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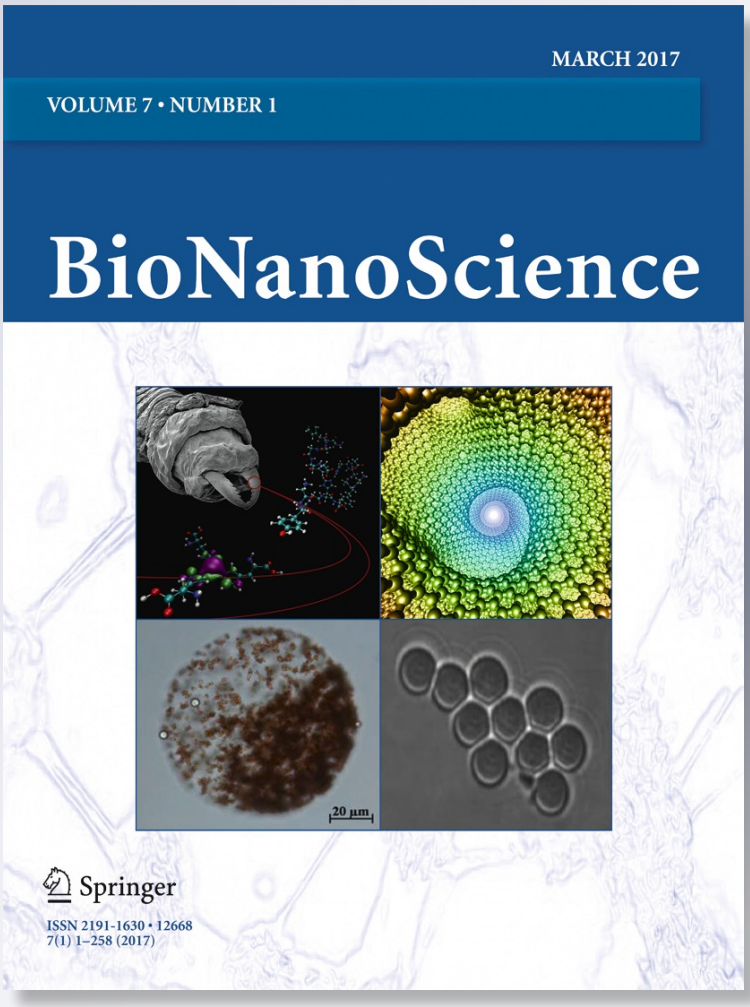

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Effects of Maternal Hyperhomocysteinemia on the Early Physical Development and Neurobehavioral Maturation of Rat Offspring

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Abstract During pregnancy, several complications have been associated with hyperhomocysteinemia (HHcy) and elevated homocysteine (Hcy) levels have been shown to play a role in the etiology of preeclampsia, placental abruption, intrauterine growth retardation, and neural tube defects and associated with the neurological consequences. In the present work, we investigated the effects of chronic maternal HHcy on the development and neurobehavioral maturation of the offspring. We analyzed classical parameters of development such as body weight, eyelid opening, ear unfolding, incisor eruption, and the appearance of hair, and subjected the pups to various tests that reflected the neurobehavioral maturation extending from 4th to 20th postnatal days (righting reflex, negative geotaxis, cliff avoidance, head shake, acoustic startle reflex, free-fall righting, cliff avoidance caused by visual stimulus, olfactory discrimination). We have shown that newborn animals were characterized by lower body weight and higher mortality. Besides, the delay in neurobehavioral maturation of the pups from the Hcy group was observed. The obtained results indicate that early developmental impairments of brain maturation induced by prenatal HHcy may underlie long-term deficits in the learning and memory behaviors.

