MODULATION OF NETWORK ACTIVITY IN DISSOCIATED HIPPOCAMPAL CULTURES BY ENZYMATIC DIGESTION OF EXTRACELLULAR MATRIX

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To investigate the role of extracellular matrix in spontaneous neuronal network activity, we used microelectrode array technology and enzymatic treatment of hippocampal culture with hyaluronidase, which digests the major component of extracellular matrix, hyaluronic acid. Studies were performed using hippocampal cells that were dissociated from embryonic C57BL6 mice (E18) and plated on microelectrode arrays (MEAs). Our findings revealed that hyaluronidase promoted seizure-like activity during two weeks after the beginning of hyaluronidase treatment in 17th day in vitro: the treatment transformed the normal network bursts to "superbursts", which lasted about 25–35 s. These superbursts appeared on the third day after hyaluronidase treatment with intersuperburst interval of 1–3 min. Seizure-like activity in hyaluronidase-treated cultures was irreversible during 2 weeks, but could be suppressed by an L-VGCC blocker and by an AMPA (alfa-amino-3-hydroxy-5-methyl-4-isoxazol-propionic acid)receptor antagonist. These results suggest that the changes in expression of hyaluronic acid can be epileptogenic and provide an in vitro model for dissection of the underlying mechanisms.

Key words: hippocampus, dissociated culture, multielectrode arrays, extracellular matrix, hyaluronic acid.

Extracellular matrix (ECM) in mammal brain consists of molecules synthesized and secreted by neurons and glial cells that form stable aggregates in intercellular space in various combinations [1]. In mature brain ECM undergoes slow changes and limits structural change, though maintaining many physiological processes including synaptic plasticity and homeostatic regulation [2].

The most expressed and studied clumps of ECM molecules in CNS are perineuronal nets [3]. They are rich in hyaluronic acid, chondroitin sulfate proteoglycans, proteins linking chondroitin sulfate proteoglycans with hyaluronic acid (link proteins) and tenascin-R that can form dimers or trimers and in this way interact with several lecticans stabilizing perineuronal nets. Perineuronal nets in cerebral cortex and hippocampus are associated mainly with GABA (gamma-aminobutyric acid) interneurons expressing calcium binding protein — parvalbumin. As hyaluronic acid is the frame of ECM in extracellular brain space, the study of its role in synaptic plasticity is most interesting. Recent studies stated the hyaluronic acid degradation by hyaluronidase in CA1-field hippocampal sections to induce CA^{2+}-signals in postsynaptic dendrites or spinules, block CA^{2+}-flows and long-term potentiality (LTP) mediated by L-type calcium channels (L-VGCC) [4]. Moreover, hyaluronic acid removal facilitates the diffusion of membrane molecules including the receptors of alfa-amino-3-hydroxy-5-methyl-4-isoxazol-propionic acid (AMPA-receptors), and increases the amplitude of responses caused by

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synapse restimulation [5]. The data prove the conclusion that perisynaptic matrix switching hyaluronic acid can raise barriers for diffusion of synaptic molecules in membrane and in this way contribute to compartmentalization of signaling synaptic mechanism.

The significance of hyaluronic acid for animal behaviour was demonstrated experimentally, when preventive administration of hyaluronidase suppressed the fear conditioning formation [6]. In addition, convulsive activity was shown to be characterized as a rule by considerable changes in ECM that proves the essential role of ECM molecules in epileptogenesis. The idea is supported by genetic researches attributing the deficiency or excess of ECM molecules to epileptogenesis in mice, as well as the reproportion of cells in neuron-glial cerebral net. Human genome was found to have some genes coding ECM molecules, the mutations of which are associated with epileptogenesis. For example, mutations in human leucine-rich glioma-inactivated gene1 (LGI1) lead to autosomal dominant epilepsy of lateral temporal lobe accompanied by auditory disorders [7]. Urokinase receptor knockout (uPAR) leads to the loss of parvalbumin expressing GABA interneurons and the development of epileptic phenotype [8]. Mutations in protein β1-subunits of potential dependant NA^+-channels (SCN1B) are related to generalized epilepsy. SCN1B protein is responsible for channel gating work, regulates the level of channel expression on plasmamembrane and acts as cell adhesion molecule when interacting with ECM regulating cell migration. In this case ECM molecules are glycoproteins including tenascin-R [9]. It should be noted that ECM change pattern induced by seizure is complex and specific in relation to brain zone and cellular subdomain. Remodeled ECM is able to cause numerous secondary long-term functional and structural changes of CNS that can govern further development of the disease.

