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Effects of Homocysteine and Its Derivatives on Spontaneous Network Activity in the Hippocampus of Neonatal Rat Pups

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Homocysteine is a sulfur-containing amino acid, which at high concentrations has neurotoxic effects and induces impairments to the development of the nervous system. Homocysteine is rapidly oxidized in the plasma, forming disulfide bonds with proteins and other low molecular weight thiols; it also undergoes transformation into the into homocysteine thiolactone. On chronic exposure, the neurotoxicity of homocysteine is therefore mediated mainly by its derivatives. The aim of the present work was to investigate the effects of homocysteine and its derivatives – homocystine and homocysteine thiolactone – on spontaneous network activity in the hippocampus of rats in the first week after birth. Giant depolarizing potentials (GDP) and multiple action potentials (MAP) were recorded using an extracellular electrode in hippocampal field CA3. All three study compounds were found to induce increases in the frequency of GDP and MAP at concentrations of 100 and 500 μM, homocystine producing the most significant increase in neuron network activity. The effects of homocysteine, homocystine, and homocysteine thiolactone on the spontaneous network activity of neurons were completely eliminated on blockade of NMDA and AMPA receptors. Thus, homocysteine and its derivatives lead to increased spontaneous network activity of hippocampal neurons in neonatal rats, which can induce impairments to the formation of the neural networks of the hippocampus in conditions of chronic hyperhomocysteinemia, and could also induce hyperexcitability and the risk of developing epilepsy in the postnatal period.

Keywords: hippocampus, homocysteine, homocystine, homocysteine thiolactone, giant depolarizing potentials, NMDA receptors, AMPA receptors.

Homocysteine is a sulfur-containing amino acid synthesized from methionine [1, 2]. Impairments to homocysteine metabolism lead to its accumulation in the blood and cerebrospinal fluid, which is termed hyperhomocysteinemia [3, 4]. Depending on the blood homocysteine concentration, hyperhomocysteinemia is classified as mild (16–30 μM), moderate (30–100 μM), and severe (>100 μM). Genetic impairments to the enzymes of homocysteine metabolism can increase the level of this amino acid to 500 μM, which leads to homocysteinuria [3]. In physiological conditions, less than 1% of total homocysteine is present in in the free reduced form, while about 10–20% is present as various oxidized forms, including a dimer, i.e., homocystine [5], and most (80%) is bound to γ-globulins and albumin [6]. Furthermore, increases in the homocysteine concentration are cytotoxic because of its conversion to homocysteine thiolactone during the process of protein synthesis [7]. Homocysteine thiolactone is a chemically reactive molecule that produces N-homocysteinylation of proteins by forming an amide bond with the amino acid lysine, leading to impairment to the structure and functional activity of proteins. This process contributes to the development of various pathologies, including atherosclerosis, thrombosis, and Alzheimer's disease [8]. The chronic action of homocysteine neurotoxicity is therefore mediated mainly by its derivatives. However, questions of which forms of homocysteine result in the development of pathological processes and are markers for them remain under study.

