ISSN 1990-7478, Biochemistry (Moscow), Supplement Series A: Membrane and Cell Biology, 2018, Vol. 12, No. 4, pp. 360–368. © Pleiades Publishing, Ltd., 2018.
Original Russian Text © L.S. Sitnikova, M.A. Ivanova, Yu.D. Stepanenko, T.V. Karelina, R. Giniatullin, D.A. Sibarov, P.A. Abushik, S.M. Antonov, 2018, published in Biologicheskie Membrany, 2018, Vol. 35, No. 4, pp. 261–270.

ARTICLES

Collapse of Neuronal Energy Balance As a Basis of *L*-Homocysteine Neurotoxicity

L. S. Sitnikova[a], M. A. Ivanova[a], Yu. D. Stepanenko[a], T. V. Karelina[a], R. Giniatullin[b], D. A. Sibarov[a], P. A. Abushik[a], and S. M. Antonov[a, *]

[a] *Sechenov Institute of Evolutionary Physiology and Biochemistry of the Russian Academy of Sciences, St. Petersburg, 194223 Russia*

[b] *University of Eastern Finland, Department of Neurobiology, P.O. Box 1627/Neulaniementie 2, 70211 Kuopio, Finland*

**e-mail: antonov452002@yahoo.com*

Received September 25, 2017; in final form, October 25, 2017

Abstract—Using fluorescence detection methods, neurotoxic effects of *L*-homocysteine (HCY), *L*-glutamate (Glu), and N-methyl-*D*-aspartate (NMDA) on primary culture of rat cerebellar neurons were compared and the agonist-evoked intracellular Ca^{2+} responses and changes in mitochondrial membrane potential were studied. Long-term (5 h) action of HCY, Glu, or NMDA caused neuronal apoptosis and necrosis that was followed by a decrease of quantity of live cells to 40%. It was revealed using Fluo-3 that neurons differed by intracellular Ca^{2+} responses to 2-min applications of HCY. In response to all studied agonists, a brief peak or gradual increase of intracellular Ca^{2+} concentration was observed. Some neurons did not respond to HCY, but all responded to Glu and NMDA. A prolonged (60 min) treatment with agonists caused a rapid or delayed Ca^{2+} overload, while only a small portion of neurons were able to compensate the intracellular Ca^{2+} elevation. Six-minute applications of HCY or Glu to neurons induced similar changes of mitochondrial potential (φ_{mit}) measured by rhodamine123. In this protocol, the ability of the NMDA receptor agonists to cause the mitochondrial dysfunction could be arranged in the following order: NMDA > Glu = HCY. After a 60-min treatment the observed difference vanished because all of the agonists reduced φ_{mit} so that an uncoupling agent FCCP did not cause any additional changes in φ_{mit}. Thus, HCY-induced neurotoxicity in cerebellar neurons is comparable to that of Glu. In this feature cerebellar neurons differ from cortical neurons, in which HCY did not significantly change φ_{mit} during short-term application. This difference could be related with peculiarities of the HCY action on NMDA receptor subtypes expressed by cerebellar neurons.

Keywords: cerebellum, homocysteine, glutamate, apoptosis, calcium, mitochondria

DOI: 10.1134/S1990747818050069

INTRODUCTION

Sulfur aminoacid *L*-homocysteine (HCY) participates in many metabolic pathways in living organism: cysteine and methionine synthesis, lipid, protein and DNA methylation, synthesis of come hormones and signaling molecules [1]. An average HCY concentration in the blood flow is ~10 μM. However, in the case of folic acid or group B vitamin deficiency, as well as in the case of C677T point mutation of methylene tetrahydrofolate reductase gene [2–4], an accumulation of HCY in blood and cerebrospinal fluid takes place, which is called hyperhomocysteinemia. There are three degrees of this pathology: moderate (16–30 μM in blood plasma), intermediate (30–100 μM), and severe (>100 μM) [1]. Hyperhomocysteinemia accompanies many cardiovascular, mental, and neurodegenerative diseases like Alzheimer disease, epilepsy, Parkinson disease, schizophrenia, lateral amyotrophic sclerosis [3, 5, 6]. It is commonly accepted that long-term hyperactivation of glutamate receptors causes continuous neuronal depolarization, disruption of ionic gradients, calcium deregulation, and consequently neuronal death [7]. Endogenous HCY is known to activate ionotropic glutamate receptors of NMDA subtype and metabotropic glutamate receptors of mGluR5 subtype [1, 8–11]. An expression pattern of these receptors differ between brain regions. Rat cortical neurons in vivo and in primary culture express NMDA receptors containing GluN2A and GluN2B subunits [12–14]. The newborn rat cerebellar neurons express mainly GluN2B subunit, which change to GluN2A and GluN2C by the time of 14 postnatal day (P14) [14, 15].

Recently it was shown that HCY activates NMDA receptors causing rapid desensitization of GluN2B but not GluN2A containing NMDA receptors [16]. Brain structures are heterogeneous by GluN2 expression profile, thus the vulnerability of different brain regions

to hyperhomocysteinemia is different. In particular, cerebellum is involved in many neurodegenerative pathologies related to hyperhomocysteinemia [17]. Therefore, it is interesting to study the neurotoxic effects of HCY at intermediate hyperhomocysteinemia concentrations and compare them to NMDA and glutamate (Glu) effects.

MATERIALS AND METHODS

Primary culture of cerebellar neurons. Primary culture of cerebellar neurons was prepared from Wistar rat embryos at 20–21 days of gestation (E20–E21). Cerebellums were enzymatically suspended with trypsin (0.04 mg/mL, Sigma–Aldrich, USA) and treated with DNAase (0.04 mg/mL, Sigma–Aldrich, USA), then with trypsin inhibitor (0.4 mg/mL, Sigma–Aldrich, USA) and with bovine serum albumin (10%, Gibco, USA). After centrifugation, cell dissociation was performed by pipetting in the media containing Neurobasal™ (Gibco, USA) with B-27 supplement (2%, Gibco, USA), antibiotic penicillin streptomycin (1%, Gibco, USA), *L*-glutamine (Gibco, USA), and 20 mM KCl to increase the chance of survival of cerebellar neurons [18]. Dispersed cells were cultured on 7- and 30-mm coverslips treated with a poly-*D*-lysine for 7–10 days (days in vitro, DIV 7–10) at 37°C and 5% CO_2. Medium in culture dishes was replaced every 2 days.

