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REVIEW

Role of Mitochondria in the Mechanisms of Glutamate Toxicity

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Abstract—Current data on glutamate-induced functional and morphological changes in mitochondria correlating with or being a result of their membrane potential changes are reviewed. The important role of Ca^{2+}, Na^+, and H^+ in the potentiation of such changes is considered. It is assumed that glutamate-induced loss of mitochondrial potential is mediated by Ca^{2+} overload resulting in the induction of nonspecific permeability of the inner mitochondrial membrane.

Key words: neurons, mitochondria, glutamate, calcium ions

During recent years, many authors have shown that hyperstimulation of glutamate receptors by excitatory amino acids is the main pathogenic factor of neuronal degeneration in brain hypoxia/ischemia. Therefore, studies of the mechanisms of destructive processes in neurons induced by toxic concentrations of glutamate (Glu) and its analogs has become especially important.

In 1957, Lucas and Newhouse showed that subcutaneous injections of glutamate to young mice caused degeneration of retinal neurons [1]. Later, these data were confirmed by Olney et al. who suggested the hypothesis explaining the neurocytotoxic effect of excitatory amino acids [2-4]. These authors proposed that prolonged and intense interaction of Glu or its analogs with Glu receptors caused stable depolarization of plasma membrane leading to altered membrane permeability and impaired ionic balance and energy producing systems that normally restore ionic homeostasis. The continuous ion influx from the external medium results in the exhaustion of energy stores, insufficient ion extrusion, and ionic imbalance, which finally leads to cell death. Later, the hypothesis explaining neurodestructive action of Glu was confirmed and supplemented by other data [5-9]. Excessive Ca^{2+} influx was regarded by a number of authors as the main reason for neuronal degeneration under the *in vitro* action of excitatory amino acids [10]. However, the reasons for Glu-induced neuronal energy imbalance remained unclear for a long time. This problem was discussed in recent reviews [11-13], but some aspects, in particular, Glu-induced changes in mitochondrial conformation due to their energy depletion were not studied. We discuss this problem in the present review.

TOXIC ACTION OF GLUTAMATE CAUSES ACCUMULATION OF CALCIUM IN NEURONAL CYTOPLASM AND MITOCHONDRIA

Present data show that during Glu toxicity, the mechanisms determining ion homeostasis, and those maintaining cell energy status are closely connected. It was shown that activated Glu receptors open Glu operated ion channels providing the influx of Na^+ and Ca^{2+} and the efflux of K^+. Membrane depolarization caused by increased intracellular Na^+ concentration results in Ca^{2+} entry via voltage-gated Ca^{2+} channels [10]. Endoplasmic reticulum and mitochondria can also release Ca^{2+} to the cytoplasm [14]. Ca^{2+} release from endoplasmic reticulum can be stimulated, for example, by inositol triphosphate [15], a product of phosphoinositol metabolism activated by binding of Glu to type 5 metabotropic receptor ($mGlu_5$) [16]. It is possible that Na^+ and Ca^{2+} neuronal overload results in the reversal of the Na^+/Ca^{2+} antiporter in plasma membrane and Ca^{2+} ions begin to enter the cell via this mechanism [17]. However, other data contradict this hypothesis [18, 19].

Earlier, it was shown that toxic action of excitatory amino acids and hyperactivation of NMDA channels closely correlate with the accumulation of intracellular Ca^{2+} [20, 21]. It is known that the concentration of cyto-