Thus, ECM change caused by convulsive activity or signaling pathways suppression by the changed matrix can represent effective therapeutic strategies for progressive epileptogenesis suppression. Currently, there is no information on the role of hyaluronic acid in neuron network activity formation.

The aim of the investigation is to study the effect of hyaluronidase on neuroelectricity of hippocampal primary culture of mice embryos.

Materials and Methods. The researches were carried out on hippocampal cells dissociated from 18-day embryos of C57BL/6 mice according to the basic rules of experimental animals management and care represented in the Order of the Ministry of Health and Social Development of Russian Federation dated August 23, 2010, No. 708н "Approval of laboratory practice rules" and as agreed upon with Ethical Committee of Nizhny Novgorod State Medical Academy.

Hippocampal dissociated cells culture. The cells were dissociated by treating hippocampal tissues by 0.25% trypsin (Invitrogen, USA). The cells were resuspended in Neurobasal™ (Invitrogen) with bioactive supplement B27 (Invitrogen), glutamine (Invitrogen), embryonic calf serum (PanEco, Moscow) and cultivated on multi-electrode matrixes under previously developed protocol [10] within 30 days in vitro (DIV). Starting density of cell culture was 9000 cells per mm^2. Culture viability was maintained in CO_2-incubator at 35.5°C and gas mixture containing 5% CO_2. Polyethylenimine (Sigma, USA) was used as a reference substance for neurons culture.

Registration and bioelectricity analysis. Spontaneous neuroelectricity was registered using multi-electrode matrixes MED64 (Alpha MED Sciences, Japan). The matrix consisted of 64 planar square electrodes, each 50x50 micrometer in size, interelectrode distance being 100 micrometer. To obtain and treat extracellular potentials (spikes) there was used Conductor — software suite of multi-electrode system (Alpha MED Sciences, Japan). The obtained data were analyzed using software package Matlab and MEAMAN. Spikes were detected by conventional method using standard deviation (boundary 8-12, and σ — root-mean-square deviation). Small network bursts were detected using the technique described above [11].

There were examined basic characteristics of bioelectricity of neuron net of dissociated hippocampal culture: duration of small pulse burst, c; interburst interval, c; spike frequency in a small burst, Hz; small burst frequency, Hz.

Experimental design. In experimental group, the culture medium was treated once by 75 U/ml of hyaluronidase (Streptomyceshyalurolyticus, Sigma, USA) on the 17 day in vitro. In a day after the enzyme treatment 50% of culture media was volume was changed. Just before the procedure the enzyme was dissolved in polyphosphate buffer. In control groups the culture media was treated by polyphosphate buffer or inactivated enzyme, equal in volume. Hyaluronidase was inactivated by 30-minutes boiling at standard atmospheric pressure.

Potassium channel blocker, 4-aminopyridine (4-AP, Sigma), 50 micromol, promoting seizure-like activity in vivo and in vitro was used for comparative assessment of bioelectricity induced by hyaluronidase. To study the mechanisms of modeling effect of hyaluronidase on synaptic transmission in neuron nets of hippocampal culture there was carried out pharmacological analysis including the study of diltiazem, a potential dependent L-type calcium channels blocker (L-VGCC), 10 micromol (Sigma), 6-cyano-7-nitroqino-xaline-2,3-dione (CNQX), 10 micromol (Sigma), as AMPA-receptors antagonist, and 3-(2-carboxypiperazine-4-il)propyl-1-phosphoric acid (CPP), 10 micromol (Sigma), as N-methyl-D-aspartate (NMDA) receptors competitive antagonist.

Neuroelectricity of dissociated hippocampal culture was studied 2 h later, and every 24 h within 11 days after treatment. Record time was 10 min.

Reliability of differences between the groups was estimated using analysis of variance (ANOVA). The differences were considered statistically significant in $p<0.05$.

