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Glutamine Effect on Cultured Granule Neuron Death Induced by Glucose Deprivation and Chemical Hypoxia

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Abstract—Using a specific fluorescent probe of mitochondrial membrane potential (tetramethylrhodamine ethyl ester), we have shown that glucose deprivation (GD) of cultured cerebellar granule neurons (CGN) for 3 h lowers mitochondrial membrane potential in these cells. Longer glucose starvation (24 h) causes CGN death that is not prevented by blockers of ionotropic glutamate receptors (MK-801 (10 μM) and NBQX (10 μM)). Glutamine or pyruvate (2 mM) maintain membrane potential of mitochondria and decrease CGN death under GD conditions. In the presence of glucose the mitochondrial respiratory chain blocker rotenone induces neuron death potentiated by glutamine. The potentiation effect is completely prevented by blockers of ionotropic glutamate receptors. These results show that glutamine under conditions of GD can be utilized by mitochondria as substrate, but at the same time, in the case of mitochondrial function deterioration, metabolism of this amino acid results in glutamate accumulation to toxic level.

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Glutamate is one of the most widespread neuromediators in the brain. However, functioning of glutamatergic neurons in the central nervous system is also dependent on another amino acid – glutamine. Mitochondrial glutaminase converts the latter to glutamate. The involvement of glutamate in nervous system pathology has been shown in numerous works, whereas the role of glutamine in destructive processes is far from being unambiguous. An increase in extracellular glutamate concentration in the case of ischemia can be mediated not only by its enhanced release from neurons, but by its synthesis from glutamine as well. Under pathological conditions (during hypoxia or ischemia) glutaminase activity significantly increased [1, 2]. These data are supported by results of Goldberg et al. [3] showing that hypoxic damage of cultured cortical neurons is enhanced in the presence of glutamine in the culture medium and is in proportion with its concentration. This is evidently also valid for other pathological conditions associated with glutamate toxicity, for example, the toxic effect of paraquat on cultured cerebellar granule neurons does not develop in the absence of glutamine in the cell culture medium [4].

However, at the same time glutamine can probably be used by neuronal mitochondria as an energy substrate, which is important for neuronal survival under conditions of glucose deficiency [5, 6].

This work deals with investigation of the effect of glutamine on maintenance of membrane potential in CGN (cultured granule neurons) mitochondria under conditions of glucose deficiency and on neuronal survival under conditions of glucose deprivation and chemical hypoxia induced by rotenone.

MATERIALS AND METHODS

All media and additives used for cell cultures were obtained from Biochrom KG (Germany). Tetramethyl-

Abbreviations: APV, aminophosphonovalerate; CGN, cultured granule neurons; GD, glucose deprivation; NBQX, 2,3-dioxo-6-nitro-1,2,3,4-tetra-hydrobenzoquinoxaline-7-sulfonamide; NMDA, *N*-methyl-D-aspartate; MK-801, (+)-5-methyl-10,11-dihydro-5H-dibenzo(a,d)cycloheptene-5,10-imino-maleate; TMRE, tetramethylrhodamine ethyl ester.

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rhodamine ethyl ester (TMRE) was from Molecular Probes (USA). Other reagents were from Sigma (Germany).

Primary cerebellar cultures. Cerebellar granule neurons grown in cultures for 7-9 days were used in this work. These neurons were obtained from the brain of 8-day-old Wistar rats by enzyme–mechanical dissociation carried out as follows: isolated cerebelli were transferred into a plastic Petri dish filled with calcium- and magnesium-free phosphate buffer. Tissue fragments were incubated for 15 min at 37°C in phosphate buffer containing 0.05% trypsin and 0.02% EDTA. After incubation, the tissue was washed in two changes of phosphate buffer and once in culture medium, after which the tissue was mechanically dissociated in culture medium. The culture medium contained 10% fetal calf serum, 2 mM glutamine, and 10 mM Hepes buffer, pH 7.2-7.4. The cell suspension was centrifuged for 1 min at 1000 rpm, the supernatant was discarded, and the pellet was resuspended in culture medium. Cells were grown in 96-well plastic plates covered with polylysine. Samples (0.1 ml) of cell suspension were added into each well. Cultures were developed in a CO_2-incubator at 36.5°C and relative humidity 98%. On the second day *in vitro*, the culture medium was replaced by fresh medium with 25 mM KCl, and cell growth continued to 7-8 days *in vitro*. To prevent proliferation of non-neuronal cells, on the second day of *in vitro* growth arabinoside monocytoside was added to final concentration 1 μM.