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plasmic Ca^{2+} at rest is below 0.1 μM, while after stimulation of Glu receptors its concentration can reach 8-16 μM [22]. At the same time, it was shown that excessive Ca^{2+} induced by Glu stimulation could be stored in neuronal mitochondria [23-26]. Under the conditions of phosphate excess, these organelles can store 200 nmol Ca^{2+}/mg protein [27]. Ca^{2+} accumulation by mitochondria depends on their membrane potential, which points to electrogenic character of Ca^{2+} transport in mitochondria. Thus, mitochondrial membrane potential can be regarded as a factor contributing to intracellular Ca^{2+} regulation in neurons, which definitely occurs during activation of Glu receptors. Khodorov et al. [26] showed that antimycin, an inhibitor of the 3rd complex of the electron transport chain, combined with the inhibitor of ATP synthase, oligomycin, significantly inhibits Ca^{2+} uptake into mitochondria. The latest data show that Ca^{2+} uptake into mitochondria depends on the degree of their association with cytoskeleton. It was shown that Ca^{2+} accumulation by mitochondria decrease by 53% in vinblastine-treated hippocampal neurons after activation of NMDA receptors [28]. Notably, calcium homeostasis of the neurons can be restored much quicker after Ca^{2+} influx stimulated by K^+ depolarization of the plasma membrane compared to Glu-induced Ca^{2+} entry. This indicates that Ca^{2+} accumulated in the neuronal cytosol due to opening of Glu activated channels and Ca^{2+} entering the cell via other channels may initiate different processes [29].

Budd and Nicholls assumed that glutamate toxicity directly depends on the ability of mitochondria to store Ca^{2+} [30] since Ca^{2+} binding sites were discovered on the cytoplasmic part of NMDA receptors [31] and NMDA channels were inactivated by Ca^{2+}. Mitochondria are located closely to these Ca^{2+} binding sites and can chelate Ca^{2+}, decreasing its local concentration and thus preventing inactivation of NMDA channels. This local Ca^{2+} uptake does not prevent Ca^{2+} accumulation in other parts of a neuron, which results in Ca^{2+} overload of the cytoplasm and consequently, mitochondria. Budd and Nicholls assume that under normal physiological conditions, Ca^{2+} uptake into mitochondria can prevent untimely inactivation of NMDA channels [30].

High concentrations of intracellular Ca^{2+} inevitably cause neuronal death initiated by hyperstimulation of NMDA receptors even under conditions of electrogenic Ca^{2+} transport in mitochondria [32]. This points to the leading role of mitochondria in initiation of Glu-induced neuronal death. Probably, destruction of mitochondria caused by Ca^{2+} overload and dysfunction of the respiratory chain and oxidative phosphorylation is the key factor initiating neuronal death. This assumption is confirmed by the fact that synchronous inhibition of the mitochondrial respiratory chain by rotenone and ATP synthase by oligomycin protects neurons from death caused by hyperstimulation of Glu receptors, prevents the decrease of ATP levels, and normalizes the level of free cytosolic Ca^{2+} [30].

GLUTAMATE-INDUCED CALCIUM ACCUMULATION IN NEURONAL MITOCHONDRIA IMPAIRS THE MORPHOLOGY AND FUNCTIONAL STATE OF THESE ORGANELLES

Numerous studies have shown that Glu-induced calcium overload of a neuron leads to the collapse of mito-