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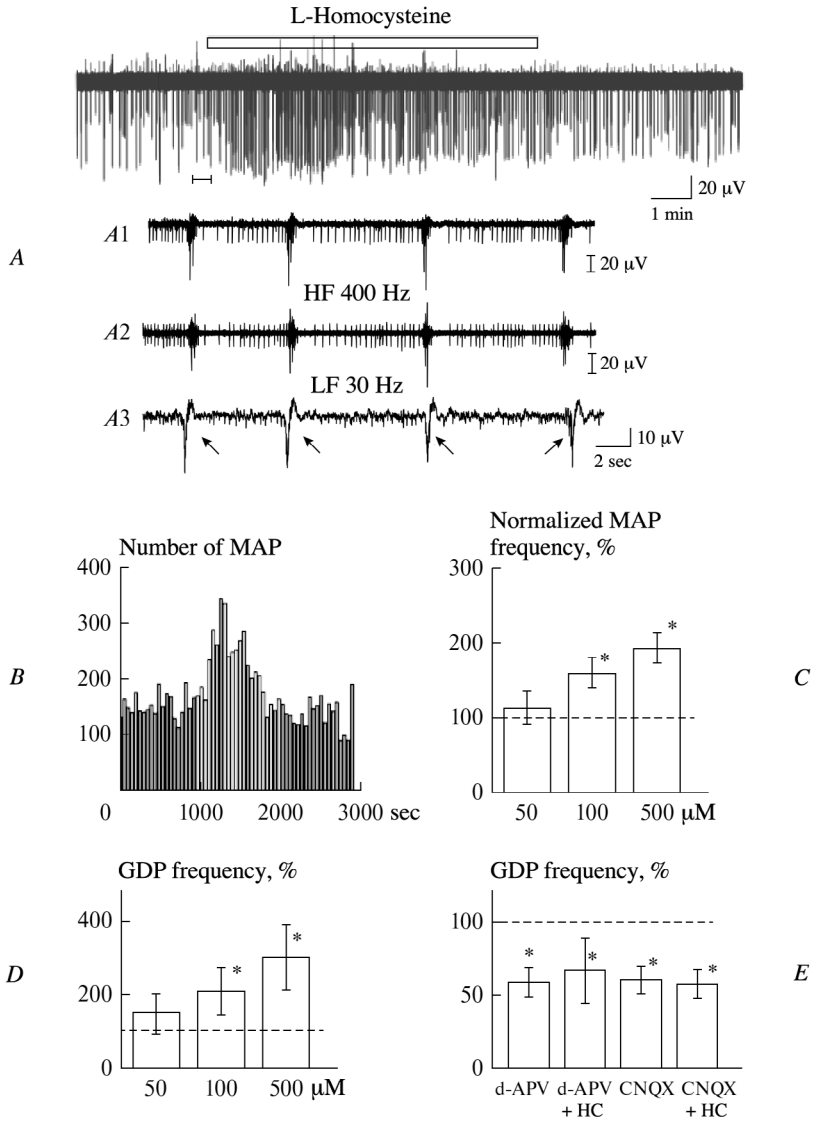

Fig. 1. Effects of homocysteine on the spontaneous network activity of neurons in rat hippocampal slices. *A*) Example of extracellular recording of hippocampal neuron population activity, which is organized as GDP and MAP. Traces with expanded time scale (dark rectangle) with GDP (indicated by asterisks) (*A*1); MAP after filtration of trace with high-frequency filter (>400 Hz) (*A*2); local field potentials (arrows) after processing of native traces with a low-frequency filter (30 Hz) (*A*3). Application of 100 μM homocysteine (white bar in *A*) leads to increases in GDP and action potential frequencies. *B*) Changes in the number of MAP in one experiment (from trace *A*, step 50 sec). Substance actions are shown as white columns. Histograms of averaged MAP (*C*) and GDP (*D*) frequencies on exposure to homocysteine at concentrations of 50, 100, and 500 μM. *E*) Effects of homocysteine (HC, 100 μM) on GDP frequency in conditions of inhibition of NMDA receptors (20 μM d-APV) and AMPA receptors (10 μM CNQX). GDP frequency in controls is taken as 100%. *$p < 0.05$ compared with controls.

Even small increases in homocysteine levels in plasma correlate with developmental cognitive impairments, neurodegenerative and cerebrovascular diseases, as well as the development of epilepsy [9–11]. Increases in homocysteine levels during pregnancy lead to toxic effects on the fetus, as homocysteine is able to cross the placenta, which leads to developmental pathologies [12, 13]. The mechanisms of the neurotoxic action of homocysteine include activation of ionotropic and metabotropic glucocorticoid receptors in neurons, excessive stimulation of which induces hyperexcitability, as well as increases in calcium levels, resulting in apoptosis [14–18].

The nervous system in neonatal animals has a number of functional characteristics. A major form of network activity is observed in many parts of the brain, including the hippocampus – giant depolarizing potentials (GDP) [19], which take part in forming neural networks supporting synchronous activation of pre- and postsynaptic neurons, which is one of the mechanisms of synaptic plasticity [20, 21].

