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COMPARATIVE AND ONTOGENIC PHYSIOLOGY

Homocysteine-Induced Membrane Currents, Calcium Responses and Changes in Mitochondrial Potential in Rat Cortical Neurons

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Abstract—Homocysteine, a sulfur-containing amino acid, exerts neurotoxic effects and is involved in the pathogenesis of many neurodegenerative disorders. In contrast to well-studied glutamate excitotoxicity, the mechanism of homocysteine neurotoxicity is not clearly understood. Using whole-cell patch-clamp, calcium imaging (fluo-3) and measurements of mitochondrial membrane potential (rhodamine 123), we studied *in vitro* in cultured rat cortical neurons transmembrane currents, calcium signals and changes in mitochondrial membrane potential induced by homocysteine versus responses induced by NMDA and glutamate. L-homocysteine (50 μM) induced inward currents that were completely blocked by the selective antagonist of NMDA receptors, AP-5. In contrast to NMDA-induced currents, homocysteine-induced currents exhibited a smaller steady-state amplitude. Comparison of calcium responses to homocysteine, NMDA or glutamate demonstrated that in all cortical neurons homocysteine elicited fast oscillatory-type calcium responses, whereas NMDA or glutamate induced a "classical" sustained elevation of intracellular calcium. In contrast to NMDA, homocysteine did not cause a drop in mitochondrial membrane potential at the early stages of its action. However, after its long-term effect, as in cases of NMDA and glutamate, changes in mitochondrial membrane potential arose comparable with its complete drop caused by protonophore FCCP-induced uncoupling of the respiratory chain. Our data suggest that in cultured rat cortical neurons homocysteine at the initial stages of its action induces *in vitro* neurotoxic effects due to the activation of NMDA-type ionotropic glutamate receptors followed by a massive calcium influx through the channels of these receptors. The long-term effect of homocysteine may lead to mitochondrial dysfuction manifested as a drop in mitochondrial membrane potential.

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Key words: homocysteine, glutamate, calcium, mitochondrial potential, cortical neurons.

Abbreviations: $[Ca^{2+}]_i$—intracellular calcium concentration, $\Delta\varphi_{mit}$—mitochondrial membrane potential, FCCP—carbonyl cyanide-p-trifluoromethoxyphenylhydrazone, NMDA—N-methyl-D-aspartate, Rho123—rhodamine 123.

INTRODUCTION

An endogeneous amino acid homocysteine (2-amino-4-mercaptobutanic acid) is involved in normal cellular metabolism, including methionine and cysteine synthesis, leading to the production of a key factor of antioxidant defense, glutathione. However, due to the folic acid and B-group vitamin deficiency or genetic polymorphism—the cytosine (C) substitution by thymine (T) in C677T nucleotide of the 5, 10-methylenetetrahydropholate reductase gene [1], the blood plasma concentration of homocysteine may increase (above the normal level of 16 μM). This condition, called hyperhomocysteinemia, is coupled to the pathogenesis of many cardiovascular [2] and neurodegenerative diseases such as the Alzheimer's and Parkinson's diseases [3, 4] and amyotrophic lateral sclerosis [5]. Recently, the evidence has been obtained that homocysteine may also participate in the pathogenesis of such a widespread neurological disorder as a migraine with aura [6, 7].

A migraine with aura is known to be associated with cortical spreading depression [8, 9], an increase in the excitability of cortical neurons due to excess glutamate in the extracellular space [10]. Glutamate, by activating ionotropic and metabotropic receptors, causes neuronal depolarization and is potentially neurotoxic. It is well known that NMDA receptors are essential for the generation of cortical spreading depression [11]. However, despite homocysteine can also activate cortical neurons [12], the role of this amino acid in migraine hyperexcitability and concomitant neurotoxicity is practically unexplored.

There are several studies that demonstrated the interaction of homocysteine with the glutamate-binding site of NMDA receptors [12, 13] and group I metabotropic glutamate receptors [14–16]. In this respect, glutamate receptors may be regarded as a potential target for high homocysteine concentrations [17].

Hyperactivation of NMDA receptors is known to be frequently associated with oxidative stress in neurons [18, 19]. On the other hand, it is known that homocysteine can also trigger oxidative stress in some experimental models [20]. However, it has been shown that in endothelial cells homocysteine rather decreases the oxidative effect [21] and sometimes exhibits the reducing properties [22, 23]. Thus, the issue of the homocysteine involvement in oxidative stress remains open being one of the foci of the present study.

MATERIALS AND METHODS

The studies were conducted on the primary culture of the Wistar rat cortical neurons at the temperature of 23–26°C. The method of preparing neuronal culture was described in detail previously [24, 25]. Briefly, the culture was derived from the embryos at days 16 or 17 of prenatal development (E16–17). Cells were cultivated in a medium on cover slips pretreated with poly-D-lysine.

Experiments on the primary cultured cells of the rat brain cortex were conducted *in vitro* on days 7–10 (DIV). The physiological solution contained 140 mM NaCl, 2.8 mM KCl, 10 mM HEPES, 2 mM $CaCl_2$, 1 mM $MgCl_2$ (pH 7.2–7.4). Since in neurons extracellular Mg^{2+} blocks the NMDA receptor channels, in experiments with NMDA the physiological solution free of Mg^{2+} was used [26].

