelial cells, vascular smooth muscle cells, and blood cells, upon increasing HC concentrations in the blood [2, 18, 20]. Despite the neurotoxic effects of HC described in the literature, it is considered a weak neurotoxin [60]. The cytotoxic effect in vitro is described only at a HC concentration exceeding 0.1 mmol/L [29, 54]. It is also possible that this model of HHC does not lead to persistent violation of methionine cycle in adult and in old rats. The normal function of enzymes involved in methionine metabolism, even with long-term excessive methionine intake, gradually metabolizes excess HC in the body and prevents its spontaneous oxidation, avoiding the accumulation of homocysteic acid, homocysteine-thiolactone, and other products that mediate marked toxic effects of HHC [25, 26].

Furthermore, under the influence of methionine loading, we found no association with changes in the biogenic amine content in the hypothalamus structures. However, there is evidence that a reduced dopamine content was observed in the cerebral cortex in rats with a high methionine consumption [19]. It should be noted, for example, that another neurotoxic compound, dimethyl hydrazine, the metabolism of which results in a large amount of free radicals that have a damaging effect on the brain and spinal cord cells [47], only slightly changed the biogenic amine content in these structures, disrupting their normal daily dynamics [38].

The relationship of biogenic amines and the level of ROS generation also remains poorly investigated. There is a lot of information that catecholamines in the brain, in particular, dopamine, undergoing autooxidation and enzymatic oxidation under the influence of monoamine oxidase, may be a source of ROS [11, 59]. The fact that mainly H_2O_2 is normally formed during oxidation of biogenic amines can explain the correlation between the contents of biogenic amines and ascorbic acid, which we have detected. Ascorbic acid is known to have several functions in the brain and neurons. As an antioxidant, it catalyzes the reduction of iron present in the brain in a large amount. The bivalent ions react rapidly with peroxide radicals. In addition, ascorbic acid enhances catecholamine biosynthesis [49], because, on the one hand, it is an electron donor for the dopamine-β-hydroxylase [12], and, on the other, it promotes regeneration of tetrahydrobiopterin, which is a cofactor of tyrosine hydroxylase in the synthesis of L-3,4-dihydroxyphenylalanine [36, 49]. Our results also confirmed that a change in these parameters has a distinct correlation with age. In the literature, there is evidence that in MPA, as in other structures of the hypothalamus, a significant reduction in the biogenic amine content occurs with age [39, 52], which is probably associated with a change of reproductive steroids.

CONCLUSIONS

These data allow a detailed analysis of the effects and mechanisms of influence of the aging process and hyperhomocysteinemia on changes in the biogenic amine content in the hypothalamic structures responsible for the regulation of the reproductive function. It was established that methionine loading in the proposed experimental scheme has no significant impact on the studied parameters, whereas age is the determining factor affecting the biogenic amine content in hypothalamic structures. The results showed that a change in their content correlated primarily with the amount of ascorbic acid. The changes in other parameters of oxidative stress studied in the hypothalamus are not dependent on age but exhibit some correlation with the biogenic amine content, including dopamine in the hypothalamic medial preoptic area, which should be taken into account in the experiments involving development of oxidative stress.

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