Results. Beginning with the 8–10 days dissociated hippocampal cultures exhibited spontaneous network burst bioelectricity. By the 17th day cultivated spontaneous burst activity became stable. Activity level registered in the 17th day of development in vitro before hyaluronidase treatment

was considered to be basal. According to literature [12–14], extracellular matrix can form on the 16–17th days in hippocampal primary culture.

Single addition of hyaluronidase (75 U/ml) into medium was noted to cause the change of bioelectricity of dissociated hippocampal cultures (Fig. 1).

Short small network bursts lasting 0.3–1 s (Fig. 1, *a*) were transformed into long-term superbursts of complex pattern (Fig. 1, *c*) lasting 10–35 s with intersuperburst interval from 60 to 205 s. the changes were stable and registered starting with the 3rd–7th day after hyaluronidase addition. Due to long intervals between bursts, 30-second records of spike activity were chosen to study the activity.

The comparative analysis of statistical parameters of neuron net activity of hippocampal primary cultures revealed complex activity pattern in the form of superburst to consist of a great number of small bursts characterize by less spikes in a burst, if the duration is the same and interburst interval is very short (Fig. 2).

Next step was the comparison of spontaneous bioelectricity of neuron net of hippocampal primary culture induced by hyaluronidase, and the activity caused by 4-AP.

4-AP added into culture medium, as expected, promotes short-term seizure-like activity. However, the pattern of neuron network activity induced by 4-AP differs from the activity pattern after hyaluronidase effect (Fig. 3).

To study the mechanisms of 4-AP-induced activity and hyaluronidase-induced activity, there was performed pharmacological analysis using ion channel blockers and glutamate receptors antagonists. 4-AP-induced activity was noted to develop in potential dependent L-type calcium channels (L-VGCC) blocked by diltiazem, benzothiazepine derivative (Fig. 4, *a*, n=3; statistical significance of differences with control of “small bursts rate” parameter,