Manipulations with animals and experimental procedures were carried out in accordance with the Instructions of the European Community Counsel 86/609/EEC concerning the use of animals for experimental investigations, and they were approved by the Moscow State University Commission on Ethics.

Glucose deprivation and chemical hypoxia. To initiate glucose deprivation (GD), cultured cells were washed twice with balanced salt solution (154 mM NaCl, 25 mM KCl, 2.3 mM $CaCl_2$, 1 mM $MgCl_2$, 23.8 mM $NaHCO_3$, 0.35 mM Na_2HPO_4, and 10 mM Hepes, pH 7.2-7.4) and incubated for 3-24 h in the CO_2-incubator at 36.5°C and relative humidity 98%.

Control cultures were incubated in balanced salt solution with glucose addition (5 mM). Cultures were treated with rotenone (1 μM, 2 h) in balanced salt solution containing glucose.

Measurement of mitochondrial membrane potential. To analyze mitochondrial membrane potential, cells were incubated with 0.1 μM TMRE (fluorescence excitation at 530 nm, emission at 640 nm) for 15 min at 36.5 ± 0.5°C and washed three times with balanced salt solution. TMRE is a penetrating cation and enters only functionally active mitochondria having potential on their inner membrane. Its fluorescence was registered using an Olympus CKX41 inverted fluorescence microscope equipped with a highly sensitive digital chamber. The TMRE fluorescence intensity, indicating the level of membrane potential, was measured in separate mitochondria of 10 cells from a field of vision using programs for computerized image analysis. All manipulations with cells were carried out at 34-36°C.

Estimation of neuron survival. After experiments cultures were fixed in ethanol–formaldehyde–acetic acid mixture (7 : 2 : 1) and stained with trypan blue. The surviving neuron percentage was estimated by calculation of morphologically intact CGN nuclei in five fields of vision under objective magnification ×40. The neuron survival in untreated control cultures was taken as 100%, and survival in experimental cultures was expressed as percent of control.

Statistic analysis. The ANOVA test with Bonferroni posttest was used for statistical analysis. Intergroup distinctions were considered as reliable at $p < 0.05$. Results are expressed as mean ± SEM. All data were obtained on nine cultures in three independent experiments.

RESULTS

Effect of glutamine on membrane potential of CGN mitochondria under glucose deprivation. Results of living culture observations and investigations of histological preparations have shown that the neuronal population of cultures obtained from cerebelli of 7-8-day-old rats was represented by essentially a single type of neurons, granule cells, while no other types of cerebellum neurons were present in the dissociated cultures. CGN were localized on glial monolayer.

Energization of mitochondria in living CGN was visualized by recording the fluorescence of TMRE, which is accumulated in mitochondria depending on the level of their membrane potential. After incubation for 3 h in the glucose-containing balanced salt solution, mitochondria of neurons actively accumulated TMRE, which exhibited red fluorescence upon irradiation with green light (Fig. 1a). In CGN (whose size is only 7-10 μm) the main part of the cell is occupied by the nucleus surrounded by a thin ring of cytoplasm, the highest amount of which is concentrated in the points of process origins. These cells are slightly spread over the substrate and are often almost spherical in shape. Owing to these morphological features of CGN, when cultures are examined using light optics, mitochondria are seen as small luminous rods or points (indicated by arrows in the figure) surrounding the dark nucleus. In sister cultures incubated for the same time in the glucose-free balanced salt solution, the accumulation of TMRE in mitochondria of neurons decreased (Fig. 1b) to 45 ± 1.8% compared to the control (Fig. 1). The morphological situation under these conditions seems diffuse. In the case of complete absence of mitochondrial membrane potential, we observed total absence of fluorescence when the presence of CGN in the field of vision can be