Keywords Maternal hyperhomocysteinemia · Neonatal development · Rat · Neurobehavioral maturation

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1 Introduction

Homocysteine (Hcy) is a sulfur-containing amino acid that is generated during methionine metabolism [1]. Hcy is normally removed by two key processes: (1) the methionine cycle that synthesizes methionine from the Hcy by methylene tetrahydrofolate reductase (MTHFR) and (2) irreversible transsulfuration that converts Hcy to cystathionine and eventually to cysteine by cystathionine β-synthase (CBS). Genetic mutations in MTHFR and CBS and nutritional deficiencies of vitamin co-factors (folate, B12, and B6) are the primary causes of elevated plasmas Hcy level—hyperhomocysteinemia (HHcy) [1].

Maternal HHcy is an independent and significant risk factor for pregnancy complications [1, 2]. In animals models, the maternal HHcy induces persistent functional disabilities, learning deficits in offspring at early and late stages of postnatal development [3–7]. In our study, we have used a battery of tests in order to evaluate the classical parameters of physical development and neurobehavioral maturation extending from 2nd to 20th postnatal days (PND) to explore the effects of maternal HHcy on the neurobehavioral maturation of rat offspring.

2 Material and Methods

Experiments were carrying out on Wistar rats in accordance with EU Directive 2010/63/EU for animal experiments and Local Ethical committee KFU (protocol no. 8 from 5 May 2015). Pregnant rats were divided into two groups as follows: one group of animals was assigned as control ($n = 6$) and the second group was assigned as Hcy group ($n = 9$) and received daily methionine (7.7 g/kg body weight) with food starting 3 weeks prior to pregnancy and 2 weeks after

delivery [8]. Total Hcy level in plasma was determined using glass carbon electrode modified with multi-walled carbon nanotubes (MWNT/GCE) under conditions of square-wave voltammeter (Eco Chemie B.V., The Netherlands) [9]. Concentration of Hcy in the plasma in the control group was 7 ± 1 μM, in the experimental group—124 ± 23 μM indicating on the severe HHcy [3].

After delivery, the litter size and the number of live offspring were recorded. The analysis of the physical development and reflexes was started at PND2 and was carried out daily between 12 and 17 p.m. until PND 20 according the previous studies [10–12]. Body weight was measured daily until PND20. The following physical features were observed: eye opening, ear unfolding, incisor eruption, and the hair appearance (Table 1) [11]. Pups were tested daily for the number of neurological reflexes (Table 2) [10–12].

In negative geotaxis reflex, the rat was positioned with the head downward on an inclined plane with a 45 % slope. Positive reaction was recorded if the pup turned 180° in with a fixed time (60 s). In the righting reflex, the time (in s) necessary to come back to a quadruped position was measured. In head shake reflex, the number of head rotations per minute was analyzed. In cliff avoidance test with a fixed time (10 s), positive reaction was recorded if the pup turned and crawled away when placed on a bench top with its forepaws extending over the edge. In acoustic startle reflex, the day of the startle response (body shaking) to a clapping sound was recorded. In cliff avoidance caused by visual stimulus test, the animal was placed on a platform, raised to a height of 45 cm above the surface and avoiding the drop is taken as a positive decision. The experiments were performed after eye opening (PND 12–20, Tables 1 and 2). In free-fall righting (PND 12–20) the pup's ability to turn over in the air from supine position and fall down onto four limbs was recorded. In the test olfactory discrimination (PND 12–20), each rat was placed in the center of an empty corridor in a neutral position. On one side, the fresh sawdust and on the other side the bedding from the home cage (including several pieces of feces) was placed. The day when the rat reached the bedding from its homecage (with their mother's odor) was measured. Positive reaction was recorded if the pup reached the bedding from its homecage in less than 60 s [10–12]. The physical features and parameters of neurological reflexes of the pups from the control group were comparable with the results of other studies [10–12].

Data are expressed as mean ± standard error of the mean (SEM). Statistical analysis was performed using Mann–Whitney *U* tests, *p* level of significance: *in case of significance Pu ≤ 0.05, **when Pu ≤ 0.01.