To evoke neurotoxic stress, there were used the saturating concentrations of glutamate receptor agonists: 50 μM L-homocysteine, 30 μM NMDA and 300 μM glutamate (Sigma-Aldrich, USA). During NMDA and homocysteine applications, glycine (a NMDA receptor co-agonist) was added regularly at a concentration presented in a growth medium (30 or 100 μM).

For fluorimetric measurement of $[Ca^{2+}]_i$, a fluorescent probe Fluo-3 (Invitrogen, USA) was used allowing relative concentrations of $[Ca^{2+}]_i$ to be obtained in a micromolar range [27]. Fluorophore was introduced to cells as acetoxymethyl ether, AM (4 μM, 45 min, darkness, 23–25°C). Then cells were incubated in darkness for 15 min in physiological solution for de-etherification with the intracellular formation of membrane-impermeable Fluo-3.

For fluorimetric measurement of mitochondrial membrane potential ($\Delta\varphi_{mit}$), a vital fluorescent dye, rhodamine 123 (Rho123, Invitrogen, USA), was used. Cells were loaded with fluorophore (5 μM) for 30 min in complete darkness at room temperature (23–25°C). As a control for $\Delta\varphi_{mit}$ measurements, a protonophore carbonyl cyanide-

p-trifluoromethoxyphenylhydrazone (FCCP, 4 μM; Sigma-Aldrich, USA) was used that leads to complete uncoupling of the respiratory chain (i.e. drop in $\Delta\varphi_{mit}$) eliciting thereby a maximal Rho123 emission [28].

For fluorimetric experiments with Fluo-3 or Rho123 on the inverted scanning confocal microscope Leica TCS SP5 MP (Leica Microsystems, Germany), cover slips with cells were transferred into the registration/perfusion chamber POCmini Chamber System(LaCon, Germany) connected to the general and local fast perfusion system. During the whole experiment (10–90 min), cells were perfused by general perfusion of physiological solution at a rate of 1 ml/min. Reactants were applied onto the cells by a local fast perfusion at the same rate. Complete substitution of the solution around the neurons occurred in less than 1 s.

Rho123 and Fluo-3 were excited by argon laser at a wavelength of 488 nm. Fluorochrome emission was registered in the spectral range of 500–560 nm. Scan frequency was 0.03 frame/sec. The resultant confocal images with Rho123 or Fluo-3 fluorescence were digitized using the Leica LAS AF software, and time dependences of single cell fluorescence intensities were plotted.

Integral neuronal currents were recorded by the method of local potential fixation in a whole-cell configuration [29]. Microelectrodes were filled with the solution containing 9 mM NaCl, 17.5 mM KCl, 121.5 mM K-gluconate, 1 mM $MgCl_2$, 10 mM HEPES, 0.2 mM EGTA, 2 mM MgATP, 0.5 mM NaGTP [30]. Recording patch-pipettes were made from borosilicate glass capillaries with an external diameter of 1.5 mm and internal filament (Sutter Instrument #BF150-86-10, Sutter Instruments Company, USA). Currents were recorded using the MultiClamp 700B amplifier with the Digitata 1440A data collection system and pClamp v10.2 software (Molecular Devices, USA). Frequency discretization was 20 000 measurements/sec with preliminary analog filtration (equivalent to the order 8 Bessel filter) with a section frequency of 1.4 kHz to remove high-frequency noises. Microelectrode tip was positioned by the MP-85 micromanipulator (Sutter Instruments Company, USA) under visual control on the Nikon Diaphot TMD inverted microscope (Nikon, Japan). Test substances were applied using the system for quick solution replacement based on BPS-4 (Ala Scientific Instruments, USA). Recording of the agonist-induced currents as well as their analysis were implemented using the pClamp Clampfit 10.2 software.

For statistical analysis, the Origin 6.1 and GraphPad Prizm 5 softwares were used. To evaluate the significance of differences, the Student's *t*-test was applied.

RESULTS

To explore the interaction of homocysteine with glutamate receptors in rat cortical neurons, a series of experiments was conducted in which the nature of the homocystein-activated inward currents was studied and their kinetics was compared with that of the responses to the specific NMDA agonist. For this purpose, we studied the kinetics of inward currents recorded by the local potential fixation in a whole-cell configuration after quick application of 50 μM homocysteine or 30 μM NMDA (the agonists were applied with 30 μM glycine as a NMDA receptor co-agonist). Both agonists induced inward integral currents comparable in their amplitudes (Fig. 1a). The NMDA-induced membrane current following the peak dropped partially to a steady state (plateau). In contrast to NMDA, the current induced by homocysteine application dropped much faster leading to a smaller plateau amplitude than in the current induced in the same cell by NMDA application (Fig. 1a). These differences may either be due to the fact that the agents interact with different receptors or, alternatively, reflect different desensitization kinetics when different agonists affect the same receptor type. Our experiments with AP-5, a selective NMDA receptor blocker, confirmed that homocysteine activates NMDA receptors because AP-5 blocked the homocysteine-induced current in the cortical neurons almost completely excluding thereby the contribution of other ionotropic glutamate receptors.