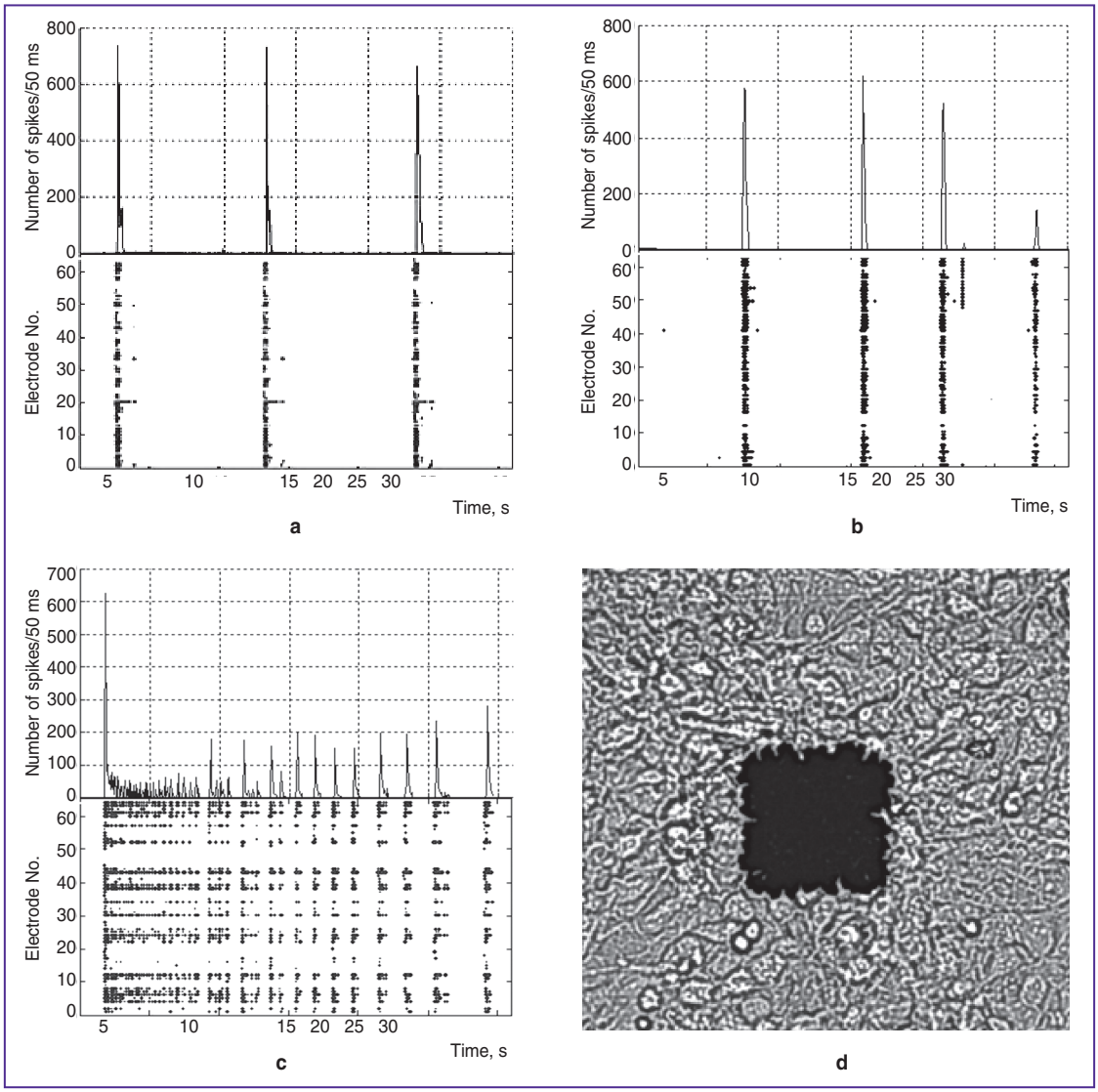

Fig. 1. Screen graph of bioelectricity of hippocampal dissociated culture (bottom graphs) and the number of spikes within 50 ms (top graphs) on the 23rd day in vitro; *a* — activity in control culture — No.1 (PBS); *b* — activity in control culture — No.2 (inactivated hyaluronidase); *c* — hyaluronidase-induced changes in activity; *d* — photomicrograph of high density of primary hippocampal culture on the 23rd day in vitro, in the area of MED64 matrix electrode, scale 50 micrometer