Despite intense studies of the of the mechanisms of the neurotoxic effects of homocysteine on the developing nervous system, there are no data on the effects of homocysteine and its derivatives on the early rhythmic activity of hippocampal neurons. The aim of the present work was to ana-

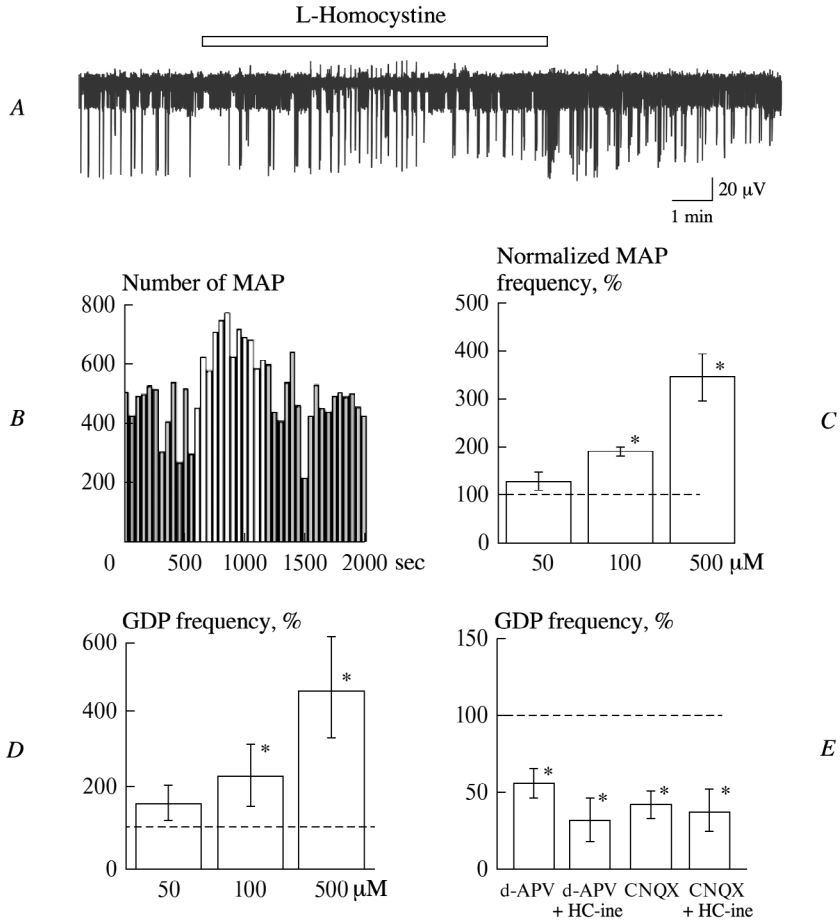

Fig 2. Effects of homocystine on the spontaneous network activity of neurons in rat hippocampal slices. *A*) Example of extracellular recording of hippocampal neuron population activity. Application of 100 μM homocystine is shown by the white rectangle. *B*) Changes in the number of MAP in one experiment (from trace *A*, step 50 sec). Substance actions are shown as white columns. Histograms of averaged MAP (*C*) and GDP (*D*) frequencies on exposure to homocystine at concentrations of 50, 100, and 500 μM. *E*) Effects of homocystine (HC-ine, 100 μM) on GDP frequency in conditions of inhibition of NMDA receptors (20 μM d-APV) and AMPA receptors (10 μM CNQX). GDP frequency in controls is taken as 100%. $*p < 0.05$ compared with controls.

lyze the effects of homocysteine and its derivatives – homocystine and homocysteine thiolactone – on the spontaneous network activity of hippocampal neurons in rat pups during the first week of postnatal development.