Experimental protocol. As the time-dependent maturation of neurons in primary culture [19] occurs in a way similar to that in intact animals, experiments were performed at DIV 7–10 [20], which approximately corresponds to the first week of in vivo postnatal development of newborn rats. Mg^{2+} was removed from the culture media to avoid the block of NMDA receptors [21]. Thus the medium of the following composition was used (mM): 140 NaCl, 2.8 KCl, 10 HEPES, 2 $CaCl_2$, (pH 7.2–7.4). Excitotoxic stress was evoked by the following concentrations of the glutamate receptor agonists: 50 μM of HCY (recording of Ca^{2+} and φ_{mit}), 100 μM of HCY (study of neuronal survival), 30 μM of NMDA, and 100 μM of Glu (Sigma–Aldrich, USA). HCY concentrations of 50 and 100 μM correspond to moderate and intermediate hyperhomocysteinemia [4] and are enough to activate both GluN2A and GluN2B subunits of NMDA receptors [16]. The concentration of 30 μM for NMDA exceeds the EC_{50} for GluN2B subunit containing NMDA receptors, having the highest calcium permeability of all glutamate receptors. Extracellular glutamate concentration of 100 μM can be observed in ischemia and brain trauma. Glycine as the NMDA receptor co-agonist was always co-applied with agonists. It was present at 100 μM in a growth media and in cell survival experiments. Glycine at 30 μM (receptor saturating concentration) was applied during recordings of intracellular Ca^{2+} and φ_{mit}. Single experiment (*n*) corresponds to a treatment of one coverslip with neuronal culture with agonists. The incubation in growth media was used as control in 5-h neuronal survival tests. Physiological solution without agonists was used as a control in Ca^{2+} or φ_{mit} measurement experiments.

Neuronal survival test. To measure the ratio of live, necrotic, and apoptotic cells, neurons we consequently stained with acridine orange (AO, 10 μg/mL, Sigma–Aldrich, USA) and ethidium bromide (EB, 40 μg/mL, Sigma–Aldrich, USA). Fluorescence evoked with 488 nm laser was captured using scanning confocal microscope Leica TCS SL (Leica Microsystems, Germany). Emission in the green spectral region 500–560 nm indicated living neurons stained with AO. Emission in the red spectral region >600 nm revealed necrotic cellular nuclei stained with EB. Cytoplasm acidification in apoptosis shifted the AO emission to yellow [14, 15], which overlapped red and green spectral regions simultaneously [22, 23]. Images were analyzed using ImageJ software (https://imagej.nih.gov/ij/) as described previously [23]. The quantities of live, necrotic, and apoptotic cells obtained from 3 images captured from one coverslip were averaged and taken as a single measurement (*n*).

Recording of intracellular Ca^{2+} responses and mitochondrial membrane potential (φ_{mit}). Relative values of $[Ca^{2+}]_i$ were recorded in submicromolar range using fluorescent dye Fluo-3AM (Invitrogen, USA) [24]. This dye was loaded into the cells as acetoxymethyl (AM) ester derivative (1 μM, 60 min, in the dark, 23–25°C). Then cells were incubated for 15 min in the dark for de-esterification with intracellular esterases and production of membrane-impermeable Fluo-3.

Changes of mitochondrial inner membrane potential ($\Delta\varphi_{mit}$) were detected using rhodamine123 dye (Rho123, Invitrogene, USA) [25]. This dye (5 μM) was loaded into the cells during 30-min incubation at room temperature (23–25°C). The protonophore carbonyl cyanide-4-(trifluoromethoxy) phenylhydrazone (FCCP, 4 μM; Sigma–Aldrich, USA) was used as a reference to visualize a full drop of mitochondrial membrane potential (φ_{mit}) and total uncoupling of mitochondrial oxidative phosphorylation, which is accompanied with a highest possible Rho123 fluorescence [25].

Fluorimetric experiments with Fluo-3 and Rho123 were conducted using inverted confocal scanning microscope Leica SP5 MP (Leica Microsystems, Germany). Coverslips with cells were placed inside a perfusion chamber POCmini Chamber System (LaCon, Germany) and connected to overall perfusion and to local fast perfusion system BPS-8 (Ala Science, USA). The overall perfusion speed in 10–90-min experiments was 1 mL/min. Agonists were applied using fast local perfusion via the tube placed 1 mm away from the region of interest. The medium was completely changed within ≤1 s.