As is known, an inward current, along with transmembrane transfer of sodium and potassium ions, is determined by the influx of calcium ions via the NMDA receptor channel. Since there are differences in the kinetics of inward currents induced by the effect of NMDA versus homocysteine on

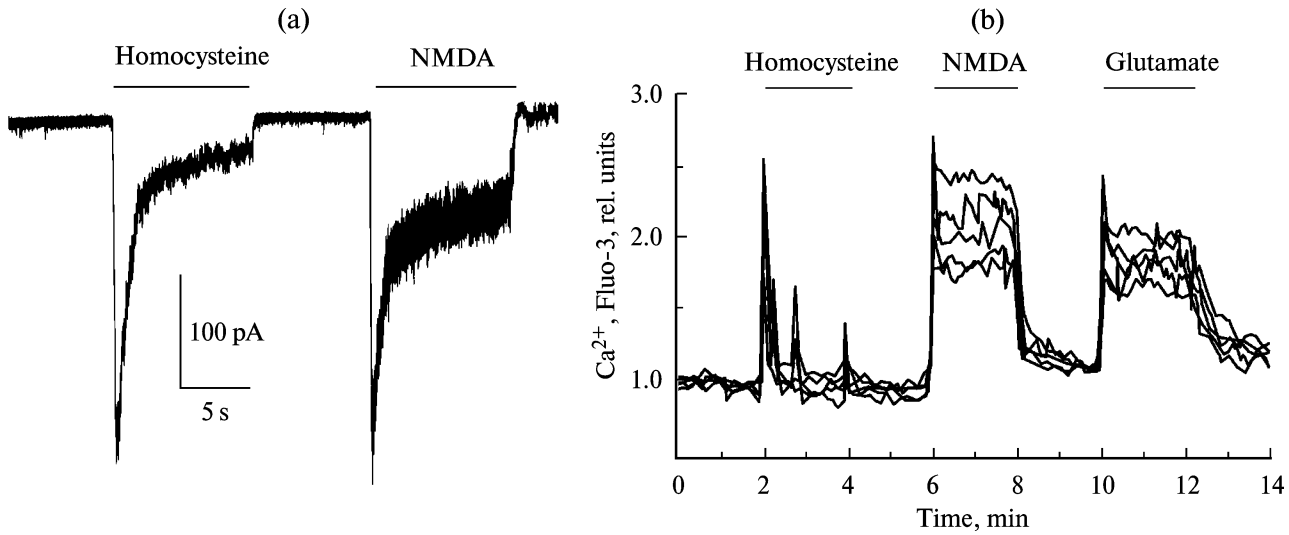

Fig. 1. (a) Inward integral currents induced by 50 μM homocysteine and 30 μM NMDA in presence of co-agonist, glycine, at the membrane potential –70 mV in rat cortical neurons. The onset of application and agonist effect durations are shown by the lines above the graphs. Presented are characteristic records from one of the total number of cells tested (57). (b) Intracellular calcium responses induced by 50 μM homocysteine, 30 μM NMDA and 300 μM glutamate in rat cortical neurons. *Ordinate*: relative fluorescence intensity (control fluorescence is taken as 1). The onset of application and agonist effect durations are shown by the lines above the graphs. Each curve represents a fluorescent Ca^{2+} response recorded in the soma of one neuron. Presented are data of one experiment. Total number of experiments—3.

NMDA receptors, it was of interest to explore the nature of Ca^{2+} responses after the short-term (6 min) action of these glutamate receptor agonists and to compare them with the responses to glutamate itself. In the neurons of the primary rat cortex culture, the dynamics of Ca^{2+} responses after the 6-min action of the agonists was similar in all neurons: 50 μM homocysteine induced typical oscillatory Ca^{2+} responses [16], whereas 30 μM NMDA and 300 μM glutamate induced a rapid increase in $[Ca^{2+}]_i$ reaching plateau and persisting over the whole period of the agonist presence (Fig. 1b). These findings supported our results obtained by recording membrane currents and indicating a rapid desensitization of NMDA receptors by homocysteine. Specifically, the calcium signal decay in response to homocysteine appears to be due to a low amplitude of the current evoked by homocysteine in the steady state (Fig. 1a).

Long-term Ca^{2+} dysregulation is known to cause mitochondrial dysfunction, disruption of the respiratory chain, triggering of oxidative phosphorylation and, eventally, the death of neurons [31].

Because homocysteine, NMDA and glutamate can exert the neurotoxic effect on CNS and PNS neurons [16], it was of interest to explore the effect of these glutamate receptor agonists on $\Delta\varphi_{mit}$ as a mitochondrial state marker. The fluorophore Rho123 allows a drop in $\Delta\varphi_{mit}$ to be detected. As a control to determine a maximal $\Delta\varphi_{mit}$, the protonophore FCCP was applied at a concentration of 4 μM 2 min following the effect of the agonists; this led to a complete uncoupling of the respiratory chain and as complete drop in the membrane potential evoking thereby a maximal Rho123 emission.

Figure 2 shows $\Delta\varphi_{mit}$ at short-term (6 min, Fig. 2 (i)) and long-term (60 min, Fig. 2 (ii)) effects of 50 μM homocysteine (Fig. 2a), 30 μM NMDA (Fig. 2b) or 300 μM glutamate (Fig. 2c) on the rat cortical neurons. Homocysteine (50 μM) after 6 min of action did not induce a considerable drop in $\Delta\varphi_{mit}$ as compared with FCCP (Fig. 2 (i), (a)), while 30 μM NMDA (Fig. 2 (i), (b)) during first 6 min of its action, conversely, induced a drop in $\Delta\varphi_{mit}$ comparable with that induced by FCCP.