Methods. Experiments were carried out on neonatal rat pups (P3–P7, where P0 is the day of birth). All experimental protocols complied with ethical norms for the humane treatment of animals which applies at Kazan Federal University (approved by the Ethics Committee of Kazan Medical University No. 9, 2013). Before the experiment, animals were anesthetized with isoflurane (4%). After extraction, rat pups' brains were placed in cooled oxygenated artificial cerebrospinal fluid (aCSF) containing 126 mM NaCl, 3.5 mM KCl, 2.0 mM $CaCl_2$, 1.3 mM $MgCl_2$, 25 mM $NaHCO_3$, 1.2 mM NaH_2PO_4, and 11 mM glucose, (pH 7.4, 310 mOsm/liter). Horizontal brain slices of thickness 400 μm were cut using an HM 650 V vibroslicer (Microm International, Germany). Slices were placed in oxygenated aCSF and held for at least 1 h at room temperature until the experiment started. GDP, characterized by negative deviation (local field potential, LFP), and population activity of hippocampal neurons – multiple action potentials (MAP) – were recorded in all slices, including the control and experimental groups (Fig. 1, *A*) [22]. Extracellular recording of LFP and MAP was carried out using an electrode made from tungsten wire (diameter 50 μm, California Fine Wire, USA) located in field CA3 of the pyramidal layer of the hippocampus. Amplification and digitization of the recorded signals were carried out using a DAM-80 amplifier (WPI, USA; ×1000, 0.1–3 kHz filter) and a Digidata 1440 ADC (Axon Instruments, USA). Signals were analyzed using Axon software package (Molecular Devices, USA), Mini Analysis (Synaptsoft Inc., USA), Origin 8.5 (Microcal Software, USA), and user functions in MatLab (MathWorks, USA). The frequencies of GDP and MAP were determined. MAP were identified using a high-frequency filter (RC single pole, >400 Hz), where all negative events exceeding the noise level by 2.5 standard deviations were taken as action potentials