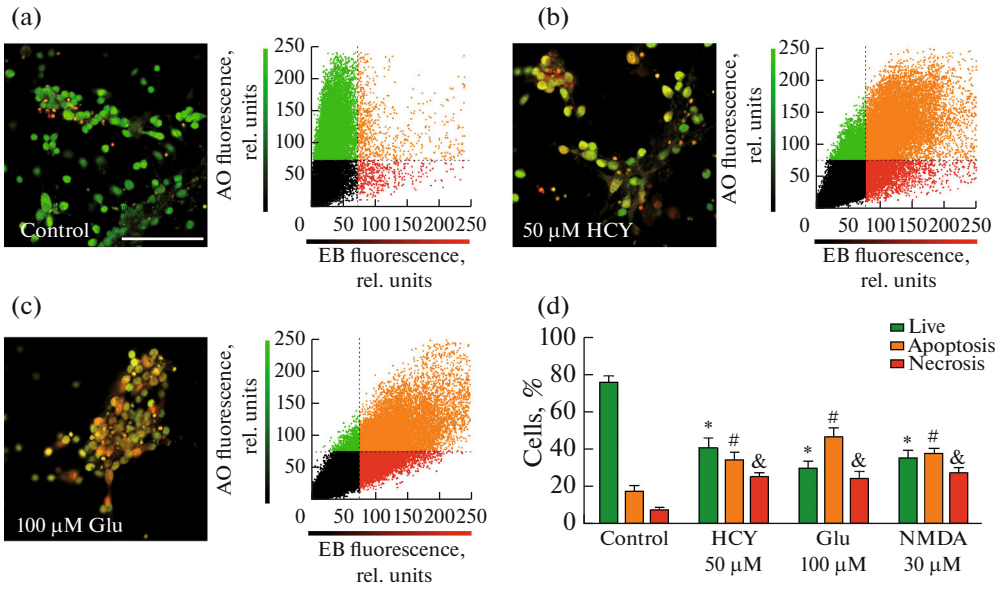

Fig. 1. Neurotoxic effect of long-term in vitro treatment of rat cereballar neurons with *L*-homocysteine (100 μM), *L*-glutamate (100 μM) and NMDA (30 μM). (a) Control; (b) 5-h treatment with 100 μM *L*-homocysteine; (c) 5-h treatment with 100 μM *L*-glutamate. Each panel show fluorescent images of neurons (right) and intensity correlation diagrams (left) of *green* (acridine orange, AO) and *red* (ethidium bromide, EB) fluorescence which allow quantitative evaluation of live (*green*), apoptotic (*yellow*) and necrotic (*red*) cells. (c) Averaged % values of live (*green*), apoptotic (*orange*) and necrotic (*red*) neurons after treatment with glutamate receptors agonists (concentrations and agonists names are shown above the bars). Mean ± SEM for 6–7 experiments ($n = 6–7$) are shown. Statistical difference from control values are marked for live (*), apoptotic (#) and necrotic (&) cells ($p < 0.0001$, ANOVA with Bonferroni post-hoc test).

Fluorescence of Rho123 and Fluo-3 was excited with 488-nm laser. Emission was captured at 500–560 nm every 30 s. The obtained images were analyzed in Leica LAS AF software (Leica Microsystems, Germany). The time course of the fluorescence intensities of cell bodies picked as regions of interest were plotted. Each agonist was studied in at least four coverslips with 20 to 50 neurons recorded in one field of view.

Statistical analysis. Measurements are presented as mean ± standard error of the mean. Experimental groups were compared using ANOVA with Bonferroni post-hoc analysis. Independent groups were compared using two-way Student's *t*-test. Groups were expected to be significantly different at $p < 0.05$.

RESULTS

Neurotoxic effect of HCY on cerebellar neurons. In control (5-h incubation in growth media) the majority of neurons were alive, as is testified by the prevalence of green pixels in color intensity diagrams (Fig. 1a). A 5-h treatment with 100 μM HCY (Fig. 1b) and 100 μM Glu (Fig. 1c), as well as with 30 μM NMDA resulted in a decrease of live cell percentage due to apoptosis and necrosis, which is observed as the prevalence of red and orange pixels at color intensity diagrams. In control conditions the amount of live neurons was 75.6 ± 3.5% ($n = 6$, Fig. 1d); apoptotic cells, 17.2 ± 3.0%, and necrotic cells, 7.2 ± 1.5%, which corresponds to normal values in primary culture of neurons [22, 23, 26]. After the treatment with HCY the quantity of live neurons decreased to 40.3 ± 5.5% ($n = 6$, Fig. 1d), while the quantity of apoptotic and necrotic cells increased to 34.0 ± 4.0 and 24.9 ± 2.2%, respectively (Fig. 1d). Glu treatment also caused a significant increase in apoptotic (46.2 ± 4.9%) and necrotic (24.0 ± 4.0%) cells ($n = 6$), while the quantity of live cells decreased to 29.5 ± 3.8%. NMDA application lowered neuronal viability to 35.0 ± 4.3% ($n = 7$), while 37.4 ± 2.8% of cells were apoptotic and 27.0 ± 3.0% of cells were necrotic (Fig. 1d).

Thus, a long-term application of either HCY or NMDA or Glu caused neuronal death mostly by apoptosis. This can be due to an excessive Ca^{2+} entry via pores of NMDA receptors, which can be activated by HCY [1, 9, 11, 16].

Intracellular Ca^{2+} responses caused by NMDA-receptor agonists in cerebellar neurons. The neurotoxic action of glutamate and neuronal death is now thought to be triggered by Ca^{2+} deregulation due to an increased conductance of Ca^{2+} channels and excessive entry of Ca^{2+} into the cytoplasm. Ionotropic glutamate receptors and release of Ca^{2+} from intracellular stores contributes to Ca^{2+} deregulation [7, 13, 23]. We analyzed the dynamics of intracellular Ca^{2+} responses to short-term applications of HCY as compared to effects of NMDA and Glu.

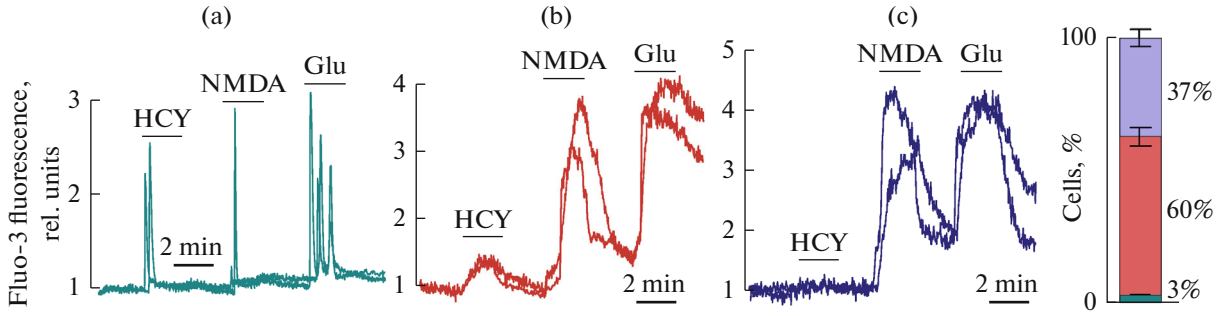

Fig. 2. Intracellular Ca^{2+} responses evoked by short-term (2 min) action of *L*-homocysteine (100 μM), *L*-glutamate (100 μM) and NMDA (30 μM). (a) Single peak Ca^{2+} responses to HCY. (b) Gradually increasing Ca^{2+} responses to HCY. (c) Neurons responding to NMDA and Gly, but not HCY. Each curve represents the intensity of single cell body fluorescence of Fluo-3. Agonists application is indicated by lines above the charts. Y-axis represents the relative fluorescence intensity of cells normalized to control baseline. The diagram on the left shows the quantitative ratio of cells sorted by response type. The diagram colors correspond to the chart colors. The data of 7 experiments are presented.