Table 1 Effects of maternal hyperhomocysteinemia on the development of physical features of pups

Parameters	Control group	Experimental group
Ear unfolding (PND)	2 ($n = 54$)	2 ($n = 78$)
The appearance of the primary hair (PND)	4 ($n = 50$)	4 ($n = 64$)
Incisors eruption (PND)	8 ($n = 47$)	8 ($n = 53$)
Eyelid opening (PND)	13 ($n = 47$)	13 ($n = 47$)

3 Results and Discussion

The average litter size of control and Hcy group was 8.6 ± 1.2 and 8.4 ± 1.6 pups (Pu ≥ 0.05, PND0), respectively. The mortality during the observation period (PND 0–20) was higher in the Hcy group (48 %) in contrast to the control group (16 %) (Fig. 1a). The body weight of pups from Hcy group was significantly lower (6.89 ± 0.44 g) than that of pups born from the control group (8.13 ± 0.48 g, Pu ≤ 0.05) at PND2, and these differences maintained during all observation period (Fig. 1b). These results were supported by previous obtained data where the average pup's weight was significantly reduced in HHcy conditions [6, 7], although in other studies, no differences or slight decrease of pup's body weight was revealed [4, 5]. Those differences could be explained by the severity of HHcy and the model of HHcy used. In most of the studies, the moderate increase of Hcy concentration in the blood plasma in the end of pregnancy was observed (20–30 μM). In our case, concentration of Hcy was 124 ± 23 μM which corresponds to severe HHcy developed in case of CBS or MTHFR deletion followed by the growth retardation and high mortality of litters [3]. Other physical features of pups from Hcy group (the day of eye opening, ear unfolding and incisor eruption) did not differ from the control values (Table 1) and this data correspond to previous findings [4, 5].

In rats, the period of 2 weeks after birth represents a critical phase in neurobehavioral maturation with a rapid brain growth which corresponds to the last months of human fetal brain growth [13]. We studied the reflexes ontogeny (righting reflex, negative geotaxis, cliff avoidance, head shake, acoustic startle reflex, free-fall righting, cliff avoidance caused by visual stimulus and olfactory discrimination) reflected the brain maturation and integrity of sensory-motor development [10]. Only in a few previous studies, the litters were analyzed at the early stage of development using the righting reflex and negative geotaxis [5]. No statistically significant difference was found between the two experimental groups in the day of negative geotaxis reflex formation (Table 2) and the onset of the righting reflex (PND6) however the time (in s) necessary to come back to a quadruped position was significantly increased in the Hcy group compared with the control group (Table 2). The same data was obtained in pups undergoing gestational vitamin B deficiency [5]. Head shake reflex was formed at

Table 2 Reflex ontogeny of litters from control and experimantal groups

Parameters	Control group ($n = 47$)	Experimental group ($n = 47$)
Negative geotaxis (the day of appearance)	6.19 ± 0.23	6.21 ± 0.17
Head shake reflex (the number of head movements per minute) PND 8	7.52 ± 0.68	2.51 ± 0.30**
Righting reflex (time in second) PND 6	1.58 ± 0.15	2.68 ± 0.12**
Cliff avoidance (the day of appearance)	5.90 ± 0.18	7.11 ± 0.29*
Acoustic startle reflex (the day of appearance)	9.74 ± 0.46	10.11 ± 0.32*
Cliff avoidance caused by visual stimulus (the day of appearance)	13.94 ± 0.35	15.91 ± 0.29*
Free-fall righting (the day of appearance)	13.3 ± 0.41	18.23 ± 0.39**
Olfactory discrimination (the day of appearance)	13.51 ± 0.16	14.71 ± 0.07*

*Pu ≤ 0.05, **Pu ≤ 0.01—Mann–Whitney *U* tests

PND8 in rat pups of both groups, but the number of head rotations per minute was significantly lower in Hcy group compare to the control group (Table 2). In the rat pups of the Hcy group, the cliff avoidance reflex was formed later (PND7) compared to the control group (PND6) (Table 2). The delay of the reflexes onset in pups of experimental group was also observed in other sensorimotor tests (Table 2).