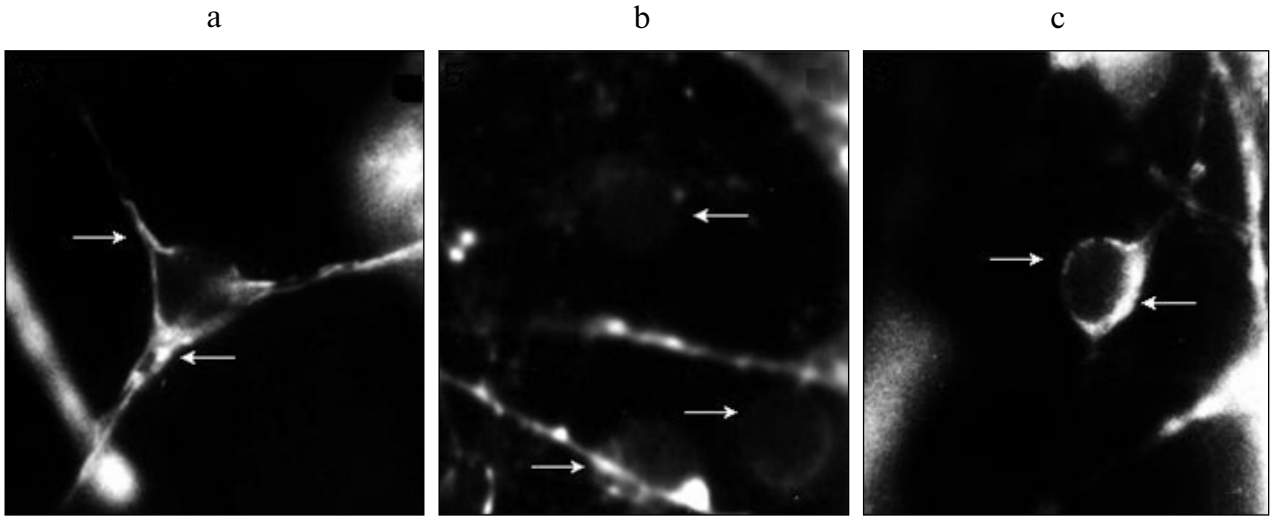

Fig. 1. Cultured cerebellar granule cells treated by rhodamine 123 (R123), an indicator of mitochondrial membrane potential: a) control, arrowheads indicate cells with intensely fluorescent mitochondria stained by R123; b) glutamate-treated (100 μM, 15 min) culture, the arrows point out cells with energy depleted mitochondria; c) culture treated by glutamate (100 μM, 15 min) in the presence of 2 mM $CoCl_2$; intensely fluorescent energized mitochondria are shown by arrowheads (from [34]).

chondrial membrane potential (Fig. 1), decreased ATP level, and finally, to neuronal death [30, 33-38].

The collapse of mitochondrial membrane potential after short-term Glu toxic treatment was associated with different ultrastructural changes in mitochondria. Thus, mitochondria with condensed matrix as well as swollen mitochondria with decreased matrix electron density could be found in Glu-treated cerebellar granule cells (Fig. 2). These mitochondria might present different stages of degeneration [34].

Glu also induced swelling of mitochondria in cortical neurons [39, 40] and in HT4 cell line derived from hippocampal neurons [41].

It is probable that mitochondrial degeneration starts with matrix condensation, and thereafter mitochondrial swelling and destruction of their cristae occurs. However, the synchronous presence of condensed and swollen mitochondria may point to heterogeneity of the mitochondrial population. Mitochondrial heterogeneity was previously noted by other authors, who showed different