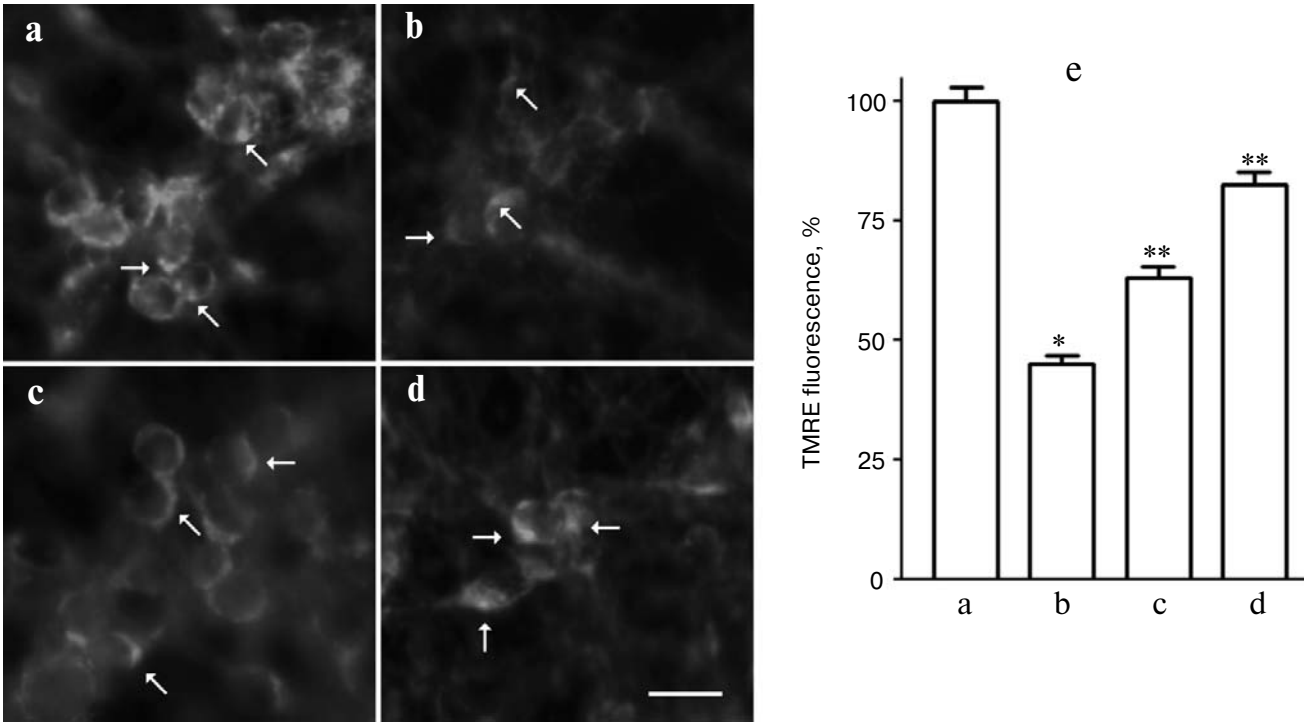

Fig. 1. Fluorescence of TMRE accumulated in mitochondria of cultured neurons in the presence of glucose (a) or in the case of 3-h glucose deprivation (b-d) (without additions (b) or in the presence of 2 mM glutamine (c) or 2 mM pyruvate (d)). e) Relative intensity of TMRE fluorescence as percentage of control; * $p < 0.001$ compared to (a); ** $p < 0.01$ compared to (b), $n = 90$, where n is number of mitochondria with measured fluorescence. Arrows point to mitochondria in CGN. Scale 10 μm.

identified only by comparison with the phase contrast image of this field of vision.

Glutamine addition (2 mM) to the glucose-free balanced salt solution maintained TMRE accumulation in mitochondria (Fig. 1c), which pointed to the existence of membrane potential in these organelles. The relative TMRE fluorescence in neuronal mitochondria under these conditions was on average 63 ± 2.2%, which was 18% higher than during GD in the absence of glutamine (Fig. 1b). Pyruvate at 2 mM concentration maintained more fully than glutamine the membrane potential in mitochondria (Fig. 1, d and e).

Effects of glutamine and pyruvate on CGN viability under glucose deprivation. In the course of these experiments the CGN existed for 24 h in the balanced salt solution. Study of histological preparations showed that no noticeable neuronal death was observed upon incubation of the culture in the glucose-containing balanced salt solution (Fig. 2a), whereas in the absence of glucose almost all CGN died (Fig. 2b). Blockers of ionotropic glutamate receptors (0.01 mM MK-801 and 0.01 mM NBQX) did not lower neuron death caused by glucose deprivation (Fig. 2c). Addition to the incubation solution of 2 mM glutamine (Fig. 2d) or pyruvate at the same concentration (Fig. 3) decreased neuronal death caused by glucose deprivation. Increasing glutamine concentration to 5 mM decreased its protective effect (Fig. 2e), while increase in pyruvate concentration to 5 mM had no reliable effect on its protective activity (Fig. 3). Negative effect of glutamine concentration increase was prevented by blocking ionotropic glutamate receptors (Fig. 2f).

