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Antihypoxic and Neuroprotective Effects of Glial Cell-Derived Neurotrophic Factor (GDNF) in Cultures of Dissociated Hippocampal Cells under Conditions of Experimental Hypoxia

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We analyzed the effect of glial cell derived neurotrophic factor (GDNF) on changes in functional bioelectric and calcium activity in dissociated hippocampal cell cultures under conditions of modeled acute normobaric hypoxia *in vitro*. GDNF (1 ng/ml) partially neutralized the negative effects of hypoxia on cell survival and parameters of functional network activity. GDNF exhibited a pronounced anti-hypoxic effect.

Key Words: *glial cell derived neurotrophic factor (GDNF); dissociated hippocampal cell cultures; hypoxia; neuroprotection*

According to modern views, neural network (NN) is a minimum functional unit of the nervous system. Memory consolidation processes, information processing and transmission occur at the level of NN [11,20,22]. Analysis of structural and functional integrity of NN in the brain under the action of stress factors is a key problem of modern neurobiology and medicine. Functioning of individual NN cannot be studied in the whole brain due to its complex structure, diverse functions, and impossibility of studying NN elements by non-invasive methods. Hippocampal NN are characterized by genetically determined locality and peculiar cell composition; this, together with relative simplicity of the structure of the hippocampus makes primary hippocampal cultures an optimal models for the study of brain NN [2,7]. Analysis of functional activity of local NN *in vitro* has became methodically possible only with introduction of innovative methods of long-term noninvasive intravital detection of neuronal network signal. The use of multielectrode systems for recording of extracellular neuronal action potentials is considered the most promising approach to studying of NN function [6]. Another promising approach for evaluation of NN activity and mechanisms of their function is the study of ionic currents in neuronal and glial cells. Functional calcium activity of neurons differs from metabolic activity and primarily characterizes changes in intracellular calcium concentration related to ligand-dependent activation of postsynaptic channels. Optical fluorescent calcium imaging is currently regarded as the most informative method of studying spatial distribution and changes in calcium concentration in cells [3,23].

Hypoxia is a stress factor that primarily affects viability and homeostasis of neurons, because these cells are highly sensitive to oxygen deprivation. Hypoxia is a key factor of ischemic injury to brain cell. The decrease in oxygen concentration in the intercellular space triggers a cascade of intracellular events associated with uncoupling of oxidative phosphorylation on mitochondrial membranes and activation of free radical processes. Oxygen supply disturbances induce significant changes in synaptic transmission leading to catastrophic metabolism rearrangement, cell death, and destruction of brain NN [9]. Glial cell-derived neurotrophic factor (GDNF) is one of the main fac-

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tors released by glial cells and maintaining viability of neurons under stress conditions. Despite high concentration of this protein in biological fluids, biological targets, mechanisms of action, and effects of this cell–cell signaling factor are poorly studied.

Here we studied the antihypoxic and neuroprotective properties of GDNF.

MATERIALS AND METHODS

Dissociated hippocampal cell culture. Cultures of dissociated hippocampal cells were isolated from 18-day embryos of C57Bl/6 mice.The animal were maintained in accordance with Standards of Laboratory Practice in the Russian Federation (Order No. 267 of the Ministry of Health Care of the Russian Federation, June 19, 2003). The study was approved by the Ethics Committee of Nizhny Novgorod State Medical Academy. The hipocampal cells were dissociated by enzymatic digestion with 0.25% trypsin (Invitrogen), resuspended in Neurobasal medium containing bioactive supplement B27, glutamine (all components were from Invitrogen), and fetal calf serum (PanEco), and cultured on coverslips (18×18 mm) or multielectrode arrays (Alpha Med Science) as described previously [6]. The initial cell density was 9000 cells/mm^2. Viability of cultures was maintained in a CO_2 incubator (MCO-18AIC Sanyo) at 35.5°C and 5% CO_2.

Hypoxia modeling. On day 14 of culturing, the medium was replaced with a medium with low O_2 content for 10 min. Oxygen was displaced from the medium via saturation of the culture medium with an inert gas. The experiment was performed in a sealed chamber in which the air was replaced with an inert gas. GDNF was added to the culture medium 20 min before hypoxia modeling. The parameters characterizing the response of primary hippocampal cultures to hypoxia were recorded in 2 h and then every 24 h over 7 days after hypoxia [1].