In contrast to the short-term action of the agonists, after 60 min of their action no significant differences between them were detected. In all cases, at 50 μM homocysteine, 30 μM NMDA or 300 μM glutamate, beginning from the 30th min

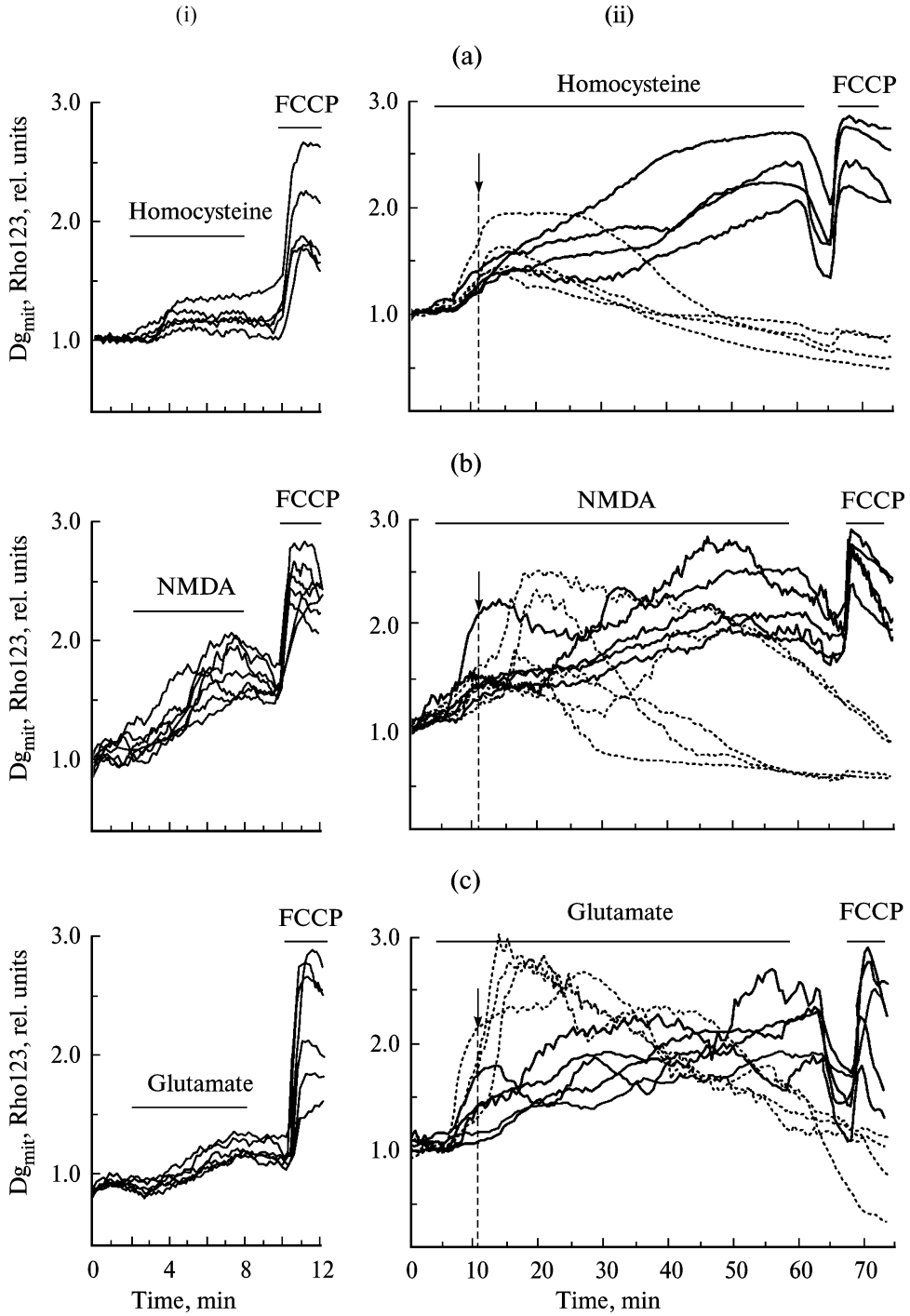

Fig. 2. Changes in mitochondrial membrane potential ($\Delta\varphi_{mit}$) detected using Rho123 in rat cortical neurons under effects of 50 μM homocysteine (a), 30 μM NMDA (b) and 300 μM glutamate (c) for 6 min (i) and 60 min (ii). *Upright dotted line* on (ii) marks 6 min of agonist effect. As a control of a maximal drop in $\Delta\varphi_{mit}$, 4 μM FCCP were applied for 2 min. The onset of application and agonist effect durations are shown by the lines above the graphs. *Ordinate*: relative fluorescence intensity (control fluorescence is taken as 1). Each curve represents a fluorescent Rho123 response recorded in the soma of one neuron. Total number of experiments—3–5.

of their action, the agonists induced equally well a conspicuous drop in $\Delta\varphi_{mit}$ (Fig. 2 (ii)) which reached its maximum by the 50–60th min of the agonists' action and was comparable with com-

plete uncoupling of the respiratory chain induced by FCCP. It is important to note that during the long-term action of NMDA, homocysteine or glutamate, in each experiment there were found the cells which exhibited a sharp increase in the intensity of Rho123 fluorescence during the first 20 min of the agonists' action indicative of a sharp drop in $\Delta\varphi_{mit}$. Later on, these cells responded with a drop in the intensity of Rho123 fluorescence and did not respond to FCCP (Fig. 2 (ii), responses are marked with dotted line). Apparently, it is exactly these cells that die first from the neurotoxic effect of homocysteine, glutamate or NMDA [16, 32–34].