Effect of glutamine on CGN viability during rotenone-induced chemical hypoxia. Chemical hypoxia of neurons was induced by addition for 2 h to glucose-containing balanced salt solution of the mitochondrial respiratory chain inhibitor rotenone. Quantitative analysis of the experimental data showed that 89 ± 3.7% of the neurons survived in the presence of rotenone, but if the incubation solution contained glutamine survival decreased to 31 ± 5.7%. Antagonists of ionotropic glutamate receptors MK-801 (0.01 mM) and NBQX (0.01 mM) completely prevented CGN death caused by combined effect of rotenone and glutamine (Fig. 4).

DISCUSSION

Despite acute glucose deficiency in the brain during hypoglycemic coma, energy metabolism during this pathological state is not completely blocked and is about 25% of normal [7]. Because of this, utilization of alternative energy substrates (especially glutamine) by neurons during glucose deficiency has been actively studied in recent years [6, 8]. Compared to other amino acids, the

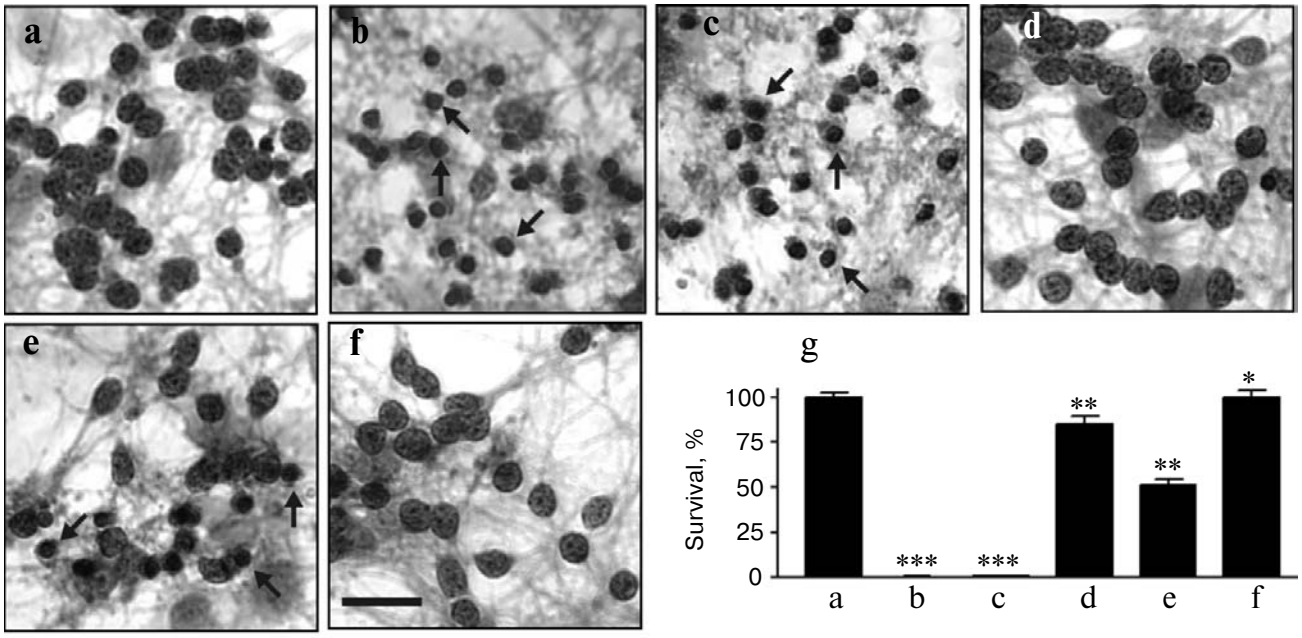

Fig. 2. Effect of glutamine on viability of cultured granule neurons: a) control in the presence of glucose; b-f) 24 h glucose deprivation (without additions (b) or in the presence of blockers of ionotropic glutamate receptors MK-801 (0.01 mM) and NBQX (0.01 mM) (c), 2 mM glutamine (d), 5 mM glutamine (e), 5 mM glutamine and blockers of ionotropic glutamate receptors MK-801 (0.01 mM) and NBQX (0.01 mM) (f)). g) Quantitative estimation of neuron survival; *** compared to (a), ** compared to (b), * compared to (e), $p < 0.001$, $n = 45$, where n is the number of fields of vision. Cultured cerebellar granule neurons stained with trypan blue (a-f). Arrows point to pycnotic nuclei of dead neurons. Scale 20 μm.

glutamine and glutamate content in the brain is high [9], but it quickly decreases during hypoglycemia [10, 11].