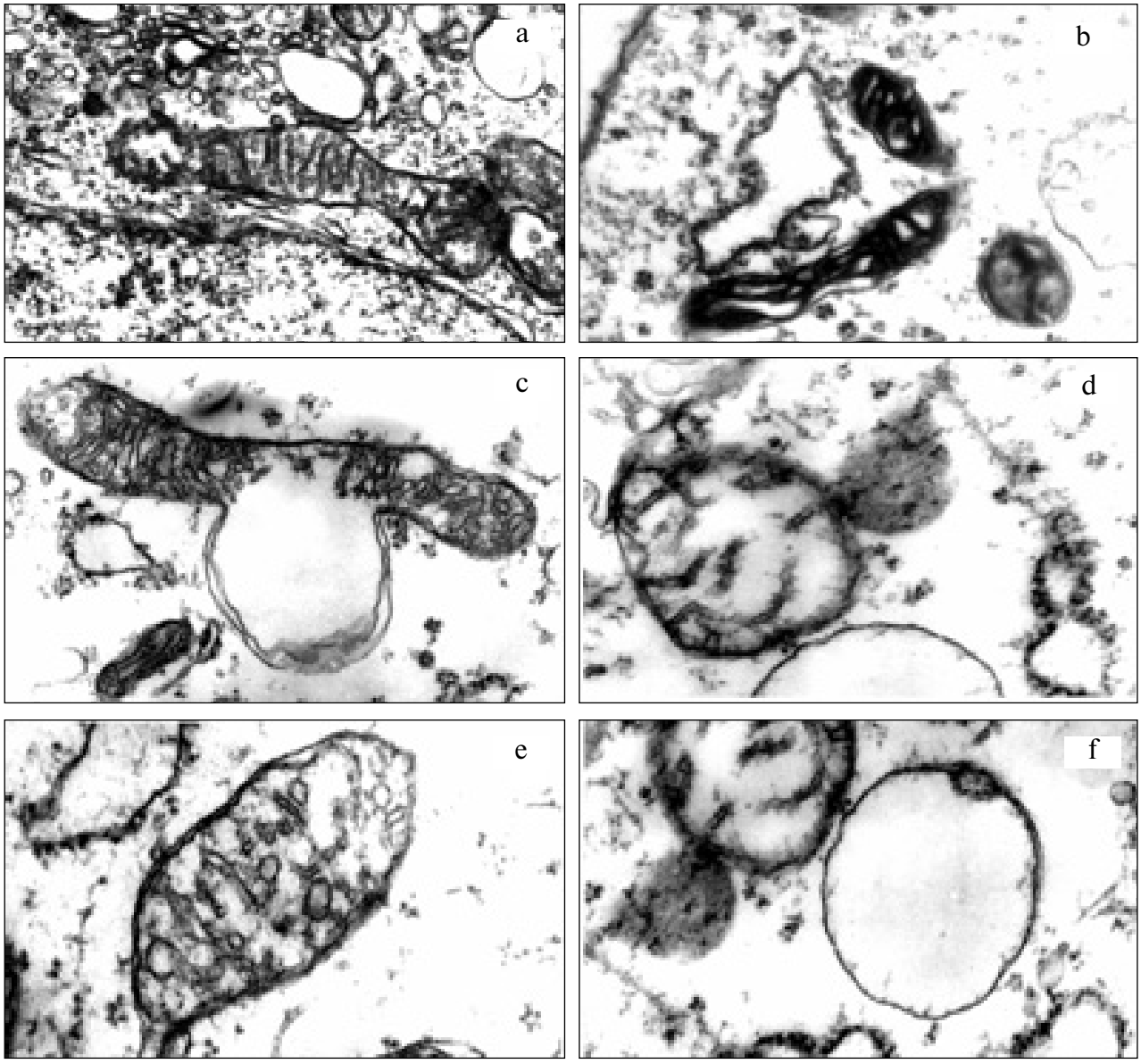

Fig. 2. Ultrastructure of mitochondria in glutamate-treated (100 μM, 15 min) cultured cerebellar granule cells: a) normal mitochondria in untreated cell; b) condensed mitochondria with decreased section area, enlarged intracristae and intermembrane spaces, decreased matrix volume associated with its high electron density; c) mitochondrion with local swelling and preserved in general ultrastructure; d) swollen mitochondrion with low electron density of the matrix, but preserved cristae and membranes; e) swollen mitochondrion with decreased matrix electron density and swollen cristae; f) swollen mitochondrion with destroyed cristae; two membrane layers are still seen in the upper part of the mitochondrion. Magnification ×40,000 (data of the authors).

activity of mitochondrial enzymes under normal conditions and various morphological changes in these organelles under pathological conditions [42]. It is possible that mitochondria with condensed matrix retain the ability to restore their normal ultrastructure after the action of Glu. Notably, the degree of swelling and energy loss sharply increased under the conditions of toxic Glu treatment in solution with isoosmotic substitution of Na^+ and Cl^- by sucrose [43]. No mitochondria with condensed matrix were found [44] (Fig. 3). Activation of Glu receptors results in the influx of calcium and sodium ions, and the latter can activate Na^+/Ca^{2+} antiporter in mitochondria, thus decreasing their Ca^{2+} buffering capacity. The recycling of Ca^{2+} in mitochondria leads to the constant discharge of the membrane potential, which can be restored by activation of mitochondrial respiration [23, 45]. Thus, during hyperactivation of Glu receptors Na^+ overload may inhibit Ca^{2+} overload of mitochondria and prevent their swelling. However, mitochondrial swelling due to the action of Glu may result not only from Ca^{2+} overload, but also from other neuron-specific mechanisms leading to the swelling of these organelles. Earlier it was shown that even 2-h incubation of PERV (porcine embryonic renal cells) cell line with Ca^{2+} ionophore A23187 cause only matrix condensation, but not mitochondrial swelling [46]. On the same cell line, it was shown that inhibition of ATP synthase by oligomycin or dicyclohexylcarbodiimide leads to mitochondrial swelling, while inhibitors of electron transport chain (rotenone and antimycin A) cause matrix condensation followed by mitochondrial swelling [47]. Similar changes in mitochondrial ultrastructure during Glu toxicity and under the action of respiration inhibitors suggest the impairment of electron transport in neuronal mitochondria during toxic action of Glu.