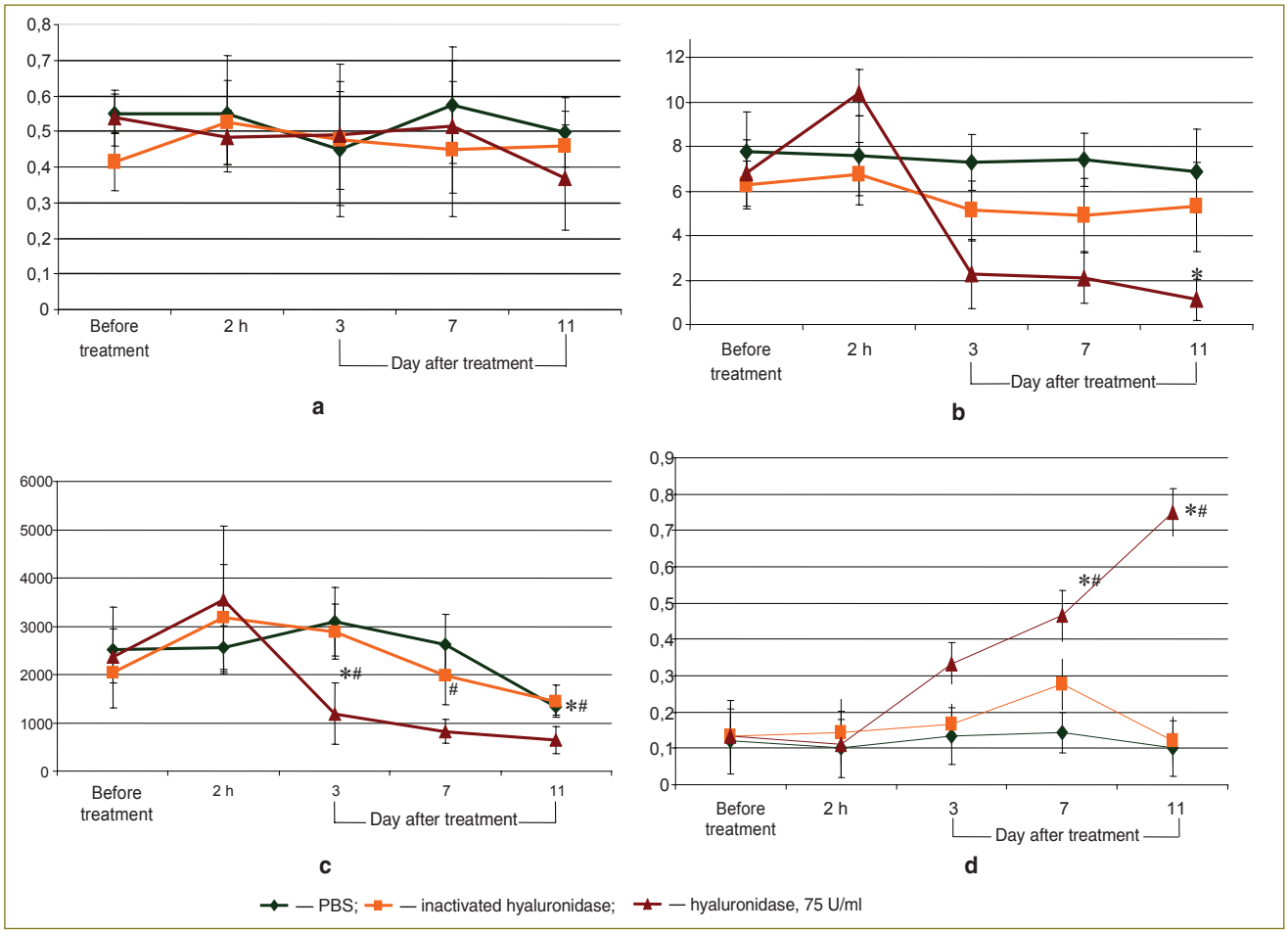

Fig. 2. Indices of 30-second record of spontaneous bioelectricity of hippocampal dissociated cultures (17–28 DIV) before 75U/ml of hyaluronidase is added in culture media; 2 h and 3–11 days after the addition: *a* — duration of a small pulse burst, s; *b* — interburst interval, s; *c* — spike rate in burst, Hz; *d* — small bursts rate, Hz. The number of repeats: in control group with PBS n=11, in the group with hyaluronidase application, 75 U/ml n=5, in the group with inactivated hyaluronidase application n=3; * — statistically significant differences with controls, p<0.05; # — statistically significant differences with the indices in the group with inactivated hyaluronidase, p<0.05