Evaluation of cell viability in dissociated hippocampal cultures. Cell viability was assessed on days 1, 3, and 7 after hypoxia modeling. To this end, cell nuclei stained with PI (dead cells) and nuclei stained with bisbenzimide (all cells culture) were counted (Sigma). The number of live cells was calculated as percentage ratio between bisbenzimide- and PI-positive cells [1].

Recording and analysis of bioelectrical activity. Changes in spontaneous bioelectrical activity were analyzed using MED64 multielectrode arrays and supplied Conductor software (Alpha Med Science). Extracellular action potentials were recorded on day 14 of culture growth *in vitro* before hypoxic exposure and over 7 days after it. The following two parameters were evaluated: the mean number of small bursts and the mean number of spikes (extracellular action potentials) per small burst. The criterion of small bursts was the presence of spikes on at least 4 different electrodes in the array with interspike intervals no exceeding 100 msec. The data were processed using MEAMAN original algorithm package developed in the MATLAB environment (State Registration Certificate for Computer Program RF No. 2012611190).

Functional calcium imaging. Functional calcium activity of NN cells was analyzed by using calcium imaging that allows recording instant changes in the content of intracellular calcium by using an LSM 510 laser scanning microscope (Carl Zeiss). Functional calcium activity of nerve cells is spontaneous change in Ca^{2+} concentration in the cytoplasm primarily determined by ligand-dependent activation of postsynaptic ionic channels and leading to short-term elevation of calcium concentration in the neuronal cytoplasm that can be detected using ion-sensitive fluorescent probes [15]. In our experiments, specific calcium dye Oregon Green 488 BAPTA-1 AM (OGB1) was used as the fluorescent probe [5]. Time series of images of OGB1 fluorescence field (indicator of free intracellular calcium) were recorded. Changes in fluorescence intensity (arb. units) show the time dependence of intracellular calcium concentration and characterize functional activity of cells in NN. Calcium oscillations detected by fluorescent confocal microscopy were analyzed using original Astroscanner software (State Registration Certificate for Computer Program RF No. 2014662670). The function of mean OGB1 fluorescence intensity in the selected field of view on time F(t) was analyzed. The following parameters were analyzed: duration (sec), frequency (number of calcium events/min), and the percent of working cells in the culture [4,3,13].

Statistical processing of the results. The data are presented as the mean (M)±standard error of the mean (SEM). Significance of differences between the groups was evaluated by ANOVA using SigmaPlot 11.0 software (Systat Software Inc.). The differences were significant at $p<0.05$.

RESULTS

Hypoxia significantly ($p<0.05$) increased the number of dead cells in the culture on day 1 after hypoxic exposure. Preventive application of GDNF considerably ($p<0.05$) reduced the number of dead cells in the culture throughout the experiment. No significant differences in cell viability after application of different concentrations of neurotrophin (0.1, 1.0, and 10 ng/ml) were revealed. However, after application of GDNF in a concentration of 10 ng/ml, significant differences from hypoxia group were found only on