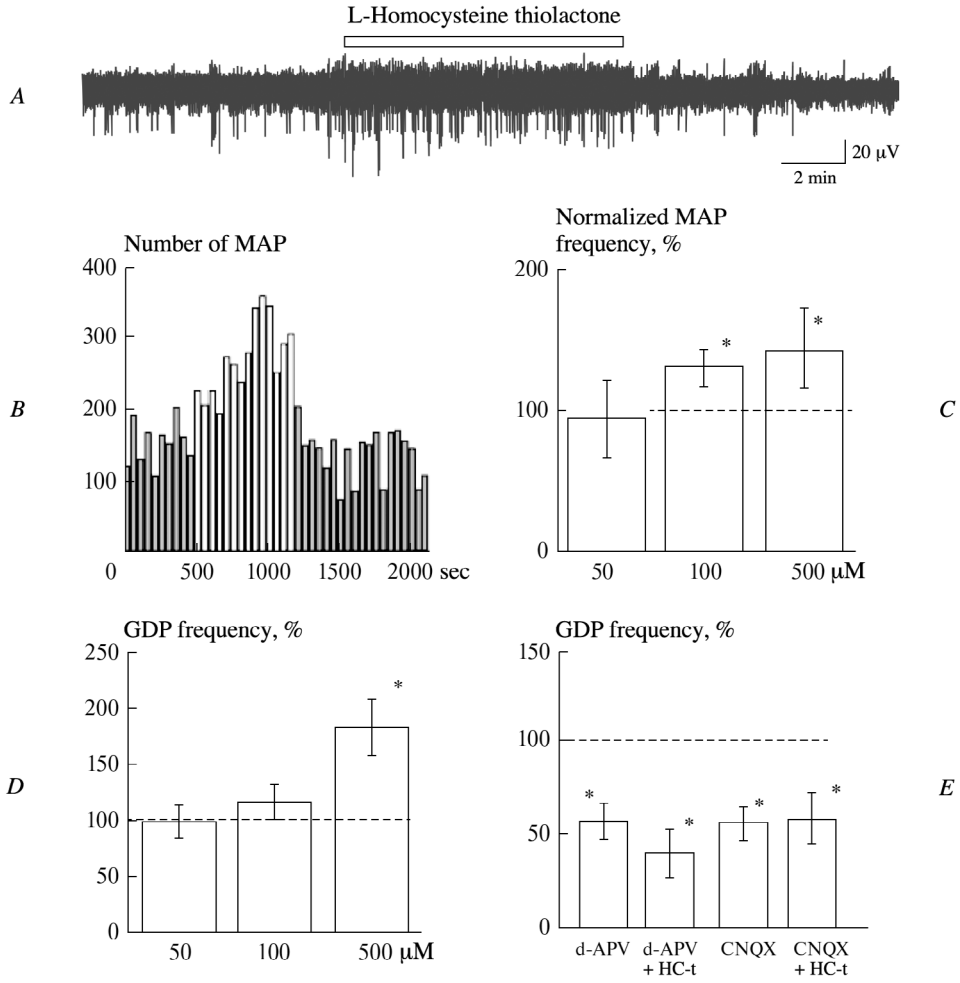

Fig. 3. Effect of homocysteine thiolactone on the spontaneous network activity of neurons in rat hippocampal slices. *A*) Example of the effect of homocysteine thiolactone (500 μM, white rectangle) on hippocampal neuron population activity. *B*) Changes in the number of MAP in one experiment (from trace *A*, step 50 sec). Substance actions are shown as white columns. Histograms of averaged MAP (*C*) and GDP (*D*) frequencies on exposure to homocysteine thiolactone at concentrations of 50, 100, and 500 μM. *E*) Effects of homocysteine thiolactone (HC–t, 500 μM) on GDP frequency in conditions of inhibition of NMDA receptors (20 μM d-APV) and AMPA receptors (10 μM CNQX). GDP frequency in controls is taken as 100%. *$p < 0.05$ compared with controls.

(Fig. 1, *A*2) [22]. LFP were analyzed by filtering native traces with a 30-Hz low-frequency filter (Fig. 1, *A*3), which provides for evaluation of LFP amplitude and latency [23].

Experiments used the following substances: L-homocysteine, L-homocystine, D,L-homocysteine thiolactone, D(–)-amino-5-phosphonopentanoate (d-APV, a selective NMDA receptor blocker), 6-cyano-7-nitroquinoxaline (CNQX, a selective α-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid (AMPA) receptor blockerr) (Sigma, USA).

Group data are presented as the mean ± standard error. Significant differences between sets were identified using the nonparametric paired Mann–Whitney U test taking $p < 0.05$ as the threshold of significance.

Results. Spontaneous synchronization of pyramidal cell activity in the hippocampus of neonatal rodents is apparent as the generation of so-called giant depolarizing potentials (GDP) – high-amplitude, long-lasting shifts in the membrane potential of nerve cells with superimposed action potentials from groups of neurons (Fig. 1, *A*) [19–21, 23]. Homocysteine, homocystine, and homocysteine thiolactone were used at concentrations of 50, 100, and 500 μM. Our experiments used concentrations of 100 and 500 μM because these plasma concentrations can be seen in severe forms of hyperhomocysteinemia in humans, and because long-term exposure leads to accumulation of the effects of low homocysteine concentrations [24].

Homocysteine, homocystine, and homocysteine thiolactone at concentrations of 50 μM did not lead to any significant changes in the spontaneous network activity of neurons (Figs. 1–3). Use of homocysteine at concentrations of 100 and 500 μM produced increases in the frequencies of GDP and MAP (Fig. 1, *A–D*). At a concentration of 100 μM, homocysteine increased the frequency of spontaneous ac-

tion potentials to 155 ± 15% (from 14 ± 3 to 21 ± 3 sec^{-1}; $n = 11$, $p < 0.05$), while at a concentration of 500 μM, the increases was to 24 ± 3 sec^{-1} (to 183 ± 16%; $n = 5$, $p < 0.05$) relative to controls (Fig. 1, *C*). Homocysteine also increased the frequency of GDP to 205 ± 45% (from 0.8 ± 0.2 to 1.3 ± ± 0.2 sec^{-1}; $n = 15$, $p < 0.05$) at a concentration of 100 μM and to 306 ± 54% (to 3.3 ± 1.4 sec^{-1}; $n = 6$, $p < 0.05$) at a concentration of 500 μM (Fig 1, *D*). The duration and amplitude of LFP did not change. The effect of homocysteine was seen for 5–8 min from substance application, after which the spontaneous activity of pyramidal neurons recovered. A change in the solution for aCSF led to recovery of the activity to baseline, though the recovery time depended on the concentration used.