Recent data indicate that Glu neurotoxic action results not only in the collapse of mitochondrial membrane potential, but in a rapid impairment of mitochondrial movement in cells [48]. These authors observed that treatment by uncouplers or through the blockade of ATP synthesis by oligomycin also stopped mitochondrial movement in the cells, but did not change three-dimensional structure of mitochondria recorded by light microscopy. The addition of uncouplers during Glu treatment prevents neither movement impairment nor changes in mitochondrial morphology, though all these parameters recovered more rapidly after removal of these reagents [48].

The mechanisms of Glu-induced mitochondrial energy depletion attract a special interest of many authors. We assume that this energy depletion results from the induction of nonspecific permeability of the mitochondrial inner membrane due to Ca^{2+} overload of these organelles [33, 34]. This is confirmed by the facts that mitochondrial depolarization is accompanied by their swelling and that Glu-induced neuronal death can be suppressed by the inhibitor of nonspecific permeability, cyclosporin A [36, 37]. Preincubation of the neurons with N-methylvaline-4-cyclosporin significantly increases the time of Ca^{2+} accumulation by mitochondria [49].

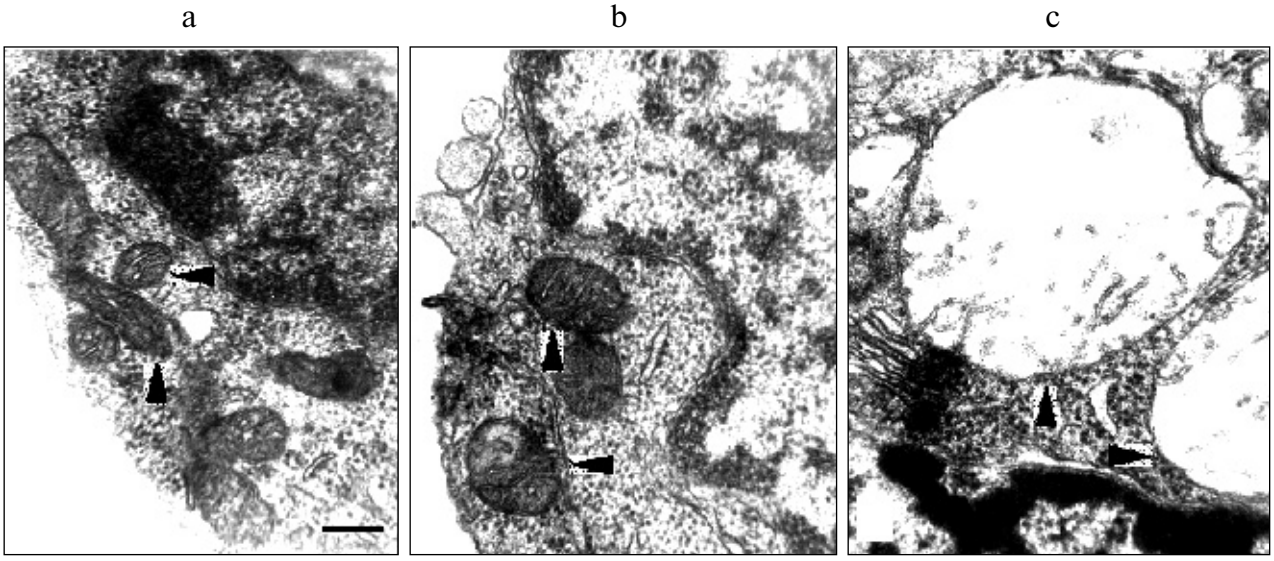

Fig. 3. Mitochondrial ultrastructure in rat cerebellar granule cells incubated in various balanced salt solutions for 20 min. Mitochondria are indicated by arrowheads. Scale bar, 0.3 μm. a) Incubation in balanced salt solution with 2.3 mM $CaCl_2$; b) incubation in low-Na^+ and Ca^{2+}-free solution. NaCl was isoosmotically substituted by sucrose. Morphology and volume of mitochondria are the same as in control cells (a); c) incubation in low-Na^+ salt solution containing 2.3 mM Ca^{2+} as in control (a). Mitochondria are strongly swollen, but separate cristae are still seen (from [44]).