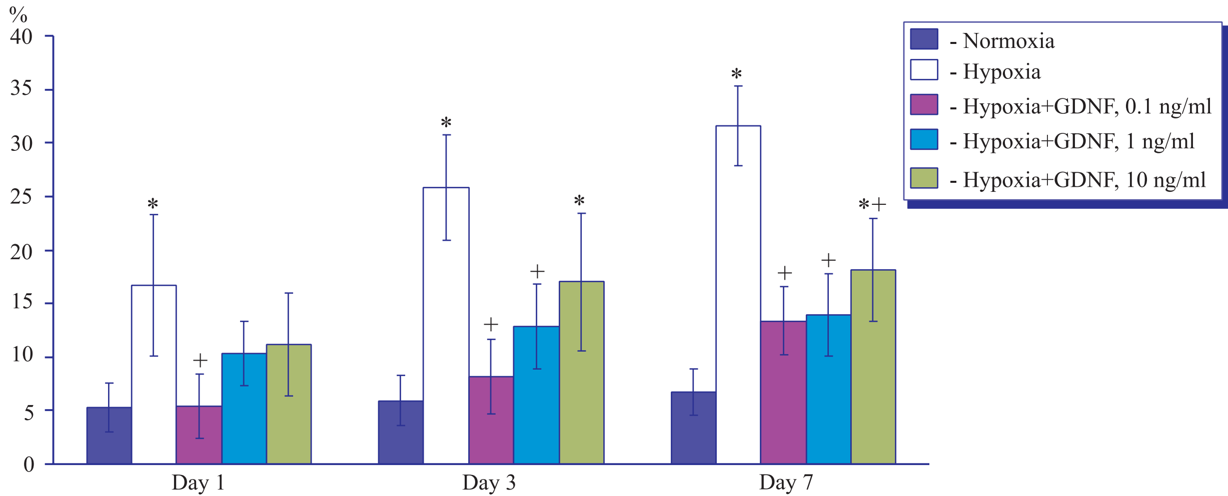

Fig. 1. Number of dead (PI+) cells in dissociated hippocampal cell cultures on days 1, 3, and 7 after hypoxia modeling. $p<0.05$ in comparison with *control, +hypoxia.

day 7 of the posthypoxic period. Thus, the maximum concentration (10 ng/ml) is not optimal and in further experiments, we used GDNF in a concentration of 1 ng/ml (Fig. 1). The number of dead (PI^+) cells in the experimental group was significantly ($p<0.05$) lower (by 2.5 times) than in the hypoxia group: 13.9% from the total number of cells. The number of dead cells in cultures not exposed to hypoxia little varied throughout during the experiment (Fig. 1).

The decrease in the number of viable cells during the posthypoxic period led to changes in spontaneous bioelectric activity. In 2 h after hypoxia modeling, we observed an irreversible decrease in the number of bursts (3.00±2.67 *vs*. 38.2±7.8 bursts 10 min prior to hypoxia, the mean number of spikes in the burst 508±232). Further observations showed that the number of bursts in spontaneous NN activity continued to decrease until day 7 after hypoxia and reached 1.52±0.75 bursts per 10 min (64.5% cultures demonstrated no spontaneous electrical activity on day 7 of the posthypoxic period).

Preventive application of GDNF (1 ng/ml) preserved spontaneous activity during the posthypoxic period (Fig. 2). Only on day 3 after normobaric hypoxia modeling, the number of network bursts significantly ($p<0.05$) decreased in comparison with both baseline and normoxia group; in parallel, the number of spikes in the burst increased. On day 7 after hypoxic exposure in the presence of GDNF (1 ng/ml), we observed recovery of the main parameters of spontaneous bio-