Generation of GDP is known to be controlled by coactivation of NMDA and GABA receptors on hippocampal neurons [20]. In addition, studies have shown that synchronization of GDP requires activation of AMPA receptors [20, 25]. As L-homocysteine is a weak NMDA receptor agonist [16], the next series of experiments analyzed the effects of homocysteine on the background of inhibition of ionotropic glutamate receptors. Prior application of the selective NMDA receptor inhibitor d-APV at a concentration of 20 μM led to a decrease in GDP frequency to 56 ± 10% (from 1.9 ± 0.4 to 1.1 ± 0.2 sec^{-1}; $n = 7$, $p < 0.05$) without any change in MAP frequency (Fig. 1, *E*). Blockade of AMPA receptors with the selective blocker CNQX at a concentration of 10 μM induced a decrease in GDP frequency to 55 ± 4% (from 1.8 ± ± 0.6 to 0.8 ± 0.24 sec^{-1}; $n = 7$, $p < 0.05$) without any change in MAP frequency. Subsequent application of homocysteine did not increase the frequency of MAP or GDP (Fig. 1, *E*). Changing the solution to aCSF led to a gradual increase in GDP frequency, with full recovery of the spontaneous network activity of neurons by 10–20 min.

In physiological conditions, homocysteine is known to exist as a dimer, i.e., homocystine [26, 27]. Use of homocystine at concentrations of 100 and 500 μM produced significant increases in MAP and GDP frequencies (Fig. 2, *A–D*). Thus, addition of 100 μM homocystine increased MAP frequency to 183 ± 9% (from 26 ± 9 to 36 ± 12 sec^{-1}; $n = 11$, $p < 0.05$), while addition of substance to a concentration of 500 μM gave an increase to 345 ± 57% (from 11 ± 6 to 32 ± 6 sec^{-1}; $n = 11$, $p < 0.05$) (Fig. 2, *C*). We also observed increases in GDP frequency on application of homocystine at concentrations of 100 μM to 257 ± 54% (from 1.8 ± 0.6 to 3.1 ± 0.8 sec^{-1}; $n = 12$, $p < 0.05$) and 500 μM to 463 ± 92% (to 5.5 ± 1.3 sec^{-1}; $n = 12$, $p < 0.05$) relative to controls (Fig. 2, *D*). The duration and amplitude of LFP of spontaneous network events in the hippocampus did not change. Substance effects were reversible.

The next series of experiments analyzed the effects of homocystine (100 μM) on the background of inhibition of NMDA or AMPA receptors. As demonstrated in fig 2, *E*, application of homocystine on the background of d-APV or CNQX did not lead to any increase in GDP or MAP frequencies.

The effects of homocysteine thiolactone on the spontaneous network activity of neurons were less marked (Fig. 3, *A–D*). At concentrations of 100 and 500 μM, homocysteine thiolactone increased MAP frequency to 128 ± 9% (from 29 ± 8 to 35 ± 4 sec^{-1}; $n = 14$, $p < 0.05$) and 143 ± 10% (from 22 ± 6 to 33 ± 7 sec^{-1}; $n = 11$; $p < 0.05$) relative to control respectively (Fig. 3, *B*, *C*). The effects on GDP were apparent only when homocysteine thiolactone was used at a concentration of 500 μM, where GDP frequency increase to 183 ± 25% (from 2.3 ± 0.4 to 4.5 ± 1.2 sec^{-1}; $n = 8$, $p < 0.05$; Fig. 3, *D*). The duration and amplitude of LFP of spontaneous network events in the hippocampus did not change. Substance effects on GDP and MAP frequency were reversible.

On the background of inhibition of NMDA or AMPA receptors, application of homocysteine thiolactone (500 μM) did not increase GDP or MAP frequencies (Fig. 3, *E*).

Discussion. The present studies on rat hippocampal slices collected in the first week of postnatal development showed that not only homocysteine, but also its derivatives homocystine and homocysteine thiolactone, had activatory effects on spontaneous network dπischarges (GDP), which were linked with activation of ionotropic glutamate receptors. These effects of homocysteine and its derivatives may contribute to pathological development of offspring on chronic exposure to high concentrations of homocysteine during the prenatal period.