However, cyclosporin A cannot be regarded as a specific inhibitor of nonspecific permeability, because it also blocks calcineurin, thus inhibiting the action of NO synthase. Calcineurin is known to induce dephosphorylation of NO synthase, activating this enzyme [50], while NO synthase may mediate toxic action of excitatory amino acids [51]. Nicotera et al. showed that a specific blocker of calcineurin, FK-506, did not prevent Glu-induced energy depletion in neuronal mitochondria [52]. These data confirm that Glu-induced energy depletion of mitochondria is mediated by Ca^{2+}-induced nonspecific permeability of the mitochondrial inner membrane. The formation of the transitional pore in the inner membrane may result in the release of Ca^{2+} into neuronal cytoplasm and thus contribute to the persistent Ca^{2+} overload of a neuron during toxic action of Glu.

MECHANISMS AND FUNCTIONAL ROLE OF ACIDOSIS OF NEURONAL CYTOPLASM DURING HYPERSTIMULATION OF GLUTAMATE RECEPTORS

In addition to changes in ionic balance of Ca^{2+}, Na^+, and K^+, Glu induces dramatic decrease in cytoplasm pH [53-55]. Our experiments using a pH-sensitive fluorescent probe, fluorescein diacetate, revealed a steep decrease of intracellular pH in cultured cerebellar granule cells by 0.3 ± 0.035 unit within 10 min incubation with 100 μM Glu. Other authors observed even stronger Glu- or NMDA-induced acidosis of neuronal cytoplasm surpassing 0.5 unit [54, 56]. The source of protons in neuronal cytoplasm during Glu toxic action has not yet been identified. Cytoplasmic acidosis may occur for many reasons such as the decreased activity of plasma Na^+/H^+ antiporter resulting from Na^+ cytoplasm overload, intensification of glycolysis and ATP hydrolysis, and activation of Ca^{2+}/H^+ antiporter due to competition between Ca^{2+} and protons for the binding sites on protein molecules. Wang and coauthors showed that Glu-induced acidosis directly correlated with Ca^{2+} transport in mitochondria, since cytoplasmic acidosis could be prevented by a specific mitochondrial Ca^{2+} transport blocker, ruthenium red [56]. Later, it was shown that NMDA-induced acidosis could be prevented by glycolysis inhibitors or the inhibitors of Ca^{2+}-ATPase, which points to the involvement of plasmalemma Ca^{2+}-ATPase in development of Glu-induced acidosis of neuronal cytoplasm [57]. It is possible that cytoplasmic acidosis prevents the formation of nonspecific permeability of the mitochondrial inner membrane, because acidification of the incubation medium is known to decelerate the formation of the transitional pore in the inner membrane [58-61].

However, the opposite view speculates that decreased cytoplasmic pH promotes the formation of Ca^{2+} permeability pore. It was shown that acidification of the incubation medium decreased the lag phase of the induction of nonspecific permeability of mitochondrial inner membrane by cumene hydroperoxide [62]. These authors assume that the induction of nonspecific permeability by pH decrease is caused by the decrease in transmembrane potential due to H^+ leak via ATP synthase, because this permeability is formed only in the absence of oligomycin.

ROLE OF MITOCHONDRIA IN THE MECHANISMS OF TOXIC ACTION OF EXCITATORY AMINO ACIDS

Mitochondria may directly mediate toxic action of excitatory amino acids. In addition to the induction of nonspecific permeability of the mitochondrial inner membrane, impairment of ATP production, and release of apoptosis-inducing substances, they can produce glutamate due to activation of mitochondrial glutaminase after long-term stimulation of Glu receptors. It was shown that under normal conditions murine cortical neurons and astrocytes produced about 30 nmol Glu per min, whereas 5-h hypoxia or exposure to 0.5 mM NMDA increased Glu production to 500-800 nmol/min. This increase in the rate of Glu accumulation in cultured neurons induced by hyperactivation of Glu receptors could be inhibited by specific inhibitors of mitochondrial glutaminase, 6-diazo-5-keto-L-norleucine and *p*-chloromercuriphenylsulfonic acid [63].