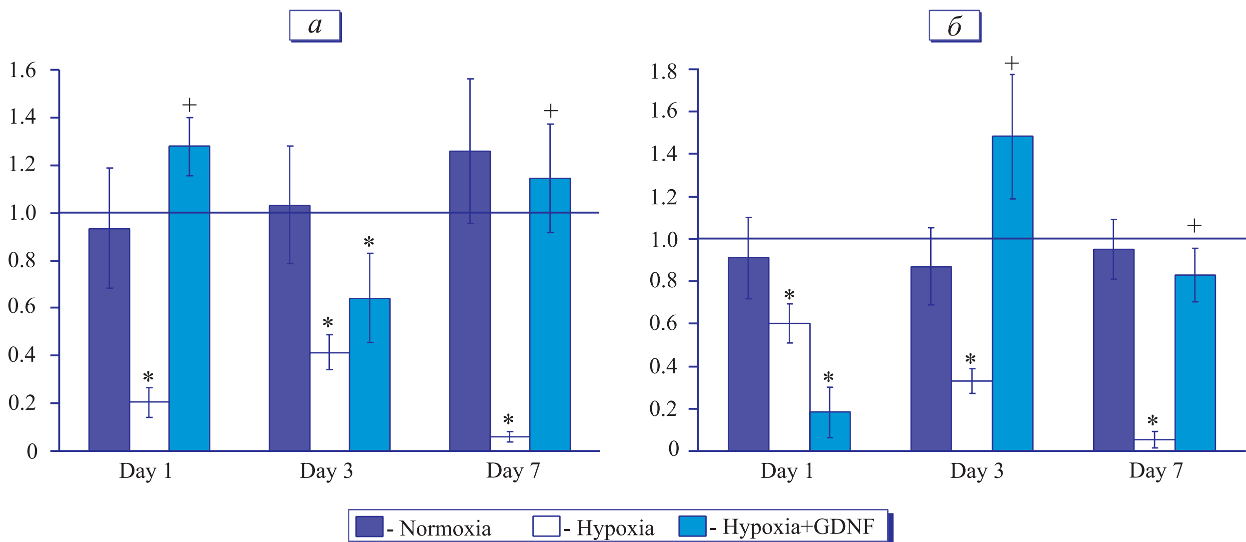

Fig. 2. Effect of GDNF (1 ng/ml) on changes in parameters of spontaneous bioelectrical activity of NN in the primary hippocampal cell culture on days 1, 3 and 7 after hypoxia modeling. *a*) Number of small bursts; *b*) number of spikes per burst. The data are standardized relative to the initial level taken as 1. $p<0.05$ in comparison with *initial level, +hypoxia.

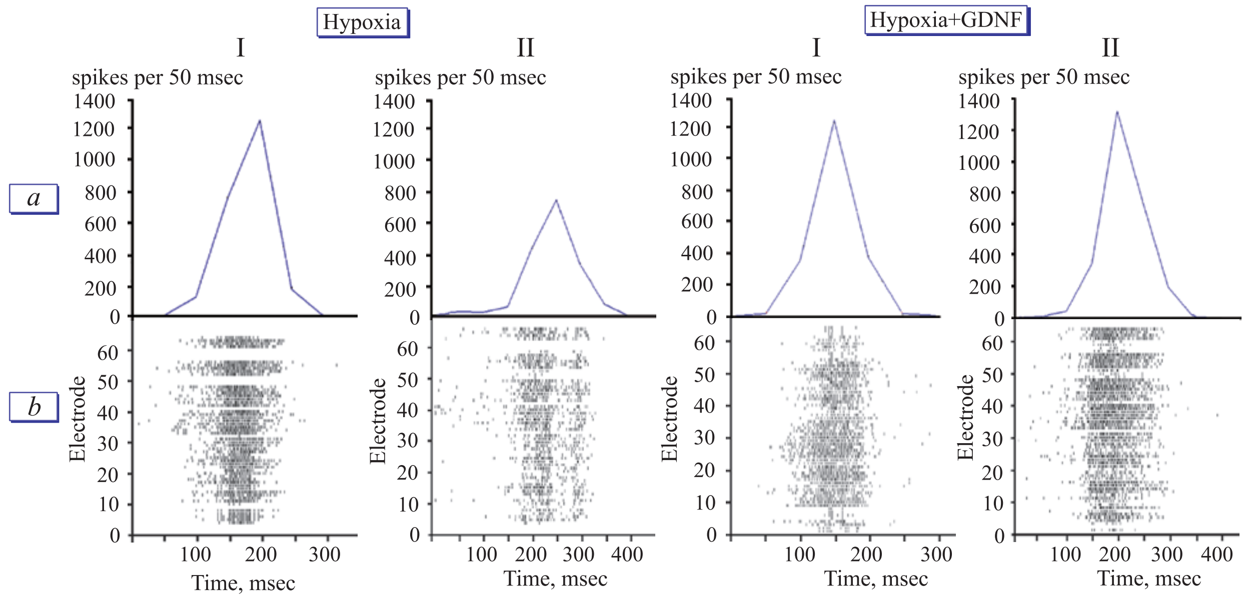

Fig. 3. Number of spikes per 50 msec (*a*) and raster plots (*b*) of neuronal network bursts in dissociated hippocampal cell culture. *I*: initial, *II*: in 7 days after hypoxic exposure.

electric network activity (number of network bursts and mean number of spikes in the burst).