To compare more precisely the drops in $\Delta\varphi_{mit}$ caused by the effect of the glutamate receptor agonists on the rat cortical neurons, we calculated the $\Delta\varphi_{mit}^{agonist}/\Delta\varphi_{mit}^{FCCP}$ ratio ($\Delta\varphi_{mit}$ for each of the agonists: 50 μM homocysteine, 30 μM NMDA or 300 μM glutamate relative to $\Delta\varphi_{mit}$ caused by FCCP) reflecting the portion of the $\Delta\varphi_{mit}$ drop due to the effect of NMDA receptor agonists of its maximal value. Figure 3 shows the $\Delta\varphi_{mit}^{agonist}/\Delta\varphi_{mit}^{FCCP}$ ratio histograms after 6 and 60 min of the agonist action. For the rat cortical neurons after 6 min of the agonist action, $\Delta\varphi_{mit}^{homocysteine}/\Delta\varphi_{mit}^{FCCP}$ was 0.13 ± 0.02, $\Delta\varphi_{mit}^{glutamate}/\Delta\varphi_{mit}^{FCCP}$—0.22 ± 0.05 and $\Delta\varphi_{mit}^{NMDA}/\Delta\varphi_{mit}^{FCCP}$—0.41 ± 0.05 relative units. The data for NMDA differed significantly from the values obtained under the effect of 50 μM homocysteine ($p < 0.01$, number of experiments = 3) and 300 μM glutamate ($p < 0.05$, number of experiments = 3). After 60 min of exposure to agonists, $\Delta\varphi_{mit}^{homocysteine}/\Delta\varphi_{mit}^{FCCP}$ was 0.44 ± 0.04, $\Delta\varphi_{mit}^{glutamate}/\Delta\varphi_{mit}^{FCCP}$—0.42 ± 0.06, $\Delta\varphi_{mit}^{NMDA}/\Delta\varphi_{mit}^{FCCP}$—0.52 ± 0.08 relative units and they did not differ significantly from one another ($p > 0.05$, number of experiments = 3–5). Thus, a 60-min exposure to NMDA receptor agonists results in almost a 2-fold drop in mitochondrial receptor potential.

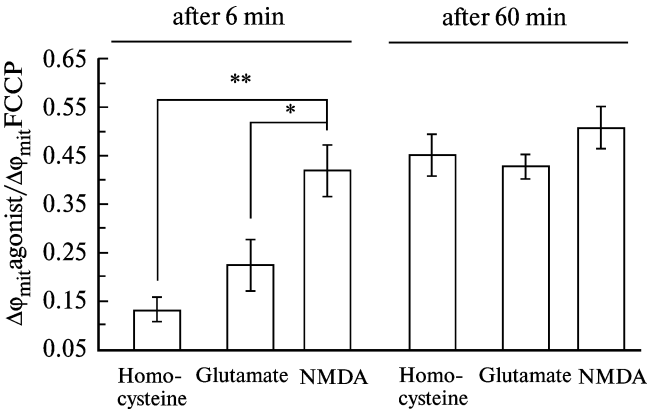

Fig. 3. Ratio of the drop in mitochondrial membrane potential ($\Delta\varphi_{mit}$) induced by 50 μM homocysteine, 30 μM NMDA and 300 μM glutamate to the complete drop in $\Delta\varphi_{mit}$ under the respiratory chain uncoupling induced by 4 μM FCCP in rat cortical neurons ($\Delta\varphi_{mit}^{agonist}/\Delta\varphi_{mit}^{FCCP}$) after 6 and 60 min of agonist action. Presented are means with standard error (*—$p < 0.05$, **—$p < 0.01$) for 3–5 experiments.

DISCUSSION

Glutamate neurotoxicity was fairly well-studied on the primary rat cortical cell culture [31–33, 35]. However, the mechanism of action of high homocysteine concentrations on the cells of the primary culture of cortical neurons is poorly understood. L-homocysteic acid, known to be structurally nearest to L-glutamate and analogous to L-homocysteine, can interact with NMDA receptors and group I metabotropic glutamate receptors stronger than homocysteine itself [14]. However, hyperhomocysteinemia is associated with the increased homocysteine level in plasma and cerebrospinal fluid [36]. In this connection, to explore the homocysteine action mechanism on CNS cells (primary rat cortical cell culture), we used L-homocysteine in concentration corresponding to medium hyperhomocysteinemia (30–100 μM).

Previously, it was shown that under the long-term effect of homocysteine on cortical and trigeminal ganglion neurons it induces the apoptotic cell death, i.e. exerts a cytotoxic effect [16] mediated via ionotropic NMDA-type glutamate receptors and metabotropic type 5 glutamate receptors [12, 14–17].