Under conditions of increased leakage of electrons from the electron transport chain to molecular oxygen and impaired Ca^{2+} homeostasis in neurons, mitochondria can serve as an additional source of free radicals. Incubation of the mitochondrial fraction of the cortex and cerebellum in medium with high Ca^{2+} and Na^+ content resulted in increased free radical production [64]. These data were confirmed later on the model of cultured rat and murine cortical cells. It was shown that NMDA treatment as well as treatment by the blocker of oxidative phosphorylation, carbonyl cyanide *p*-(trifluoromethoxy)phenylhydrazone (FCCP), stimulated the production of reactive oxygen species (ROS). ROS production depended on extracellular Ca^{2+} concentration and could be decreased by inhibitors of the electron transport chain [65-67], while FCCP enhanced the NMDA-induced ROS production [66]. At the same time, it was stated that the induction of nonspecific mitochondrial permeability was associated with enhanced ROS production [68, 69]. These data confirm that Ca^{2+}-dependent formation of nonspecific mitochondrial pore induced by hyperstimulation of NMDA receptors can promote the development of oxidative stress in neurons. Vergun et al. point to the other mechanism of participation of free radicals in Glu toxicity [70]. These authors showed that combined treatment of the neurons by various antioxidants during toxic Glu action may inhibit the influx of ions via NMDA channels.

