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# BIOPHYSICS AND BIOCHEMISTRY

# Kainate-Induced Degeneration of Hippocampal Neurons. Protective Effect of Activation of the Endocannabinoid System

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We studied the prolonged action of kainic acid on glutamatergic neurons in the dorsal hippocampus and the endocannabinoid-dependent protection against neurodegeneration. The pyramidal neurons of the CA3 field of the hippocampus, as well as granular and mossy cells of the dentate gyrus were examined. Light and electron microscopy revealed substantial damage to the components of the protein-synthesizing (rough endoplasmic reticulum, Golgi apparatus, and polyribosomes) and catabolic (lysosomes, autophagosomes, multivesicular structures, and lipofuscin formations) systems in all cells. Pyramidal and mossy neurons die mainly by the necrotic pathway. The death of granular cells occurred through both apoptosis and necrosis. The most vulnerable cells are mossy neurons located in the hilus. Activation of the endocannabinoid system induced by intracerebral injection of URB597, an inhibitor of degradation of endocannabinoid anandamide, protected the normal structure of the hippocampus and prevented neuronal damage and death induced by KA.

**Key Words:** *kainic acid; endocannabinoids; hippocampus; morphological analysis; light and electron microscopy*

Hyperexcitation of neurons and status epilepticus induced by neurotoxin kainic acid (KA) are used to create an experimental model of temporal lobe epilepsy, in particular to elucidate the mechanisms of oxidative stress in neurodegenerative diseases [14]. In temporal lobe epilepsy, neuronal connections in the hippocampus are damaged due to cell death [7]. Oxidative stress is one of the main causes of neurodegeneration caused by KA [6]. One of the mechanisms strictly controlling excitability of neurons is activation of the endocannabinoid (EC) system [1,5] that ensures functioning of the neural networks under conditions of hyperexcitability [3,7]. Experimental epilepsy is accompanied by an increase in the level of the EC anandamide and activation of EC receptors [5]. However, the lifetime of EC in the brain is short due to both their reuptake by neurons and enzymatic degradation. The level of EC and their activity can be significantly increased by suppressing their degradation [3]. To this end, we used agent URB597 [3-(3-carbamoylphenyl)phenyl]-N-cyclohexylcarbamate], an inhibitor of fatty acid amide hydrolase (FAAH).

An experimental model of epilepsy revealed significant degeneration of hippocampal neurons under the action of KA. Previously, we analyzed the dependence of the state of CA1 field neurons in rat hippocampus on the severity of status epilepticus and the role of EC in neuronal survival. As pyramidal CA3

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neurons are primarily damaged in patients with temporal lobe epilepsy and animals treated with KA [12], the study of these neurons is of greatest interest.

There is no consensus regarding the resistance of granular neurons in the dentate gyrus (DG) to neurotoxins [11,15]. Mossy cells of the DG hilus that innervate both glutamatergic granular neurons and GABAergic DG interneurons [9] also demonstrate specific sensitivity to neurotoxins.

The function of neurons and their adaptive capacity are determined by the state of the protein-synthesizing system and its relationship with the catabolic system. Using light and electron microscopy, we analyzed the degree of damage to components of these systems in the CA3 pyramidal neurons and in mossy and granular neurons of DG under the action of KA administered alone or against the background of EC system activation with URB597.

## MATERIALS AND METHODS

The experiments were carried out on male Wistar rats ($n$=26) in accordance with the Directive 2010/63/EU of the European Parliament and of the Council "On the Protection of Animals Used for Scientific Purposes" (September 22, 2010). The rats were divided into 4 groups: control rats received injection of saline followed in 24 h by injection of 10% DMSO (group 1; $n$=5) or URB597 (10 μg in 10% DMSO; group 2; $n$=5), experimental animals received injection of KA (0.6 μg in saline) followed in 24 h by injection of 10% DMSO (group 3; $n$=8) of URB597 (10 μg in 10% DMSO; group 4; $n$=8). The drugs (all from Sigma-Aldrich) were injected intraventricularly in awake rats (ML=1.5, DV=1.5) [8] with a Hamilton 75N microsyringe at a rate of 1 μl/min in equal volumes (1 μl). Injections of DMSO and URB597 in DMSO were performed daily for 10 days. Animal behavior after injection of KA was controlled visually. The intensity of status epilepticus significantly varied in different animals. The seizure severity was estimated according to modified Racine scale (stages 1-5).