A short-term 2-min application of 100 μM HCY (with 30 μM glycine) caused two types of Ca^{2+} responses. (1) A fast single-peak Ca^{2+} response, recorded in 3.3 ± 0.1% cells (n = 7, 9 cells out of 164). This dynamics was also observed for 30 μM NMDA and 100 μM Glu (Fig. 2a). (2) Slow Ca^{2+} response with lower amplitude as compared to NMDA or Glu action observed in the same cells, recorded in 96.7 ± 3.4% neurons (n = 7, 155 cells out of 164, Fig. 2b). The last group of neurons included a subgroup of cells responding to Glu, NMDA, and HCY (37.1 ± 3.4%, Fig. 2b) and cells responding to NMDA and Glu only (59.6 ± 3.6%, n = 7, 164 cells out of 275, Fig. 2c). A wide variability of cellular Ca^{2+} responses to HCY in cerebellar neurons as compared to cortical neurons can be due to heterogeneity of NMDA-receptor subtypes expressed in cerebellar cells, which results in different desensitization kinetics, calcium permeability and sensitivity to HCY [16] and other agonists [14].

A long-term (60 min) action of 100 μM HCY caused a gradual 2–4-fold increase in the intracellular Ca^{2+} concentration in all cells. Some neurons with fast uncompensated Ca^{2+} response died during the HCY treatment (Fig. 3a, red color). Other neurons survived but showed Ca^{2+} overload at the end of the HCY treatment (Fig. 3a, yellow color). Application of 100 μM Glu caused a rapid Ca^{2+} response in all neurons followed either by Ca^{2+} overload (Fig. 3b, red color) or by compensation of the Ca^{2+} rise (Fig. 3b, green and blue colors). Upon application of 30 μM NMDA there was no rapid Ca^{2+} response but neurons demonstrated a fast (Fig. 3c, red color) or delayed (Fig. 3c, yellow color) Ca^{2+} overload, which can provoke mitochondrial dysfunction [7] and neuronal death by apoptosis [22] or necrosis [27]. A separate group of few neurons did not respond to HCY or NMDA but demonstrated non-periodic short Ca^{2+} spikes (Fig. 3, blue color). These neurons responded only to Glu and possibly expressed only AMPA receptors, which cannot be activated by HCY or NMDA.

The influence of NMDA-receptor agonists on mitochondrial membrane potential (φ_{mit}) in cerebellar neurons. A long-term 1-h application of HCY caused an elevation of intracellular Ca^{2+}, and a 5-h treatment resulted in apoptosis (Fig. 1). It is interesting to compare a short (6 min) and long-term (60 min) action of HCY, NMDA, and Glu on functional state of mitochondria in neurons. Protonophore FCCP (4 μM) evoked maximal fluorescence of Rho123 as compared to control (Figs. 4a, 4b, 4c), indicating zero mitochondrial membrane potential φ_{mit} (Fig. 4c). The influence of 6-min application of agonists on mitochondrial membrane potential changes ($\Delta\varphi_{mit}$) was presented as $\Delta\varphi_{mit}^{agonist} / \Delta\varphi_{mit}^{FCCP}$, that is, a ratio of the Rho123 fluorescence change observed in the presence of NMDA-receptor agonists to the Rho123 fluorescence change in the presence of FCCP. The obtained values are presented in Fig. 5. This ratio for HCY was 0.32 ± 0.05 (n = 4); for Glu it was 0.55 ± 0.16 (n = 5), and for NMDA, 0.79 ± 0.17 (n = 4). Even a 6-min application of NMDA caused a full drop of φ_{mit}, so $\Delta\varphi_{mit}^{NMDA} / \Delta\varphi_{mit}^{FCCP}$ did not differ significantly from 1 (Fig. 5). Glu and HCY caused only a partial drop of φ_{mit}, so $\Delta\varphi_{mit}^{HCY} / \Delta\varphi_{mit}^{FCCP}$ and $\Delta\varphi_{mit}^{Glu} / \Delta\varphi_{mit}^{FCCP}$ values were significantly lower than 1 (Fig. 5) but did not differ from each other. Thus, the potency of the neurotoxic effect of studied NMDA-receptor agonists as uncouplers of mitochondrial oxidative phosphorylation was as follows: NMDA > Glu = HCY.

At the end of a long-term (60 min) application all three agonists caused a full drop of φ_{mit} ($\Delta\varphi_{mit}^{agonist} / \Delta\varphi_{mit}^{FCCP}$ = 1, Fig. 4). However, HCY effect was a bit slower that in the case of Glu or NMDA. The effects of HCY and Glu were partly reversible, as the washout of agonists restored normal fluorescence of Rho123. NMDA effect was irreversible. Thus, long-term action of agonists, especially NMDA, caused cell