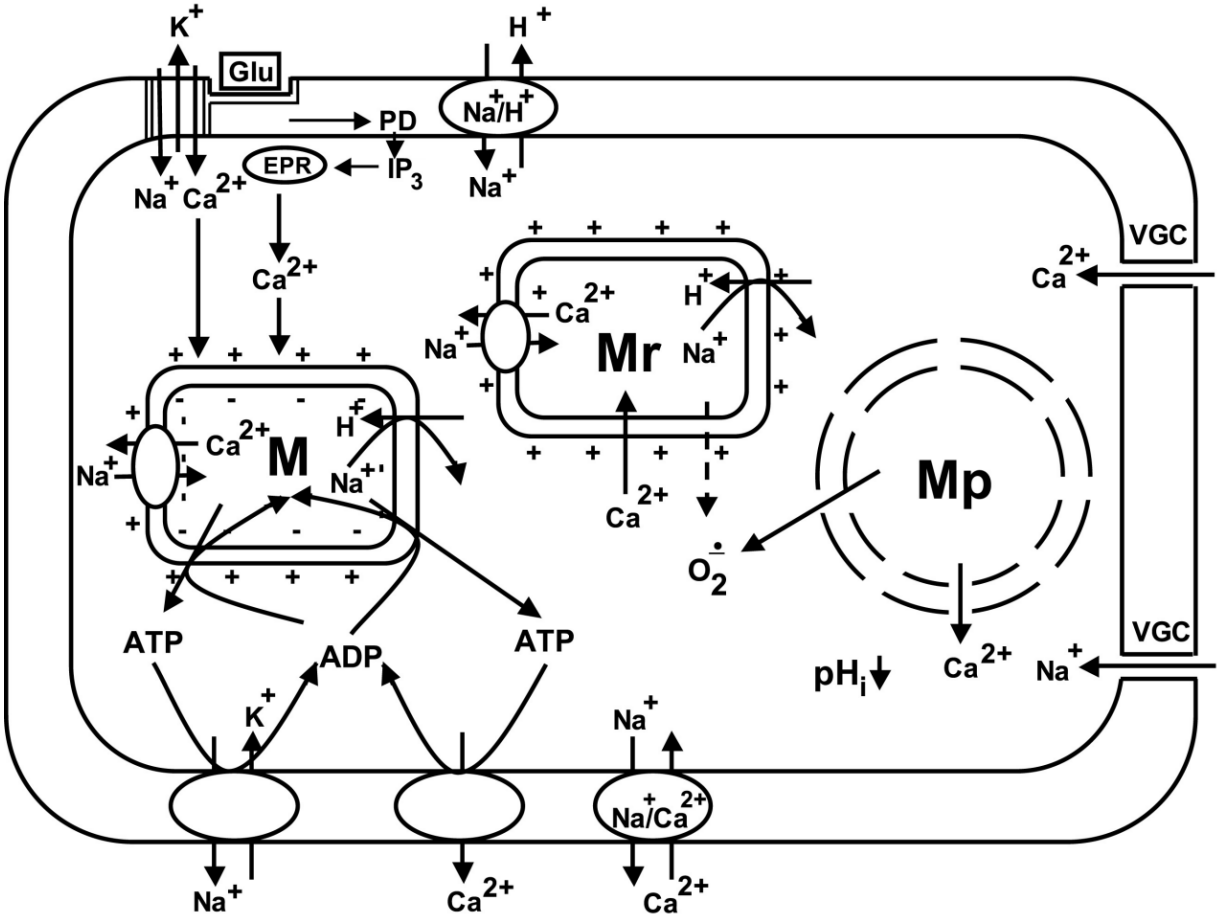

Fig. 4. Mechanisms of Glu-induced changes in Ca^{2+} and Na^+ concentrations impairing the structure and function of neuronal mitochondria. PD, phosphoinositide diphosphate; IP_3, inositol triphosphate; VGC, voltage-gated channels; EPR, endoplasmic reticulum; M, normal mitochondrion; Mr, mitochondrion with Ca^{2+} recycling; Mp, mitochondrion with an open pore.

The most recent data showed that ND2 NADH dehydrogenase subunit known as complex I component of the mitochondrial respiratory chain and encoded by a mitochondrial gene, fixed regulatory Scr protein in NMDA receptor complex. This protein interaction is critical for the regulation of NMDA receptor activity [71]. Thus, mitochondria directly participate in the intraneuronal transduction of NMDA receptor signal.

In conclusion, it should be noted that mitochondria play a significant or even critical role in the mediation of Glu-induced neuronal death. Figure 4 presents a scheme summarizing the mechanisms leading to the impairment of energy and ionic homeostasis under the toxic action of Glu.

During hyperactivation of Glu receptors, K^+ ions leave the neurons, while Na^+ and Ca^{2+} enter the cells via NMDA and kainate channels and after depolarization of the plasma membrane via voltage-gated Ca^{2+} channels. The interaction of Glu with metabotropic receptors activates phosphoinositide metabolism and production of inositol triphosphate, which causes the release of Ca^{2+} from endoplasmic reticulum. Ca^{2+} overload leads to a pronounced acidification of the cytoplasm. As shown in Fig. 4, Na^+ ions enter the cell via receptor-operated and voltage-gated channels, Na^+/H^+ and Na^+/Ca^{2+} antiporters, and are extruded by the Na^+/K^+ pump. Since the efficacy of Na^+-dependent Ca^{2+} extrusion is determined by transmembrane Na^+ gradient, any process increasing intracellular Na^+ concentration, including Na^+/H^+ exchange, inhibits Ca^{2+} extrusion via Na^+/Ca^{2+} antiporters. Activation of Glu receptors causes constant influx of Na^+ and Ca^{2+} via NMDA and voltage-gated channels. This ionic overload leads to intense ATP expense due to the activity of ATP-dependent extrusion of intracellular ions and Ca^{2+} recycling in the mitochondria. Na^+ overload leads to the impairment of Glu and glucose uptake. Excessive cytosolic Ca^{2+} is largely accumulated by mitochondria, which stimulates the production of free radicals by these organelles. Radical production combined with Ca^{2+} overload promotes mitochondrial swelling, damage of their inner membrane associated with the formation of large pores, collapse of mitochon-

drial membrane potential, inhibition of aerobic ATP production, release of cytochrome *c* and Ca^{2+}, and the cessation of mitochondrial movement. The induction of nonspecific permeability may lead to further mitochondrial swelling and increased ROS production. Under the conditions of toxic Glu action, Ca^{2+} release from the mitochondria via the nonspecific permeability pore may contribute to stable Ca^{2+} overload of a neuron. Deprived of the main ATP source, these neurons cannot recover ionic homeostasis, which leads to activation of lipolytic and proteolytic enzymes and finally, to cell death.

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