Morphological examination of the hippocampus (field CA3 and DG) was carried out 2 weeks after KA injection. Frontal sections of the dorsal hippocampus were fixed for 2 h in a 2.5% glutaraldehyde solution in 0.1 M phosphate buffer and postfixed in 2% $OsO_4$. On semithin (10 μm) sections of the CA3 field, morphologically intact pyramidal neurons containing well expressed nucleus and nucleolus were counted in 16 fields of view (735×555 μm) for each animal (objective 20×). Ultrathin sections (70-75 nm) were contrasted with uranyl acetate and lead citrate and studied using a JEM 100B electron microscope (Jeol). Qualitative analysis of the ultrastructure of hippocampal neurons was carried out on negatives digitized on an Epson V700 scanner in at least 50 fields of view.

The quantitative processing of the obtained results was carried out using Prism 5.0 software (GraphPad Software). The hypothesis of a normal distribution of the general population of data was tested using the Pearson $\chi^2$ test or the Shapiro—Wilk test for small samples at a significance level of $\alpha$=0.01. Taking into account the obtained data on the normal distribution of certain parameters, the two-sample Student's $t$ test or the nonparametric Mann—Whitney $U$ test was used to compare the groups. In the case of Student's $t$ test, the hypothesis of equality of variances was preliminarily tested using a two-sample $F$ test. Quantitative results are presented in the Table 1 as $M$±$SEM$. Differences between groups were considered statistically significant at $p$<0.05 (95%).

## RESULTS

For structural analysis, only rats demonstrating distinct behavioral responses to the introduction of KA (16 of 26) were used. A light microscopy examination showed that, two weeks after injection of KA, neurons in the CA3 field were in different morphological states. The number of morphologically intact neurons significantly decreased (Table 1).

Ultrastructural analysis revealed 4 groups of pyramidal neurons of the CA3 field depending on the level of degradation of structural components (Fig. 1). Neurons of the first group have a typical structure of intact neurons (Fig. 1, *a*) with well-developed rough endoplasmic reticulum (RER), Golgi apparatus (GA) with transport vesicles, polyribosomes, and various elements of the catabolic system (lysosomes, autophagosomes, multivesicular structures). The second group of neurons included cells with signs of degeneration (Fig. 1, *b-d*): partial fragmentation of RER, the swelling of cisternae, shedding of ribosomes from RER membranes, dissociation of polyribosomes, swelling and blurring of GA membranes. Near these structures, we observed large aggregates of electron-dense material not

**TABLE 1.** Number of Morphologically Intact Pyramidal Neurons in CA3 Area of Dorsal Hippocampus ($M$±$SEM$)

| Group | Number of morphologically intact pyramidal neurons |
|---|---|
| Control ($n$=5) | 182±4 |
| KA ($n$=8) | 51±4* |
| KA+URB597 ($n$=8) | 114±6*+ |

**Note.** $p$<0.05 in comparison with *control, +KA.

surrounded by the membrane; these aggregates most likely consisted of ubiquitin-containing protein deposits [2], which is a consequence of damage to RER and inhibition of the ubiquitin-proteasome system as a result of oxidative stress. The number of late autophagolysosomes (lipofuscin granules) markedly increased due to activation of autophagy and inhibition of the ubiquitin proteasome system [2] The presence of multivesicular formations was also noted, which may reflect damage to the structural integrity of the outer membranes [10] as a result of oxidative stress. Dysfunction of the mechanisms of synthesis and degradation under pathological conditions leads to intensive accumulation of misfolded proteins and lipofuscin granules, late lysosomes, and finally, to cell death [2,10]. Neurons of the third group were characterized by more significant degenerative processes: depletion and vacuolization of the cytoplasm, which, most likely, indicates irreversible processes (Fig. 1, *e*). The fourth group of neurons included hyperchromic cells (Fig. 1, *f*). They are characterized by increased electron density of the cytoplasm against the background of the previously noted lesions.