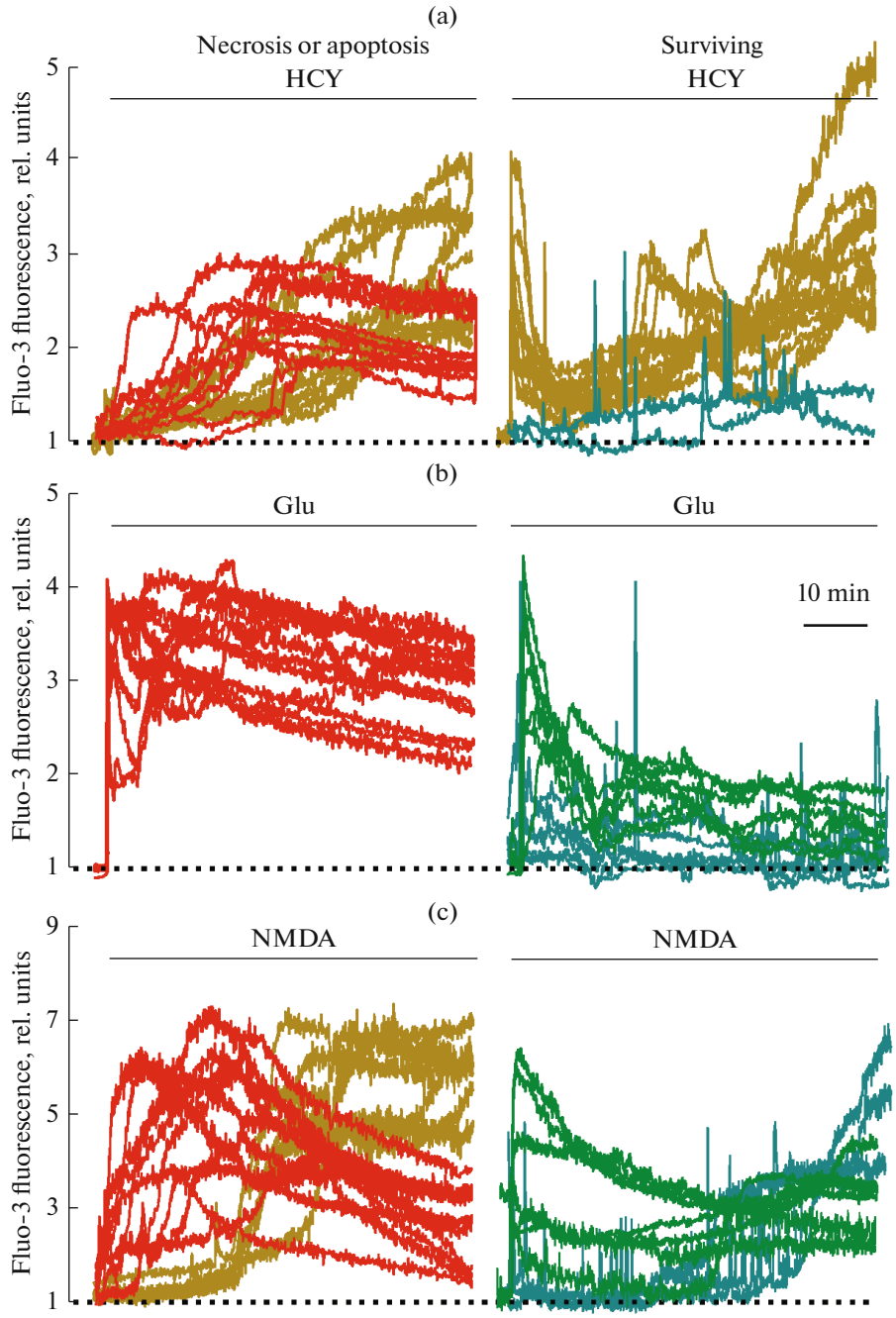

Fig. 3. Intracellular Ca^{2+} responses evoked by long-term (60 min) action of *L*-homocysteine (100 μM), *L*-glutamate (100 μM) and NMDA (30 μM). (a) Samples of Ca^{2+} responses to HCY. (b) Samples of Ca^{2+} responses to Glu. (c) Samples of Ca^{2+} responses to NMDA. Each curve represents the Fluo-3 fluorescence intensity of a single cell body. Agonist application is indicated by lines above the charts. Y-axis represents the relative fluorescence intensity of cells normalized to control baseline. Left charts (a, b, c), show dying neurons and right charts (a, b, c), living cells. *Red* color indicates early Ca^{2+} overload; *yellow* color, delayed Ca^{2+} overload; *green* color, compensation of Ca^{2+} responses, and *blue*, spiking Ca^{2+} responses of neurons. Pooled data of 3–5 experiments are presented.

swelling, loss of Rho123 staining [28], and neuronal death at the end of the treatment, so that the FCCP application did not result in further drop of φ_{mit}. The changes of φ_{mit} of neurons, which did not surviv during the treatment with agonists, are plotted in red (Fig. 4).

DISCUSSION

Many neurodegenerative diseases related with cerebellar functions [29], such as Alzheimer [30] and Parkinson [31] diseases, lateral amyotrophic sclerosis [6, 32], and other pathologies [3], are accompanied with