There are only a few electrophysiological studies of the effect of homocysteine [12, 13, 37]. We were the first to measure inward integral currents under the effect of homocysteine which were compared with NMDA-induced integral currents. In contrast to MNDA, homocysteine-activated receptors desensitize faster (Fig. 1a). Interestingly, the homocystein-induced inward current amplitude is characteristic of NMDA receptors that lack the GluN2B subunit [38]. Inward currents gener-

ated due to the activation of NMDA receptors are known to reflect the Ca^{2+} influx kinetics. In this respect, we compared intracellular Ca^{2+} responses evoked by short-term effects of homocysteine, NMDA and glutamate.

Our electrophysiological data are consistent with the results on intracellular Ca^{2+} responses. Short-term impacts of homocysteine, NMDA and glutamate induce in the rat cortical neurons Ca^{2+} responses differing in their dynamics: Ca^{2+} response to homocysteine following its maximum dropped almost to the control level and represented Ca^{2+} oscillations (Fig. 1b) dissimilar to the typical Ca^{2+} dynamics caused by the effects of NMDA or glutamate [31, 34].

The recent studies have shown that the homocysteine-induced death of embryonic cortical neurons is mediated via not only NMDA receptors, but recruits the ERK-MAPK and p38-MAPK kinase signaling pathways [17]. Other studies suggest that homocysteine is implicated in oxidative stress that leads to the death of neurons [18, 19, 39]. Meanwhile, it is generally accepted that hyperactivation of NMDA receptors is associated with oxidative stress [18, 19]. Moreover, as demonstrated on hippocampal neurons, homocysteine at high concentration (250 μM) leads to the polymerization of poly-ADP-ribose and depletion of NAD^+ reserves causing thereby mitochondrial oxidative stress and apoptosis [40]. It has been also shown that severe induced hyperhomocysteinemia causes lipid peroxidation, a cell oxidative stress marker [20]. Importantly, in the experiments addressing $\Delta\varphi_{mit}$ under the short-term effect of 50 μM homocysteine we did not reveal a potential drop in the primary rat cortical cell culture (Fig. 2 (i), (a)). As compared with homocysteine, NMDA and glutamate induced greater changes in $\Delta\varphi_{mit}$ (Fig. 2 (i), (b, c)). First of all, these differences can be accounted for the fact that homocysteine and glutamate are endogeneous glutamate receptor agonists and have individual intracellular mechanisms of utilization (glutamate transporter for glutamate and glutathione formation for homocysteine [41]). Probably, the detection of oxidative stress through the drop in $\Delta\varphi_{mit}$ requires the longer effect of homocysteine. In contrast, NMDA induces strong hyperactivation of NMDA receptors and relatively weak desensitization that jointly results in Ca^{2+} dysregulation and subsequent drop in $\Delta\varphi_{mit}$ leading to cell death.

This theory is supported by the studies of $\Delta\varphi_{mit}$ under long-term effects of the agonists (homocystein, NMDA and glutamate) (Fig. 2 (ii)). In all cases, during 60 min the agonists (including homocysteine) led to a strong depolarization of mitochondria comparable with FCCP-induced complete uncoupling of the respiratory chain that may have eventually caused the death of neurons [31].

Our results demonstrate that in the rat cortical neurons the mechanisms mediating the neurotoxic effect of homocysteine, although much less pronounced, are not basically dissimilar to the glutamate and NMDA action mechanisms. In addition to Ca^{2+} dysregulation, they include mitochondrial dysfunction that leads to uncoupling of the mitochondrial respiratory chain and triggers oxidative phosphorylation which may eventually initiate the apoptotic death of neurons.

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REFERENCES

1. Isobe, C. and Terayama, Y., A remarkable increase in total homocysteine concentrations in the CSF of migraine patients with aura, *Headache*, 2010, vol. 50, no. 10, pp. 1561–1569.
2. Brattström, L. and Wilcken, D.E., Homocysteine and cardiovascular disease: cause or effect? *Am. J. Clin. Nutr.*, 2000, vol. 72, no. 2, pp. 315–323.
3. Sachdev, P.S., Homocysteine and brain atrophy, *Prog. Neuropsychopharmacol. Biol. Psychiatry*, 2005, vol. 29, no. 7, pp. 1152–1161.
4. Boldyrev, A.A. and Johnson, P., Homocysteine and its derivatives as possible modulators of neuronal and non-neuronal cell glutamate receptors in Alzheimer's Disease, *J. Alzheimer's Dis.*, 2007, vol. 11, pp. 219–228.
5. Zoccolella, S., Bendotti, C., Beghi, E., and Logroscino, G., Homocysteine levels and amyotrophic lateral sclerosis: A possible link, *Amyotroph. Lateral. Scler.*, 2010, vol. 11, no. 1–2, pp. 140–147.
6. Lea, R., Colson, N., Quinlan, S., Macmillan, J., and Griffiths, L., The effects of vitamin supplementation and MTHFR (C677T) genotype on homocysteine-lowering and migraine disability,