Ultrastructural examination of mossy cells in the hilus revealed substantial morphological lesions and a similarity to the pyramidal neurons of the fourth group of the CA3 field, but with more pronounced signs of degeneration (Fig. 2, *a*). In addition, these cells, in contrast to other neurons, contained no autophagosomes, which is consistent with previous data [13] and explains greater vulnerability of these cells to KA in comparison with other neurons. The giant presynaptic boutons at the endings of the afferent fibers generally looked empty.

Ultrastructural analysis of granular cells in DG revealed different degree of degeneration. In degrading cells, signs of both necrosis and apoptosis were seen (Fig. 2, *b*, *c*). Damage to RER and GA, numerous dark protein aggregates near them, dissociation of polyribosomes, great number of vesicles, lipofuscin formations, and multivesicular bodies are indicators of necrosis. At the same time, many granular cells demonstrated signs of apoptosis: partial condensation of chromatin in both the karyoplasm and on the nuclear membrane, as well as the presence of free polyribosomes in the cytoplasm, which are probably

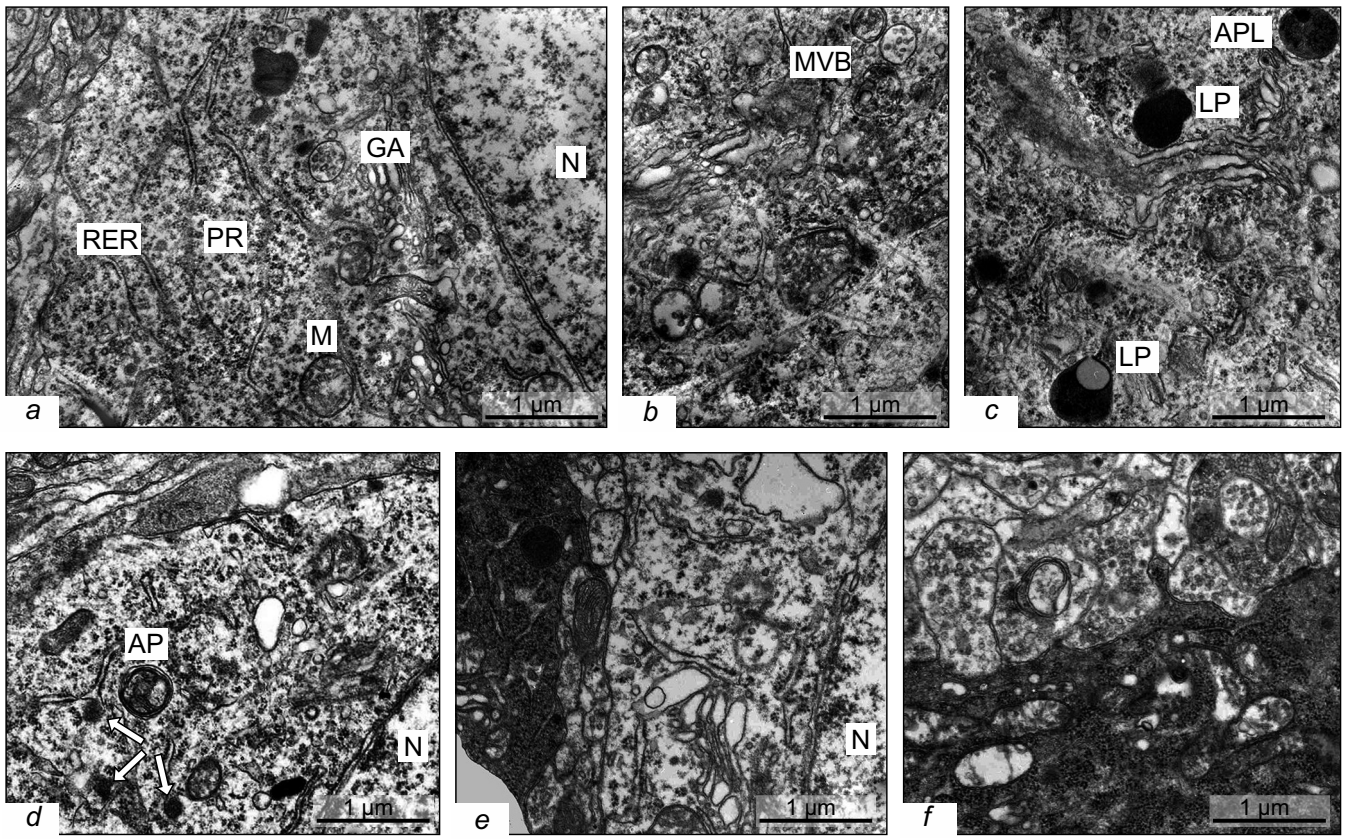


**Fig. 1.** Ultrastructure of four groups of hippocampal pyramidal neurons in 2 weeks after intraventricular injection of KA. *a*) Neurons of group 1; *b*–*d*) neurons of group 2; *e*) neurons of group 3; *f*) neurons of group 4. PR: polyribosomes; M: mitochondria; N: nucleus; MVB, multivesicular bodies; LP: lipofuscin formations; APL: autophagolysosoma; AP: autophagosoma. Arrows show accumulations of electron-dense dark material.