Glutamine is utilized by mitochondria not for immediate maintenance of oxidative phosphorylation, but after its conversion by glutaminase to glutamate that, according to Sutherland et al. [8], is utilized by mitochondria during glucose deprivation. Therefore, investigations of glutamine utilization by neurons as energy substrate during glucose deprivation have been aimed at study of the generation of aspartate from glutamate as the key event associated with maintenance of neuronal energy during pyruvate deficiency [6, 8]. A convenient model for investigation of glutamine as an energy substrate during glucose deprivation is the CGN [6]. These neurons, both under normal and pathological conditions, synthesize glutamine from glutamate more intensely than cortical neurons [12]. Glucose deprivation is accompanied by rapid increase in calcium level and decrease in mitochondrial membrane potential in the CGN cytoplasm [13]. These disturbances during at least the first hour are completely energy-dependent, because they are fully reversible after restoration of normal glucose level in the culture medium [14].

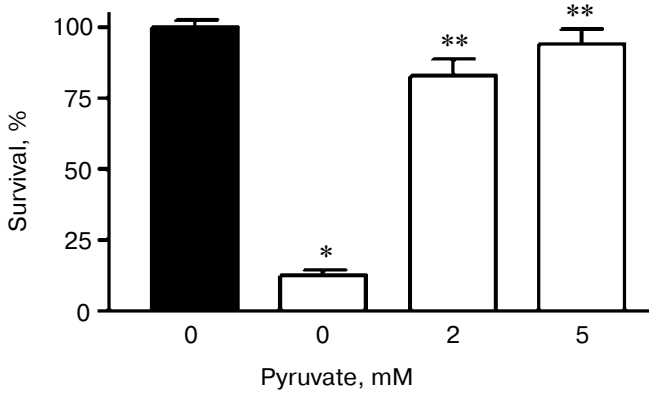

Fig. 3. Effect of pyruvate on survival of cultured granule neurons under 24-h glucose deprivation. Black column, control in presence of glucose in balanced salt solution. White columns, glucose deprivation, * $p < 0.001$ compared to control; ** $p < 0.001$ compared to glucose deprivation without additions, $n = 45$, where n is the number of fields of vision.

In our work relative change in membrane potential of CGN mitochondria upon glucose deprivation was measured using a fluorescent probe. Our data show that in the case of glucose deficiency glutamine maintains energy of mitochondria and neuron viability, while blockers of ionotropic glutamate receptors do not prevent in such conditions neuronal death caused by GD. These results support the opinion that glutamine is an alternative (to pyruvate) energy substrate maintaining neuronal survival in the brain during glucose deprivation. However, in our experiments mitochondrial membrane potential was lower in the presence of glutamine than in the pres-