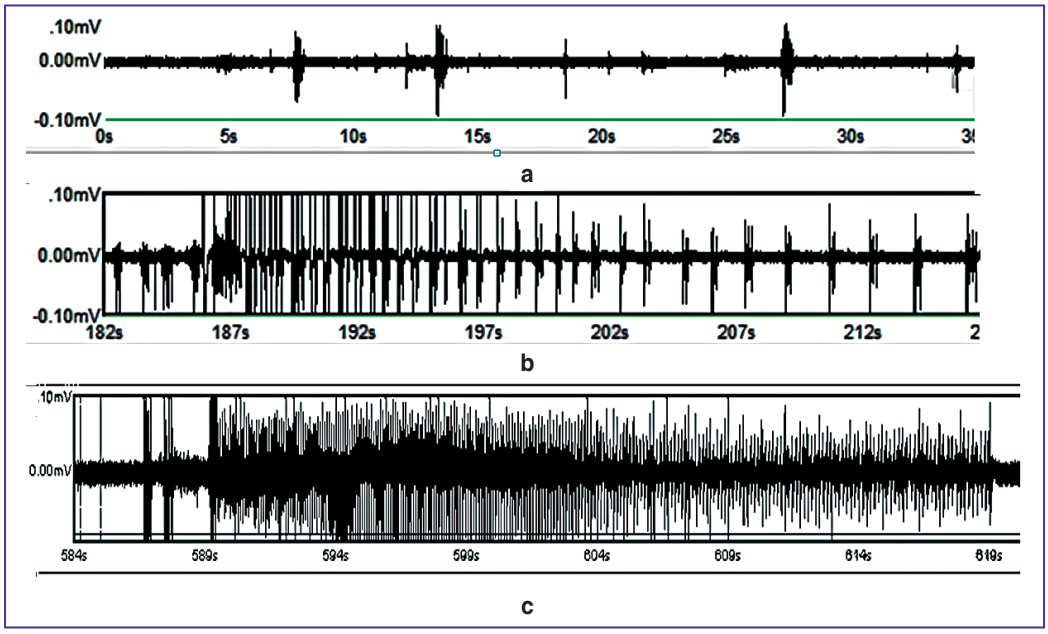

Fig. 3. Bioelectricity of hippocampal dissociated cultures: *a* — spontaneous bioelectricity; *b* — 4-aminopyridine induced activity; *c* — activity induced by hyaluronidase, 75 U/ml

p=0.461), as well as against the background of CNQX antagonist of AMPA-receptors (Fig. 4, *c*, n=3; p=0.350), but the activity did not develop in NMDA-receptors block by CPP competitive antagonist (Fig. 4, *e*, n=3; p=0.001). At the same time spontaneous activity induced by hyaluronidase was not blocked by CPP competitive antagonist of NMDA-receptors (Fig. 4, *f*, n=3; p=0.809), but was blocked by CNQX antagonist of AMPA-receptors (Fig. 4, *d*, n=3; p=0.004) and by L-VGCC blocker — diltiazem (Fig. 4, *b*, n=3; p=0.011).

Thus, hyaluronidase (75 U/ml) added on the 17th day in vitro into hippocampal primary culture caused delayed changes of spontaneous bioelectricity of neuron networks with the formation of superburst, with duration of 25–35 s, that can be characterized as seizure-like activity. The superbursts appeared 3–7 days after hyaluronidase had been added.

The mechanism of seizure-like activity formation after hyaluronidase differed from that of 4-AP-induced activity by different contribution of glutamate receptors. Seizure-like activity caused by hyaluronidase was not arrested in NMDA-receptors block, but depended on AMPA-receptors activity. By contrast to this, to maintain 4-AP-induced activity, it was necessary to have open NMDA-receptors, moreover, depolarization facilitating and initiating the work of these receptors formed at the cost of the decrease of hyperpolarizing effect of potassium channels when they are blocked by 4-AP.

Moreover, it should be noted that delayed hyaluronidase-induced seizure-like activity disappeared when calcium potential dependent channels were blocked by diltiazem. It substantiates the conclusion of the considerable contribution to the maintenance of seizure-like activity of calcium ions after enzymic breakdown of hyaluronic acid.The data received by G.Kochlamazashvili and coauthors [4] studying the role of hyaluronic acid in formation of long-term potentials of pyramidal neurons of hippocampal CA1-field proved hyaluronic acid removal by hyaluronidase within 1 h to lead