Obviously, neurotoxicity of Hcy during the prenatal HHcy is underlying the impairments of neurogenesis and the plasticity of the developing brain. The proposed mechanisms of brain maturation delay in HHcy rats includes the reduced expressions of glial fibrillary acidic protein and S100B protein in the brain [4], accumulation of Hcy in different brain structures which can trigger neuronal apoptosis [5], and oxidative stress by the generation of free radicals and inhibition of glutathione peroxidase. Moreover, it was shown that Hcy induces hyperactivation of NMDA receptors, with subsequent desensitization [14–16] and increases the activity of maxi calcium-activated potassium channels in rat GH3 cells underlying the decrease of growth hormone release [17].

Our study is in consequence with clinical studies which have shown that maternal HHcy is associated with an increased risk of pregnancy-induced hypertension and pregnancy loss, neural tube defects, and intrauterine growth restriction. Immature delivery in such cases is accompanied by high infant mortality and a large ratio of neonatal complications. The infants born by mothers under HHcy have mental and physical retardation [1, 2].

4 Conclusions

Thus, we have shown that prenatal HHcy induces the lower body weight and increased mortality of the offspring. At the same time, all other indicators of physical maturation were not different from the control. Pups from Hcy group demonstrated

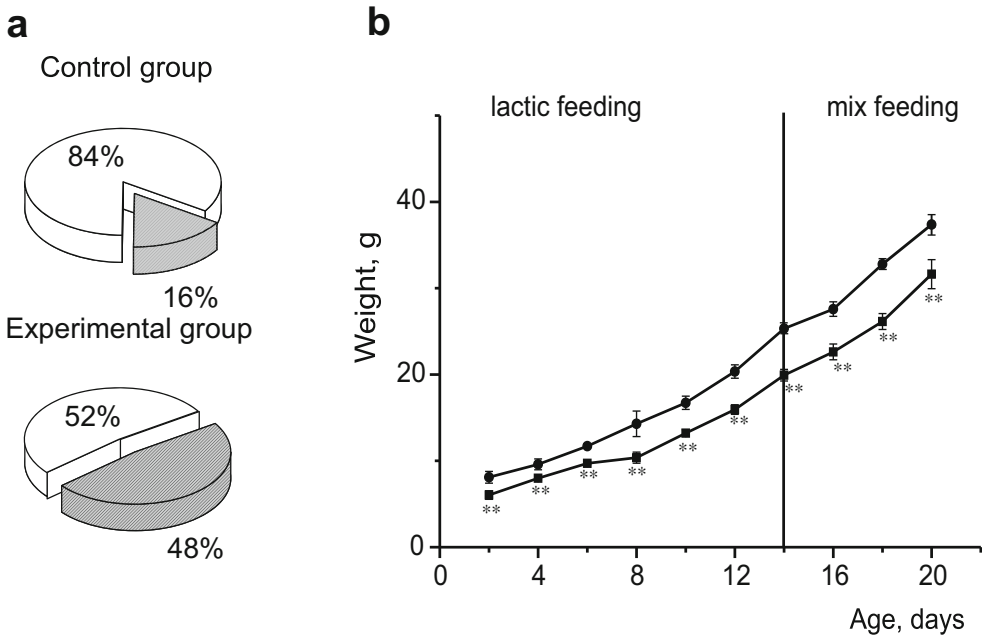

Fig. 1 Effects of chronic maternal hyperhomocysteinemia on the mortality and body weight of litters. **a** The mortality of pups (PND0-20) from control и Hcy groups. **b** Daily changes in body weight of pups from control (*black squares*) and Hcy groups (*open circles*). **Pu ≤ 0.01, *Pu ≤ 0.05 (in Mann–Whitney *U* tests)

the delay of neurobehavioral maturation tested using the battery reflexes (righting, cliff avoidance, auditory startle response, free-fall righting, olfactory reflex). Apparently, these early postnatal changes of brain maturation of rats exposed to elevated Hcy level in prenatal and early postnatal period underlie the functional impairments of the central nervous system and cognitive dysfunctions in late postnatal life.

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