Analysis of the network burst structure showed that in normoxia, the number of extracellular action potentials over 50 msec significantly ($p<0.05$) decreased relative to baseline by posthypoxic day 7. After preventive application of GDNF (1 ng/ml), network burst structure remained unchanged. The number of

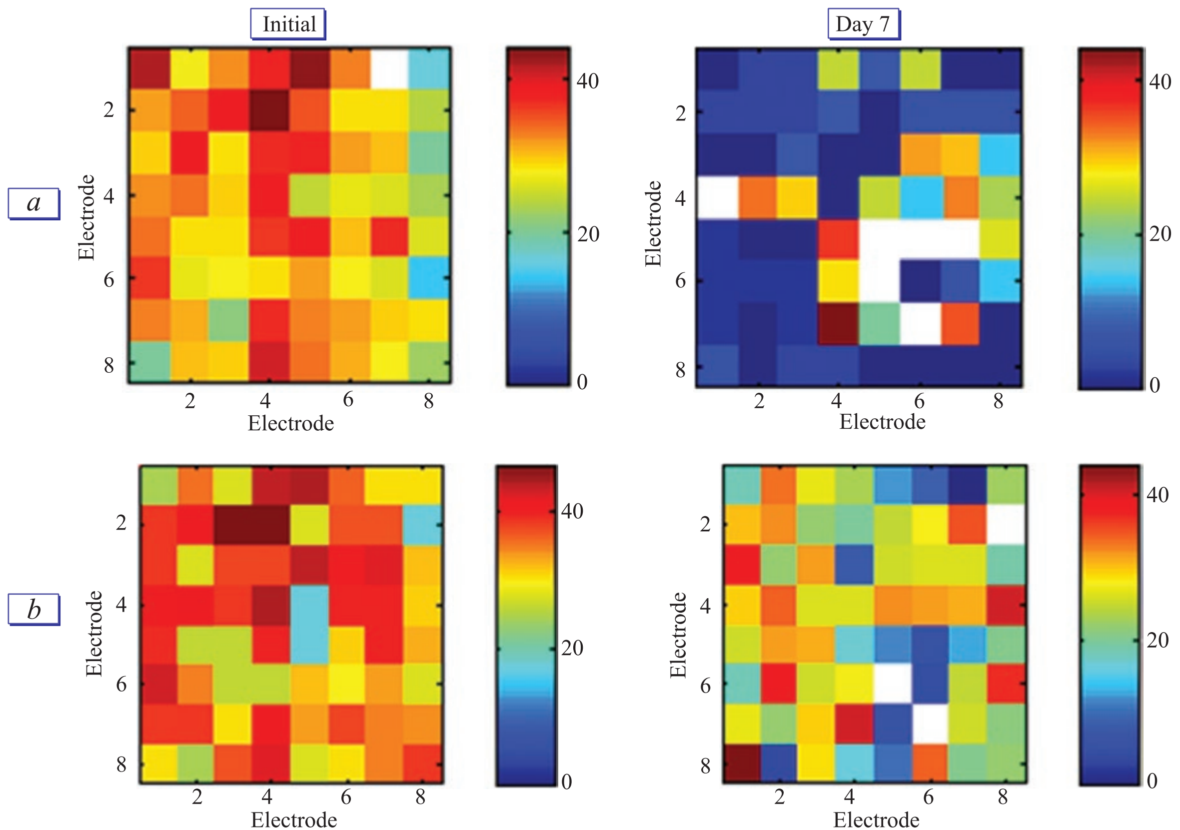

Fig. 4. Activation pattern of spontaneous bioelectrical activity of dissociated hippocampal cell cultures. Mean first spike timing in the burst. Colour chart: first spike timing in small bursts recorded on different electrodes (msec). *a*) Hypoxia, *b*) hypoxia+GDNF.