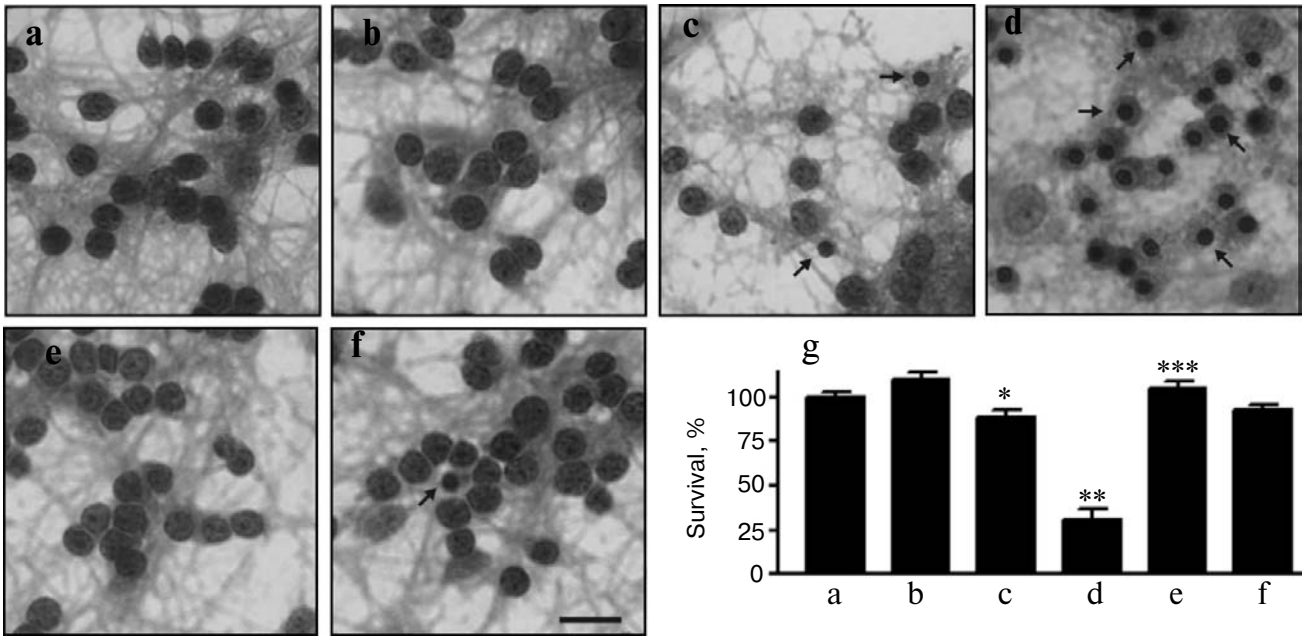

Fig. 4. Effect of glutamine (2 mM) on survival of cultured granule neurons with rotenone (1 μM, 2 h): a) control; b) 2 mM glutamine; c) rotenone; d) rotenone in presence of 2 mM glutamine; e) rotenone in presence of 2 mM glutamine with blockers of ionotropic glutamate receptors (0.01 mM MK-801 and 0.01 mM NBQX); f) rotenone with blockers of ionotropic glutamate receptors. g) Quantitative estimation of neuron survival, * $p < 0.05$ compared to (a); ** $p < 0.01$ compared to (c); *** $p < 0.01$ compared to (d), $n = 45$, where n is the number of fields of vision. Cultured cerebellar granule neurons stained by trypan blue (a-f). Scale 15 μm. Arrows point to pycnotic nuclei of dead neurons.

ence of pyruvate. This can be explained by the fact that when pyruvate is used as energy substrate in the total reaction of the tricarboxylic acid cycle, three NADH molecules and one FADH molecule are formed, while with glutamate only two NADH molecules and one $FADH_2$ are formed [8]. Besides, as was already mentioned above, aspartate formed upon the interaction of glutamate with aspartate aminotransferase can be accumulated during hypoglycemia and is able (like glutamate) to stimulate glutamate receptors. In the case of excessive glutamine concentration, aspartate together with glutamate produced by glutaminase from glutamine can be toxic due to hyperactivation of glutamate receptors. Our experiments show that during hypoglycemia, despite the above-mentioned shortcomings of glutamine as an energy substrate for neurons, this amino acid maintains viability of the neurons, but in the case of blocking of mitochondrial function, like under chemical hypoxia, the presence of glutamine in the CGN culture medium enhances neuron death even in the presence of glucose. Under conditions of anoxia, ischemia, and chemical hypoxia, like in the case of hypoglycemia, glutaminase intensely produces glutamate from glutamine [1-3]. However, unlike hypoglycemia, under these pathological conditions disturbance of oxidative phosphorylation is observed. Blocking of mitochondrial ATP synthesis results in rapid exhaustion of energy resources, which causes a disturbance of glutamate reuptake by neurons and glia, hyperstimulation of glutamate receptors, and finally, neuronal death.

The role of astrocytes in the utilization of excess glutamate in the brain should be noted. In the normal conditions, glutamate via specific carriers GLT1/EAAT2 and GLAST/EAAT1 is transported into astrocytes [15] where it is converted to glutamine by the cytoplasmic enzyme glutamine synthase in the presence of magnesium ions. Petito et al. note that enhancement of glutamine synthesis by glutamine synthase in the rat brain upon ischemia and during postischemic period can be an important factor of extracellular glutamate level normalization and protection against neurotoxic effect of excitatory amino acids [16]. However, in such pathological states this function of astrocytes can be limited, because glutamine synthesis from glutamate takes place at the expense of ATP, and therefore elimination of excess glutamate by astrocytes in the case of energy deficiency is retarded.

Our experiments have shown that under conditions of mitochondrial respiratory chain inhibition glutamine stimulates processes of neuronal damage. However, if mitochondria are functionally active glutamine is utilized as a substrate during glucose deprivation by CGN mitochondria, an alternative to pyruvate, for maintenance of cell energy and neuronal survival.

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