Increases in homocysteine levels in the maternal body are known to lead to complications of pregnancy, including fetal neural tube defects and delayed growth [4, 15, 28, 29]. The most sensitive to the toxic effects of L-homocysteine are nerve cells in the prenatal and early postnatal periods of development [1] due to efficient transfer of homocysteine into cells in the form of its dimer homocystine [30]. Homocysteine accumulation leads to oxidative stress, which produces significant neuron damage in various parts of the brain [12, 13, 31, 32].

Studies of the mechanisms of action of homocysteine in vitro have shown that acute application of homocysteine alters the process of synaptic plasticity in the hippocampus of rats aged 6–8 weeks [33, 34]. Incubation of primary and secondary cell cultures in homocysteine led to changes in the electrophysiologiπcal properties of neurons, rapid desensitization of GluN1/N2B receptors, and decreased secretion of growth hormone in GH3 cells [17, 18, 35, 36]. Our studies using hippocampal slices from neonatal rat pups with prenatal hyperhomocysteinemia demonstrated changes in the electrical properties of neurons and impairment to spontaneous network activity [37].

Homocysteine in plasma is rapidly oxidized to form a series of metabolites, including homocystine and homocysteic acid, and is bound to plasma proteins [5]. In addition, homocysteine can form an even more toxic metabolite – homocysteine thiolactone – due to the enzyme methionyl-tRNA synthetase [6, 7, 27, 38]. Chronic hyperhomocystein-

emia therefore leads to accumulation of homocysteine derivatives, which can have their own effects. However, no studies of the effects of homocysteine and its derivatives on the electrical activity of neurons in the neonatal hippocampus have yet been conducted. It should be noted that early electrical activity recorded in hippocampal slices from neonatal rats – GDP – plays important roles in the development of neural networks and their synchronization and the formation of synaptic connections and affects the proliferation, migration, and differentiation of cells, as well as the secretion of growth factors such as BDNF [39]. The mechanism by which GDP arise is linked with activation of GABA and NMDA receptors [21], and AMPA receptors take part in the synchronization of GDP in neural networks [21, 25].

Our studies showed that high concentrations of homocysteine and its derivatives (100 and 500 μM) increase GDP and MAP frequencies in the hippocampus of neonatal rats. There were no changes in the amplitude or duration of GDP, which may indicate a lack of substance effects on synaptic connections and the generation of pacemaker activity by interneurons determining GDP shape [40]. It is interesting that the most significant increase in spontaneous network activity was seen on exposure to homocystine, which is an oxidized form of homocysteine and which can be regenerated to homocysteine within cells [3]. The effects of homocysteine thiolactone on GDP were less marked and the increase in MAP frequency was apparent only on use of the concentration of 500 μM, which appears to be evidence of its lower affinity for glutamate receptors. In addition, manifestation of its effects requires longer time periods, as the known mechanism of its action is a posttranslational modification of proteins (N-homocysteinylation) which results in changes to their functions [7].

One of the mechanisms of the toxic action of homocysteine is activation of NMDA, AMPA, and metabotropic (groups I and III) glutamate receptors [31, 41]. In fact, application of selective inhibitors of NMDA and AMPA receptors in our studies completely inhibited the excitatory action of homocysteine and its derivatives on the spontaneous network activity of hippocampal neurons.

Considering the hyperexcitability of neural networks in the first week after birth due to the depolarizing action of GABA, it appears that activation of glutamate receptors and strengthening of neuronal activity may be prerequisites for the occurrence of epileptic activity in conditions of chronic exposure to homocysteine [42, 43]. In fact, administration of homocysteine thiolactone in in vivo animal experiments led to the onset of epileptic activity in both young and adult animals [3, 4, 7, 44, 45]. Increases in the spontaneous network activity of neurons on exposure to homocysteine and its derivatives may therefore make a contribution to impairments to the formation of hippocampal neural networks in conditions of chronic hyperhomocysteinemia and induce hyperexcitability and the risk of developing epilepsy in the postnatal period.

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