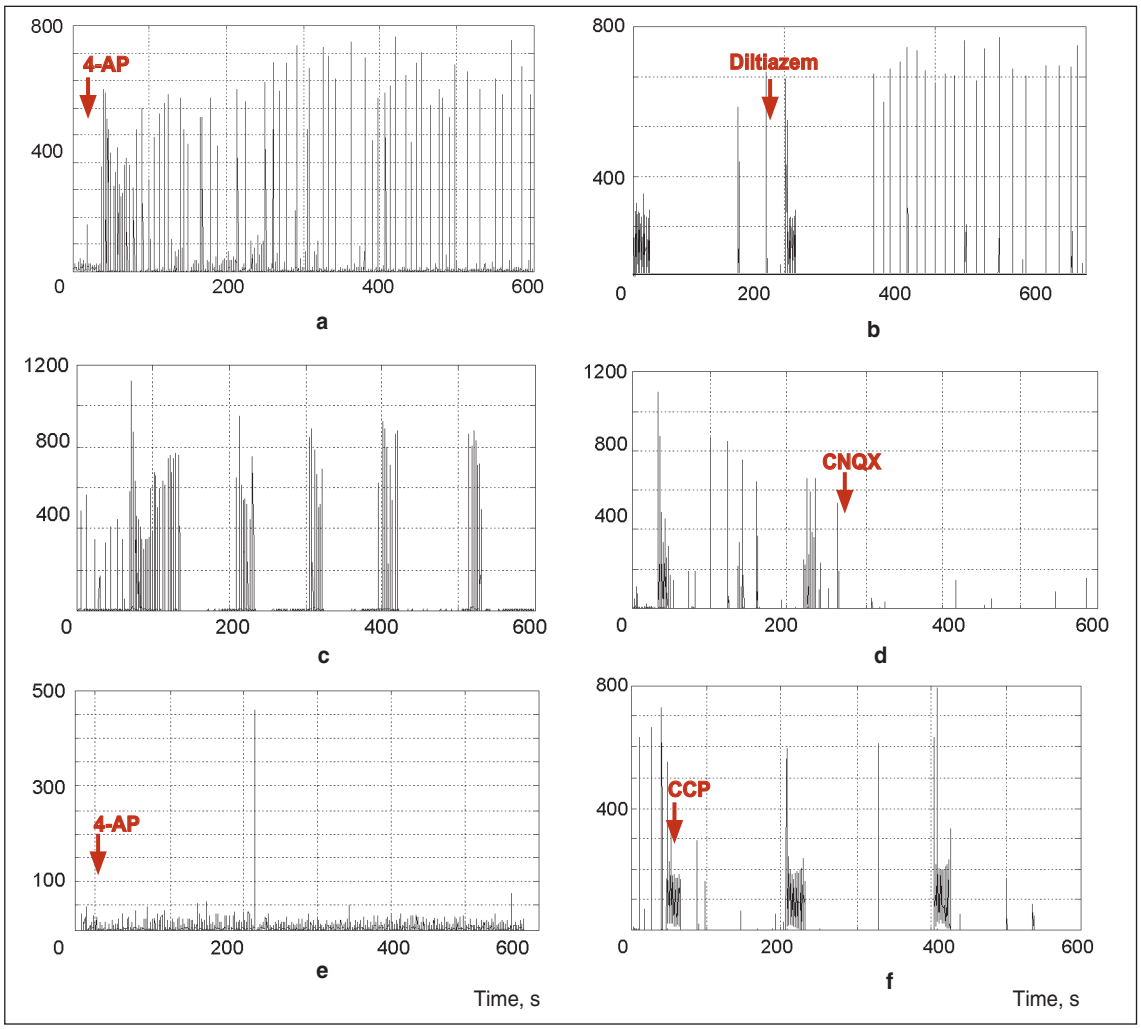

Fig. 4. Time-frequency graph of bioelectricity of hippocampal dissociated cultures (the number of spikes within 50 ms): 4-aminopyridine induced activity against the background of diltiazem, 20 micromol (*a*); against the background of CNQX, 10 micromol (*c*), and against the background of CPP, 10 micromol (*e*); effect on spontaneous hyaluronidase-induced activity of diltiazem, 20 micromol (*b*); against the background of CNQX, 10 micromol (*d*) and CPP, 10 micromol (*f*). In *a*, *c*, *e* cases, application of diltiazem, CNQX and CPP was carried out 10 minutes before 4-AP application; in *b*, *d*, *f* cases, antagonists were added against the background of spontaneous hyaluronidase-induced activity

to the decrease of operating effect of postsynaptic potential dependent L-type calcium channels (L-VGCC) damaging the formation of long-term potentials in hippocampal pyramidal cells. There was specific sensitivity of $Ca_v1.2$-containing channels to modulation by hyaluronic acid. In contrast to these researches, in our study we estimate hyaluronidase long-term effect on network level where spontaneous activity was maintained at the cost of interneurons net being a part of a general neuron net in culture of hippocampal dissociated cells. The lack of activity of $Ca_v1.2$-containing channels due to hyaluronic acid deficiency within some period of time (3–7 days) after its enzymic breakdown could lead to the reduction of inhibition process contribution to neuron net and, therefore, to epileptogenesis.

The study of molecular mechanisms underlying the seizure-like discharges, at net level, using modern neurophysiological techniques will lead to the development of selective and individually oriented antiepileptic drugs, as well as the development of new anti-eliptogenic strategies aim at epileptogenesis prevention.

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