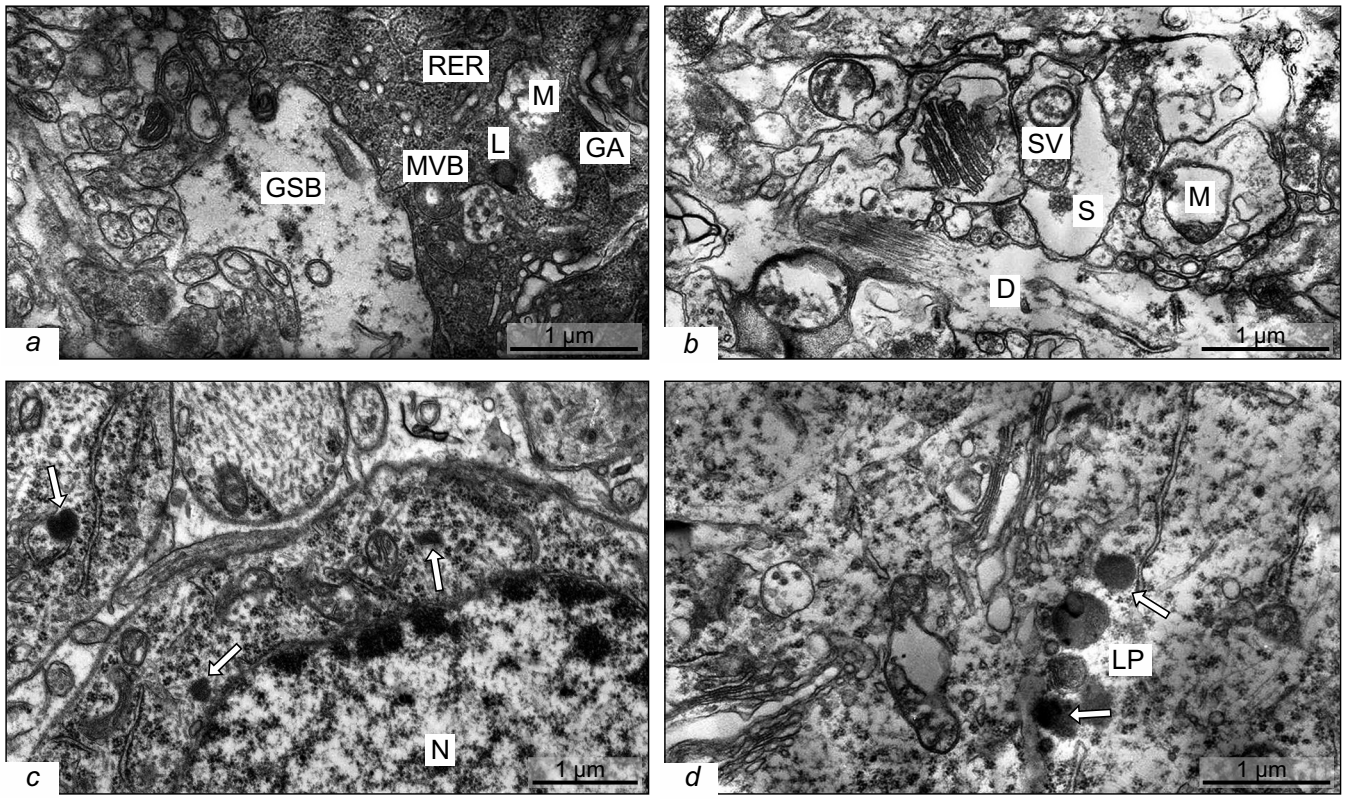


**Fig. 2.** Ultrastructure of mossy (*a, b*) and granular neurons (*c, d*) of DG in 2 weeks after intracerebroventricular injection of KA. Flooded dendrite with empty synapses (*b*); agglutinated synaptic vesicles and destroyed mitochondria are seen. GSB: a giant synaptic bouton near a mossy cell; MVB, multivesicular bodies; L: lysosome; SV: synaptic vesicles; D; dendrite; S: synapse; N: nucleus; LP: lipofuscin formations.

responsible for the synthesis of proteins necessary for apoptosis. Normally, granular cells die by apoptosis, because granular cells in adults undergo aging and are replaced with new cells [4]. Immature young granular cells after exposure to a damaging factor die mainly by necrosis [15]. According to our findings, KA against the background of apoptosis provoked considerable necrotic changes in the components of the cytoplasm in one and the same cell.

Degradation in axo-dendritic synapses in the neuropil of the CA3 field and DG should be noted (Figs. 1, 2). Synaptic endings underwent light (edematous) destruction. The axonic terminal swelled and became clear; the amount of synaptic vesicles (SV) in it decreased, and the remaining SV underwent agglutination. Empty boutons alternated with boutons filled with vesicles. All these observations indicate that pulse transmission from DG to CA3 pyramidal cells was impaired. Degeneration of mossy cells due to their greater vulnerability to hyperexcitation caused by neurotoxins is probably the most critical event in the system of morphological rearrangements.

Two weeks after the combined injection of KA and URB597, the number of morphologically intact neurons was significantly higher that after treatment with KA alone (Table 1), but lower than in the control. The morphology of many light neurons did not differ from that in the control (Fig. 3, *a*, *b*). The axo-dendritic synapses were preserved near these neurons, including mossy cells (Fig. 3, *c*-*e*).

The main effect of mossy cells on granular cells is not excitation, but inhibition via activation of inhibitory interneurons [9]; therefore, degeneration of mossy cells probably shifts the balance between the excitation and inhibition towards excitation. Activation of the EC system through inhibition of oxidative activity caused by KA contributes to the preservation of neurons and provides a stable excitation/inhibition balance in the neural network.