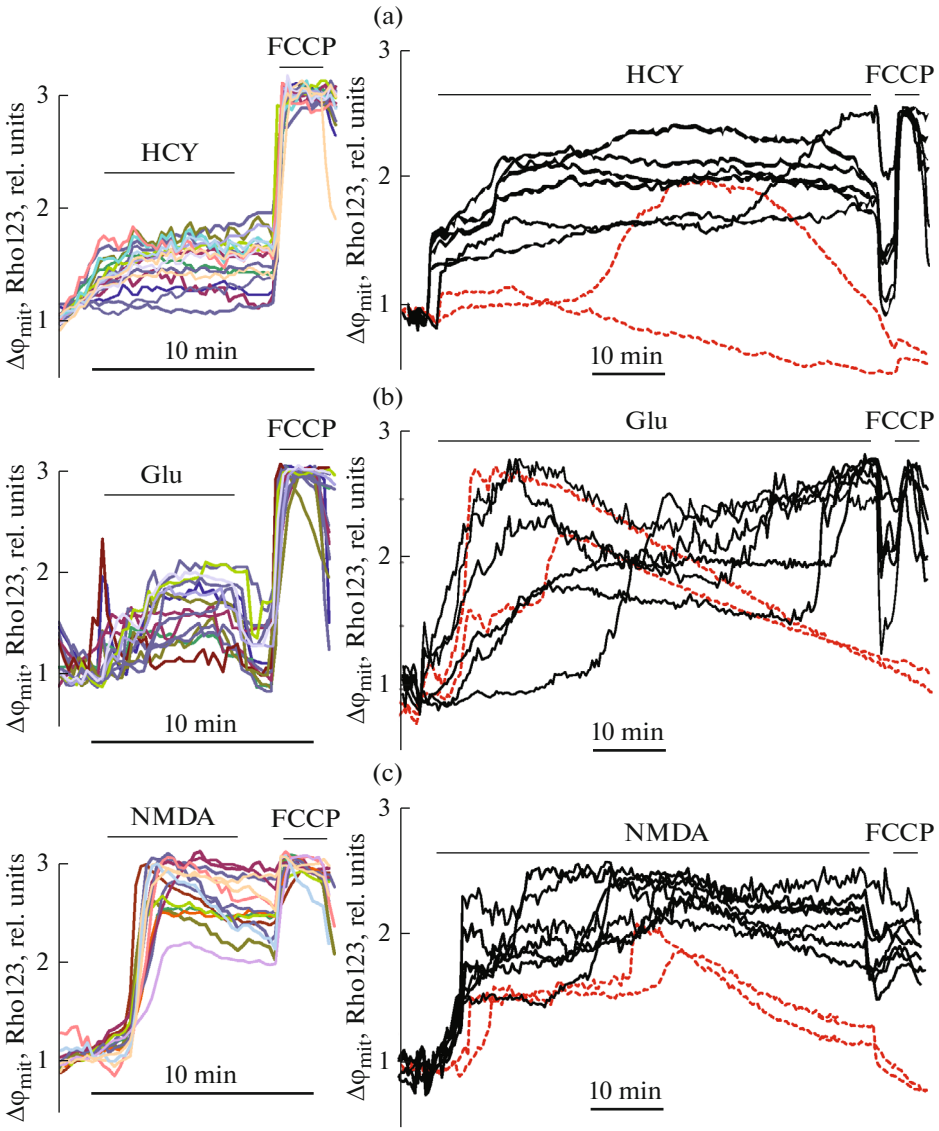

Fig. 4. Changes of mitochondrial membrane potential ($\Delta\varphi_{mit}$) measured by Rho123 fluorescence in cerebellar neurons in vitro in response to 6-min (left) and 60-min (right) applications of *L*-homocysteine (50 μM), NMDA (30 μM) and *L*-glutamate (100 μM). (a) Sample fluorescent signal of neurons showing $\Delta\varphi_{mit}$ in response to the HCY application. (b) Sample fluorescent signal of neurons showing $\Delta\varphi_{mit}$ in response to Glu application. (c) Sample fluorescent signal of neurons showing $\Delta\varphi_{mit}$ in response to NMDA application. FCCP (4 μM) application was used as a control reference of maximal drop of φ_{mit}. Y-axis represents relative fluorescence intensity of cells normalized to control baseline. Each curve represents the fluorescence intensity of a single neuronal body. Pooled data of 4–5 experiments are presented.

hyperhomocysteinemia, suggesting its involvement in pathogenesis. Our experimental results concerning the cytotoxic action of HCY on cerebellar neurons show that long-term treatment with HCY (as well as with Glu or NMDA) causes apoptosis of cerebellar neurons.

However, the comparison of Ca^{2+} responses and φ_{mit} changes during short-term action reveals the difference between agonists. All cortical neurons responded to a 2-min of HCY in the same way by a fast Ca^{2+} response [33]. Cerebellar neurons differed in their Ca^{2+} responses to 2-min HCY application. The majority of cerebellar neurons show stationary elevation in intracellular Ca^{2+} like in case of NMDA and Glu. Some neurons did not respond to HCY, while responding to NMDA and Glu. This heterogeneity can be explained by the unique pattern of expression of NMDA receptor subtypes observed in cerebellum. Cortical neurons express GluN1/GluN2B in the extrasynaptic area [34], activation of which contributes to pro-apoptotic signaling cascades [35, 36] but shows a rapid desensitization by HCY [16]. Many cerebellar neurons express GluN2C- and GluN2D-containing NMDA receptors in extrasynaptic area [37, 38], which are possibly not desensitized by HCY. This

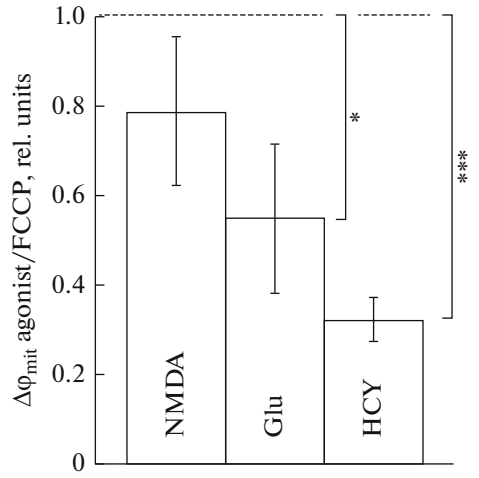

Fig. 5. The quantitative ratio of of *L*-homocysteine (50 μM), NMDA (30 μM) and *L*-glutamate (100 μM) effect on mitochondrial membrane potential. The chart show the ratio of agonists evoked change of φ_{mit} ($\Delta\varphi_{mit}$) to 4 μM FCCP evoked drop of φ_{mit} after 6-min treatment of cerebellar neurons with agonists. Mean values with standard error of mean are presented. Significant differences between agonist- and FCCP-evoked responses are indicated by asterisks: *, $p < 0.05$ ($n = 4$, one-way Student's *t*-test) and ***, $p < 0.001$ ($n = 5$, one-way Student's *t*-test).

may explain a relative tolerance of cortical neurons to the neurotoxic action of HCY [39].