Pharmacogenet. Genomics, 2009, vol. 19, no. 6, pp. 422–428.
7. Oterino, A., Toriello, M., Valle, N., Castillo, J., Alonso-Arranz, A., Bravo, Y., Ruiz-Alegria, C., Quintela, E., and Pascual, J., The relationship between homocysteine and genes of folate–related enzymes in migraine patients, *Headache*, 2010, vol. 50, no. 1, pp. 99–168.
8. Giniatullin, R.A., Neurophysiological mechanisms of migraine and new principles of pathogenetic treatment, *Kazan. Med. Zh.,* 2011, vol. 92, no. 5, pp. 728–735.
9. Moskowitz, M.A., Genes, proteases, cortical spreading depression and migraine: impact on pathophysiology and treatment, *Funct. Neurol.*, 2007, vol. 22, no. 3, pp. 133–136.
10. Pietrobon, D. and Moskowitz, M.A., Pathophysiology of migraine, *Annu. Rev. Physiol.*, 2013, vol. 75, pp. 365–391.
11. Chauvel, V., Vamos, E., Pardutz, A., Vecsei, L., Schoenen, J., and Multon, S., Effect of systemic kynurenine on cortical spreading depression and its modulation by sex hormones in rat, *Exp. Neurol.*, 2012, vol. 236, no. 2, pp. 207–214.
12. Lipton, S.A., Kim, W.K., Choi, Y.B., Kumar, S., D'Emilia, D.M., Rayuda, P.V., Arnelle, D.R., and Stamler, J.S., Neurotoxicity associated with dual actions of homocysteine at the N-methyl-D-aspartate receptor, *Proc. Natl. Acad. Sci. USA*, 1997, vol. 94, no. 11, pp. 5923–5928.
13. Ganapathy, P.S., White, R.E., Ha, Y., Bozard, B.R., McNeil, P.L., Caldwell, R.W., Kumar, S., Black, S.M., and Smith, S.B., The role of N-methyl-D-aspartate receptor activation in homocysteine-induced death of retinal ganglion cells, *Invest. Ophthalmol. Vis. Sci.*, 2011, vol. 52, no. 8, pp. 5515–5524.
14. Shi, Q., Savage, J.E., Hufeisen, S.J., Rauser, L., Grajkowska, E., Ernsberger, P., Wroblewski, J.T., Nadeau, J.H., and Roth, B.L., L-homocysteine sulfinic acid and other acidic homocysteine derivatives are potent and selective metabotropic glutamate receptor agonists, *J. Pharmacol. Exp. Ther.*, 2003, vol. 305, no. 1, pp. 131–142.
15. Yeganeh, F., Nikbakht, F., Bahmanpour, S., Rastegar, K., and Namavar, R., Neuroprotective effects of NMDA and group I metabotropic glutamate receptor antagonists against neurodegeneration induced by homocysteine in rat hippocampus: in vivo study, *J. Mol. Neurosci.*, 2013, vol. 50, no. 3, pp. 551–557.
16. Abushik, P.A., Niittykoski, M., Giniatullina, R., Shakirzyanova, A., Bart, G., Fayuk, D., Sibarov, D.A., Antonov, S.M., and Giniatullin, R., The role of NMDA and mGluR5 receptors in calcium mobilization and neurotoxicity of homocysteine in trigeminal and cortical neurons and glial cells, *J. Neurochem.*, 2014, vol. 129, no. 2, pp. 264–274.
17. Poddar, R. and Paul, S., Novel crosstalk between ERK MAPK and p38 MAPK leads to homocysteine-NMDA receptor-mediated neuronal cell death, *J. Neurochem.*, 2013, vol. 124, no. 4, pp. 558–570.
18. Tenneti, L., D'Emilia, D.M., Troy, C.M., and Lipton, S.A., Role of caspases in N-methyl-D-aspartate-induced apoptosis in cerebrocortical neurons, *J. Neurochem.*, 1998, vol. 71, no. 3, pp. 946–959.
19. Reyes, R.C., Brennan, A.M., Shen, Y., Baldwin, Y., and Swanson, R.A., Activation of neuronal NMDA receptors induces superoxide-mediated oxidative stress in neighboring neurons and astrocytes, *J. Neurosci.*, 2012, vol. 32, no. 37, pp. 12973–12978.
20. Matte, C., Monteiro, S.C., Calcagnotto, T., Bavaresco, C.S., Netto, C.A., and Wyse, A.T., In vivo and in vitro effects of homocysteine on Na^+,K^+-ATPase activity in parietal, prefrontal and cingulate cortex of young rats, *Int. J. Dev. Neurosci.*, 2004, vol. 22, no. 4, pp. 185–190.
21. Outinen, P.A., Sood, S.K., Liaw, P.C.Y., Sarge, K.D., Maeda, N., Hirsh, J., Ribau, J., Podor, T.J., Weitz, J.I., and Austin, R.C., Characterization of the stress-inducing effects of homocysteine, *Biochem. J.*, 1998, vol. 332, pt. 1, pp. 213–221.
22. Perna, A.F., Ingrosso, D., and De Santo, N.G., Homocysteine and oxidative stress, *Amino Acids*, 2003, vol. 25, no. 3–4, pp. 409–417.
23. Loureiro, S.O., Romão, L., Alves, T., Fonseca, A., Heimfarth, L., Moura Neto, V., Wyse, A.T., and Pessoa–Pureur, R., Homocysteine induces cytoskeletal remodeling and production of reactive oxygen species in cultured cortical astrocytes, *Brain Res.,* 2010, no. 1355, pp. 151–164.
24. Mironova, E.V., Lukina, A.A., Brovtsyna, N.B., Krivchenko, A.I., and Antonov, S.M., Types of glutamate receptors determining concentration dependence of its neurotoxic effect on the rat cortical neurons, *Zh. Evol. Biokh. Fiziol.,* 2006, vol. 42, no. 6, pp. 559–566.
25. Antonov, S.M. and Johnson, J.W., Voltage-dependent interaction of open-channel blocking molecules with gating of NMDA receptors in rat cortical neurons, *J. Physiol.*, 1996, vol. 493, pt. 2, pp. 425–445.
26. Antonov, S.M. and Johnson, J.W., Permeant ion regulation of N-methyl-D-aspartate receptor