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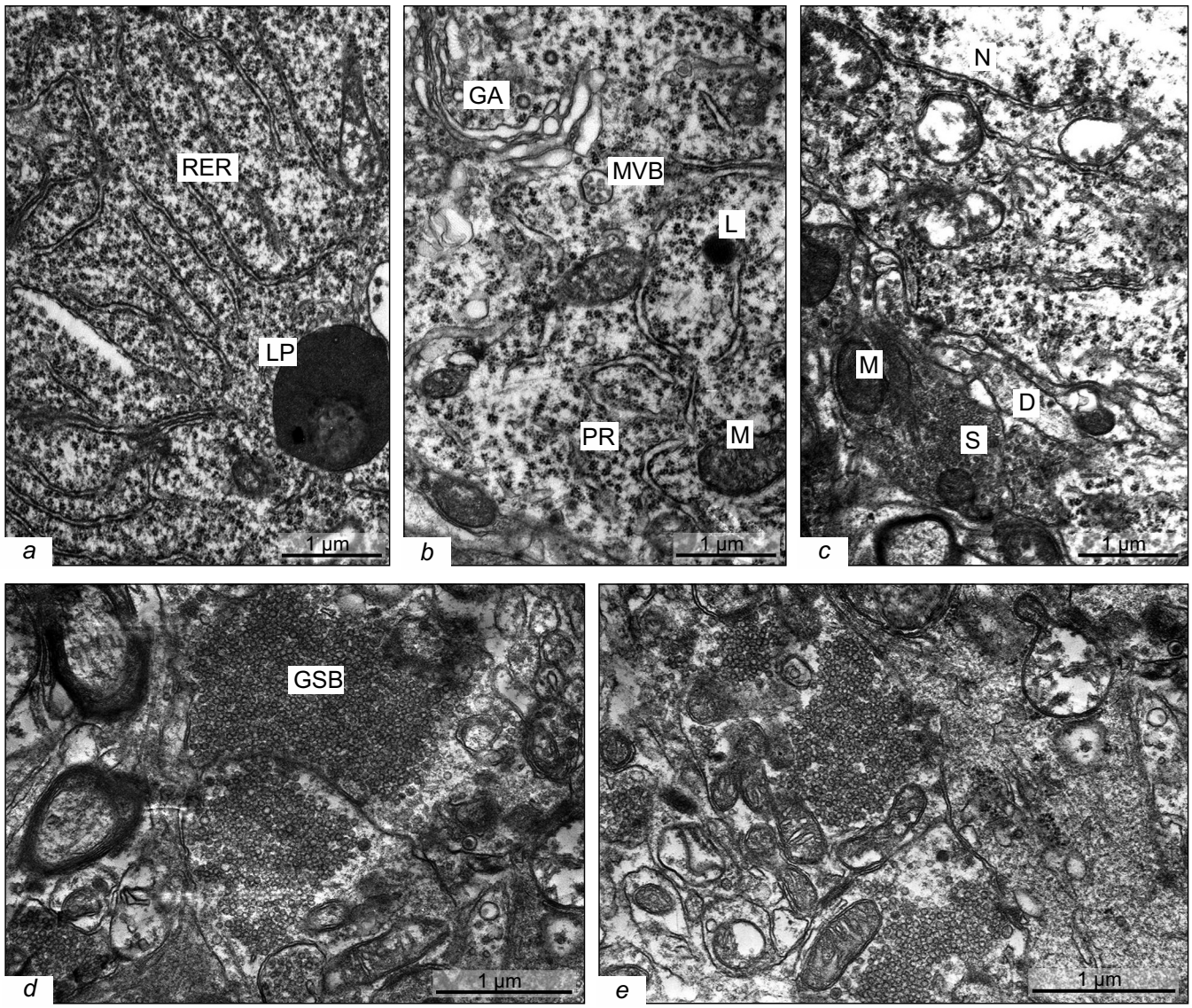


**Fig. 3.** Ultrastructure of 4 groups of hippocampal pyramidal CA3 neurons in 2 weeks after intracerebroventricular injection of KA+URB597 (*a-c*). Giant synaptic bouton uniformly filled with synaptic vesicles with well-preserved mitochondria. Ultrastructure of giant synapses of mossy cells (*d*) and synapses of granular cells (*e*). LP: lipofuscin formations; MVB, multivesicular bodies; L: lysosome; PR: polyribosomes; M: mitochondria; N: nucleus; S: synapse; D: dendrite; GSB, giant synaptic bouton near a mossy cell.

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