The comparison of mitochondrial membrane potential changes in response to different NMDA receptor agonists in cerebellar neurons shows that long-term action of HCY, NMDA and Glu results in a complete mitochondrial dysfunction, which is reversible in case of HCY. In short-term (6 min) experiments the potency of uncoupling of mitochondrial oxidative phosphorylation by NMDA receptor agonists was as follows: NMDA > Glu = HCY. NMDA was the strongest neurotoxic agent as compared to Glu and HCY because this synthetic compound cannot be utilized by membrane transporters and metabolized by cells. It also does not activate metabotropic NMDA receptors. Glu and HCY revealed the same potency to cause mitochondrial dysfunction in cerebellar neurons. In this feature cerebellar neurons differ from cortical neurons, in which HCY neurotoxicity was much weaker than of other NMDA receptor agonists [33]. In the study performed on cerebellar granule cells [40] HCY at a rather high concentration of 25 mM did not cause significant Ca^{2+} entry into neurons. This differs from our observations and can possibly be explained by a strong desensitization of GluN2B subunits of NMDA receptors at high HCY concentrations [16]. Probably, HCY-induced neurotoxicity at millimolar range is due to activation of metabotropic glutamate receptors.

It should be noted that in both short- and long-term experiments NMDA show the strongest effect on mitochondrial potential drop resulting in irreversible mitochondrial dysfunction. NMDA selectively activates NMDA receptors, but not metabotropic glutamate receptors. It cannot be captured by glutamate transporters. Obviously, endogenous glutamate receptor agonists (Glu and HCY) exert weaker effects on mitochondrial functions. Possibly their neurotoxic effect strongly depends on glutamate metabotropic receptor activation. This coincides with the observation that mGluR5 antagonist MTEP protects neurons against HCY neurotoxicity [11].

Thus, in cerebellar neurons a long-term HCY action causes Ca^{2+} deregulation, mitochondrial dysfunction, and apoptosis due to a specific expression profile of NMDA receptor subtypes. In consequence, an increase in the endogenous HCY in hyperhomocysteinemia can provoke cerebellar neurodegeneration with significant functional disorders.

ACKNOWLEDGMENTS

The study of intracellular calcium responses and mitochondrial potential was supported by the Russian Science Foundation (project no. 16-15-10192). The study of neuronal survival was supported by the Russian Foundation for Basic Research (project no. 16-04-00653).

COMPLIANCE WITH ETHICAL STANDARDS

Conflict of interests. The authors declare that they have no conflict of interest.

Statement on the welfare of animals. Experiments involving animals were performed in accordance to FELASA guidelines and were approved by local regulations of IEPHB RAS.

REFERENCES

1. Shi Q., Savage J.E., Hufeisen S.J., Rauser L., Grajkowska E., Ernsberger P., Wroblewski J.T., Nadeau J.H., Roth B.L. 2003. *L*-homocysteine sulfinic acid and other acidic homocysteine derivatives are potent and selective metabotropic glutamate receptor agonists. *J. Pharmacol. Exp. Ther.* **305** (1), 131–142.
2. Rozen R. 1997. Genetic predisposition to hyperhomocysteinemia: Deficiency of methylenetetrahydrofolate reductase (MTHFR). *Thromb. Haemost.* **78** (1), 523–526.
3. Sachdev P.S. 2005. Homocysteine and brain atrophy. *Prog. Neuropsychopharmacol. Biol. Psychiatry.* **29** (7), 1152–1161.
4. Isobe C., Terayama Y. 2010. A remarkable increase in total homocysteine concentrations in the CSF of migraine patients with aura. *Headache.* **50** (10), 1561–1569.
5. Sachdev P. 2004. Homocysteine, cerebrovascular disease and brain atrophy. *J. Neurol. Sci.* **226** (1–2), 25–29.
6. Zoccolella S., Bendotti C., Beghi E., Logroscino G. 2010. Homocysteine levels and amyotrophic lateral

sclerosis: A possible link. *Amyotrophic Lateral Sclerosis*. **11** (1–2), 140–147.