channel block by Mg^{2+}, *Proc. Natl. Acad. Sci. USA*, 1999, vol. 96, no. 25, pp. 14571–14576.
27. Grynkiewicz, G., Poenie, M., and Tsien, R.Y., A new generation of Ca^{2+} indicators with greatly improved fluorescence properties, *J. Biol. Chem.*, 1985, vol. 260, no. 6, pp. 3440–3450.
28. Duchen, M.R., Mitochondria, calcium-dependent neuronal death and neurodegenerative disease, *Pflügers Arch.*, 2012, vol. 464, no. 1, pp. 111–121.
29. Hamill, O.P., Marty, A., Neher, E., Sakmann, B., and Sigworth, F.J., Improved patch-clamp techniques for high-resolution current recording from cells and cell-free membrane patches, *Pflügers Arch.*, 1981, vol. 391, no. 2, pp. 85–100.
30. Han, E.B. and Stevens, C.F., Development regulates a switch between post- and presynaptic strengthening in response to activity deprivation, *Proc. Natl. Acad. Sci. USA*, 2009, vol. 106, no. 26, pp. 10817–10822.
31. Khodorov, B., Glutamate-induced deregulation of calcium homeostasis and mitochondrial dysfunction in mammalian central neurons, *Progr. Biophys. Molec. Biol.*, 2004, vol. 86, no. 2, pp. 279–351.
32. Abushik, P.A., Sibarov, D.A., Eaton, M.J., Skatchkov, S.N., and Antonov, S.M., Kainate-induced calcium overload of cortical neurons in vitro: Dependence on expression of AMPAR GluA2-subunit and down-regulation by subnanomolar ouabain, *Cell Calcium*, 2013, vol. 54, no. 2, pp. 95–104.
33. Evstratova, A.A., Mironova, E.V., Dvoretskova, E.A., and Antonov, S.M., Apoptosis and receptor specificity of its mechanisms in neurotoxic effect of glutamate, *Ross. Fiziol. Zh. im. I.M. Sechenova,* 2008, vol. 94, no. 4, pp. 380–393.
34. Mironova, E.V., Evstratova, A.A., and Antonov, S.M., A fluorescence vital assay for the recognition and quantification of excitotoxic cell death by necrosis and apoptosis using the confocal microscopy on neurons in culture, *J. Neurosci. Methods*, 2007, vol. 163, no. 1, pp. 1–8.
35. Sibarov, D.A., Bolshakov, A.E., Abushik, P.A., Krivoi, I.I., and Antonov, S.M., Na^+,K^+-ATPase functionally interacts with the plasma membrane Na^+,Ca^{2+} exchanger to prevent Ca^{2+} overload and neuronal apoptosis in excitotoxic stress, *J. Pharmacol. Exp. Ther.*, 2012, vol. 343, no. 3, pp. 596–607.
36. Surtees, R., Bowron, A., and Leonard, J., Cerebrospinal fluid and plasma total homocysteine and related metabolites in children with cystathionine beta-synthase deficiency: the effect of treatment, *Pediatr Res.*, 1997, vol. 42, no. 5, pp. 577–582.
37. Bolton, A.D., Phillips, M.A., and Constantine-Paton, M., Homocysteine reduces NMDAR desensitization and differentially modulates peak amplitude of NMDAR currents, depending on GluN2 subunit composition, *J. Neurophysiol.*, 2013, vol. 110, no. 7, pp. 1567–1582.
38. Traynelis, S.F., Wollmuth, L.P., McBain, C.J., Menniti, F.S., Vance, K.M., Ogden, K.K., Hansen, K.B., Yuan, H., Myers, S.J., and Dingledine, R., Glutamate receptor ion channels: structure, regulation and function, *Pharmacol. Rev.*, 2010, vol. 62, no. 3, pp. 405–496.
39. Sibrian-Vazquez, M., Escobedo, J.O., Lim, S., Samoei, G.K., and Strongin, R.M., Homocystamides promote free-radical and oxidative damage to proteins, *Proc. Natl. Acad. Sci. USA*, 2010, vol. 107, no. 2, pp. 551–554.
40. Jara-Prado, A., Ortega-Vazquez, A., Martinez-Ruano, L., Rios, C., and Santamaria, A., Homocysteine–induced brain lipid peroxidation: effects of NMDA receptor blockade, antioxidant treatment, and nitric oxide synthase inhibition, *Neurotox. Res.*, 2003, vol. 5, no. 4, pp. 237–243.
41. Lu, S.C., Regulation of glutathione synthesis, *Mol. Aspects Med.*, 2009, vol. 30, no. 1–2, pp. 42–59.