7. Khodorov B. 2004. Glutamate-induced deregulation of calcium homeostasis and mitochondrial dysfunction in mammalian central neurones. *Prog. Biophys. Mol. Biol.* **86** (2), 279–351.
8. Beard R.S. Jr., Reynolds J.J., Bearden S.E. 2011. Hyperhomocysteinemia increases permeability of the blood-brain barrier by NMDA receptor-dependent regulation of adherens and tight junctions. *Blood*. **118** (7), 2007–2014.
9. Poddar R., Paul S. 2009. Homocysteine-NMDA receptor-mediated activation of extracellular signal-regulated kinase leads to neuronal cell death. *J. Neurochem*. **124** (4), 558–570.
10. Yeganeh F., Nikbakht F., Bahmanpour S., Rastegar K., Namavar R. 2013. 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*. **50** (3), 551–557.
11. Abushik P.A., Niittykoski M., Giniatullina R., Shakirzyanova A., Bart G., Fayuk D., Sibarov D.A., Antonov S.M., Giniatullin R. 2014. The role of NMDA and mGluR5 receptors in calcium mobilization and neurotoxicity of homocysteine in trigeminal and cortical neurons and glial cells. *J. Neurochem*. **129** (2), 264–274.
12. Zhong J., Russell S.L., Pritchett D.B., Molinoff P.B., Williams K. 1994. Expression of mRNAs encoding subunits of the N-methyl-*D*-aspartate receptor in cultured cortical neurons. *Mol. Pharmacol*. **45** (5), 846–853.
13. Abushik P.A., Sibarov D.A., Eaton M.J., Skatchkov S.N., Antonov S.M. 2013. Kainate-induced calcium overload of cortical neurons *in vitro*: Dependence on expression of AMPAR GluA2-subunit and down-regulation by subnanomolar ouabain. *Cell Calcium*. **54** (2), 95–104.
14. Paoletti P., Bellone C., Zhou Q. 2013. NMDA receptor subunit diversity: Impact on receptor properties, synaptic plasticity and disease. *Nat. Rev. Neurosci*. **14** (6), 383–400.
15. Akazawa C., Shigemoto R., Bessho Y., Nakanishi S., Mizuno N. 1994. Differential expression of five N-methyl-*D*-aspartate receptor subunit mRNAs in the cerebellum of developing and adult rats. *J. Comp. Neurol*. **347** (1), 150–160.
16. Sibarov D.A., Abushik P.A., Giniatullin R., Antonov S.M. 2016. GluN2A subunit-containing NMDA receptors are the preferential neuronal targets of homocysteine. *Front. Cell. Neurosci*. **10** (246), doi 10.3389/fncel.2016.00246
17. Jadavji N.M., Wieske F., Dirnagl U., Winter C. 2015. Methylenetetrahydrofolate reductase deficiency alters levels of glutamate and γ-aminobutyric acid in brain tissue. *Mol. Genet. Metab. Rep*. **3**, 1–4.
18. Hockberger P.E., Tseng H.Y., Connor J.A. 1989. Development of rat cerebellar Purkinje cells: Electrophysiological properties following acute isolation and in long-term culture. *J. Neurosci*. **9** (7), 2258–2271.
19. Weber A., Schachner M. 1984. Maintenance of immunocytologically identified Purkinje cells from mouse cerebellum in monolayer culture. *Brain Res*. **311** (1), 119–130.
20. Karelina T.V., Stepanenko Yu.D., Abushik P.A., Sibarov D.A., Antonov S.M. 2016. Downregulation of Purkinje cell activity by modulators of small conductance calcium-activated potassium channels in rat cerebellum. *Acta Naturae*. **8** (3), 91–99.
21. Antonov S.M., Johnson J.W. 1999. Permeant ion regulation of N-methyl-*D*-aspartate receptor channel block by Mg^{2+}. *Proc. Nat. Acad. Sci. USA*. **96** (25), 14571–14576.
22. Mironova, E.V., Evstratova A.A., Antonov S.M. 2007. A fluorescence vital assay for the recognition and quantification of excitotoxic cell death by necrosis and apoptosis using confocal microscopy on neurons in culture. *J. Neurosci. Methods*. **163** (1), 1–8.
23. Sibarov D.A., Bolshakov A.E., Abushik P.A., Krivoi I.I., Antonov S.M. 2012. 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*. **343** (3), 596–607.
24. Grynkiewicz G., Poenie M., Tsien R.Y. 1985. A new generation of Ca^{2+} indicators with greatly improved fluorescence properties. *J. Biol. Chem*. **260** (6), 3440–3450.
25. Duchen M.R. 2012. Mitochondria, calcium-dependent neuronal death and neurodegenerative disease. *Pflügers Archiv*. **464** (1), 111–121.
26. Mironova E.V., Lukina A.A., Brovtsyna N.B., Krivchenko A.I., Antonov S.M. 2006. Glutamate receptors types defining neurotoxic glutamate action on rat cortex neurons. Zh. Evol. Biokhim. Fiziol. (Rus.). **42** (6), 559–566.
27. Surin A.M., Krasilnikova I.A., Pinelis V.G., Khodorov B.I. 2014. Study of the relationship between glutamate-induced delayed calcium deregulation, mitochondrial depolarization and subsequent neuronal death. *Pathogenesis*. **12**, 40–46.
28. Duchen M.R., Surin A.M. 2002. On the role of mitochondria and calcium in glutamate-induced neurotoxicity in hippocampal neurons in culture. Biol. Membrany (Rus.). **19**, 97–109.
29. Prell T., Grosskreutz J. 2013. The involvement of the cerebellum in amyotrophic lateral sclerosis. *Amyotroph. Lateral Scler. Frontotemporal Degener*. **14** (7–8), 507–515.
30. Boldyrev A.A., Johnson P. 2007. Homocysteine and its derivatives as possible modulators of neuronal and non-neuronal cell glutamate receptors in Alzheimer's disease. *J. Alzheim. Disease*. **11** (2), 219–228.
31. Kuhn W., Hummel T., Woitalla D., Müller T. 2001. Plasma homocysteine and MTHFR C677T genotype in levodopa-treated patients with PD. *Neurology*. **56** (2), 281–282.
32. Valentino F., Bivona G., Butera D., Paladino P., Fazzari M., Piccoli T., Ciaccio M., La Bella V. 2010. Elevated cerebrospinal fluid and plasma homocysteine levels in ALS. *Eur. J. Neurol*. **17** (1), 84–89.
33. Abushik P.A., Karelina T.V., Sibarov D.A., Stepanenko Y.D., Giniatullin R.A., Antonov S.M. 2015. Homocysteine-induced membrane currents, calcium

responses and changes in mitochondrial potential in rat cortical neurons. *J. Evol. Biochem. Physiol.* **51**, 296–304.

34. Scimemi A., Fine A., Kullmann D.M., Rusakov D.A. 2004. NR2B-containing receptors mediate cross talk among hippocampal synapses. *J. Neurosci.* **24**, 4767–4777.
35. Hardingham G.E., Bading H. 2010. Synaptic versus extrasynaptic NMDA receptor signalling: Implications for neurodegenerative disorders. *Nat. Rev. Neurosci.* **11**, 682–696.
36. Stark D.T., Bazan N.G. 2011. Synaptic and extrasynaptic NMDA receptors differentially modulate neuronal cyclooxygenase-2 function, lipid peroxidation, and neuroprotection. *J. Neurosci.* **31**, 13710–13721.
37. Misra C., Brickley S.G., Farrant M., Cull-Candy S.G. 2000. Identification of subunits contributing to synaptic and extrasynaptic NMDA receptors in Golgi cells of the rat cerebellum. *J. Physiol.* **524**, 147–162.
38. Brickley S.G., Misra C., Mok M.H.S., Mishina M., Cull-Candy S.G. 2003. Identification of subunits contributing to synaptic and extrasynaptic NMDA receptors in Golgi cells of the rat cerebellum. *J. Neurosci.* **23**, 4958–4966.
39. Abushik P.A., Bart G., Korhonen P., Leinonen H., Giniatullina R., Sibarov D.A., Levonen A.L., Malm T., Antonov S.M., Giniatullin R. 2017. Pro-nociceptive migraine mediator CGRP provides neuroprotection of sensory, cortical and cerebellar neurons via multi-kinase signaling. *Cephalalgia*. **37** (4), 1373–1383.
40. Ziemińska E., Stafiej A., Łazarewicz J.W. 2003. Role of group I metabotropic glutamate receptors and NMDA receptors in homocysteine-evoked acute neurodegeneration of cultured cerebellar granule neurones. *Neurochem. Int.* **43**, 481–492.

Translated by D. Sibarov