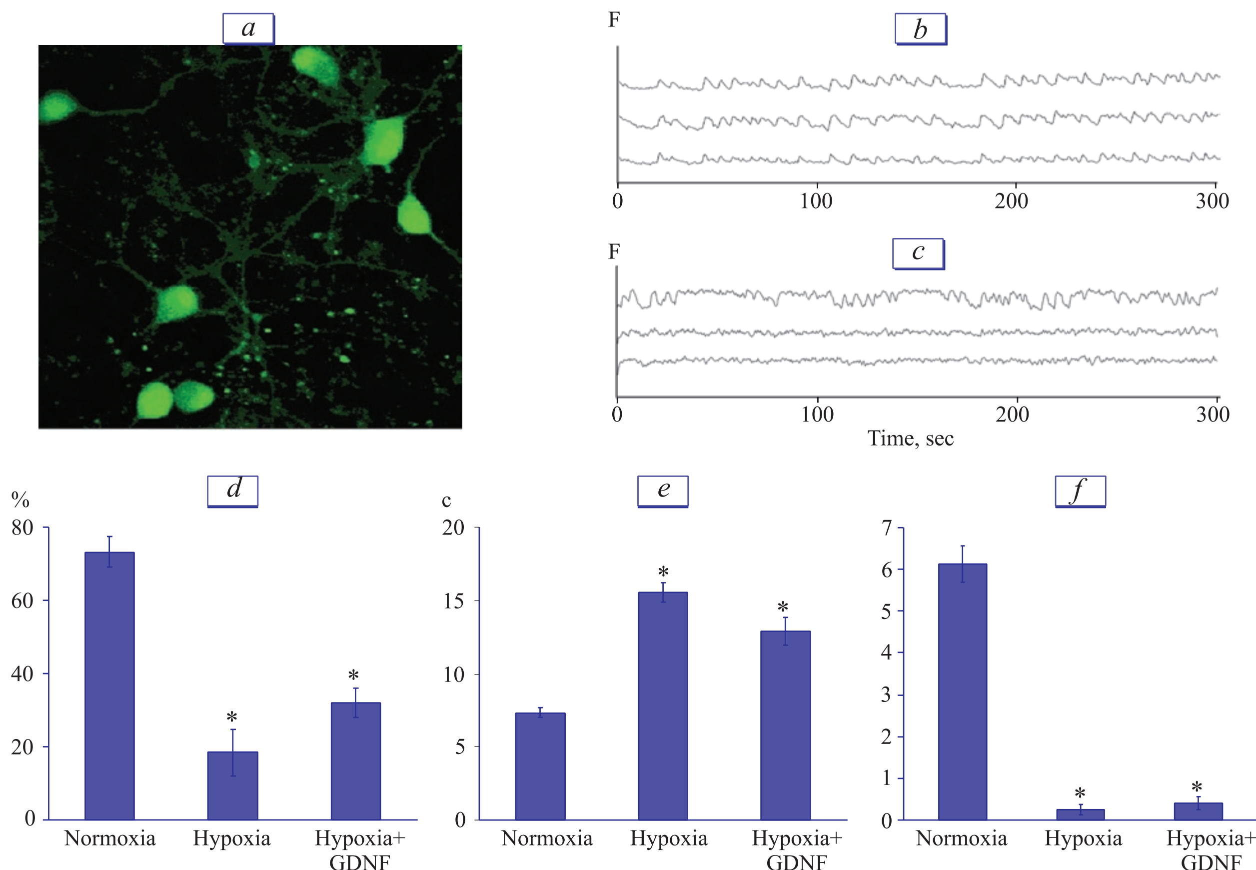

Fig. 5. Effect of GDNF (1 ng/ml) on changes in parameters of spontaneous calcium activity in the primary hippocampal cell culture on days 7 after modeling of acute normobaric hypoxia. *a*) Dissociated cells labeled with fluorescent dye OGB1; *b*, *c*) typical calcium activity profiles in cells of dissociated hippocampal cultures under normoxic conditions (*b*) and in 7 days after hypoxic exposure (*c*); F: fluorescence intensity, arb. units; *d*) percentage of working cells in culture; *e*) duration of calcium oscillations; *f*) frequency of calcium oscillations (per minute). *$p<0.05$ in comparison with normoxia.

spikes over 50 msec and raster plots of spontaneous bioelectrical activity of dissociated cultures remained practically unchanged relative to the initial level of activity (Fig. 3). However, raster diagrams do not allow quantitative comparison of functional characteristics of the network burst. For detailed analysis of functional structure of network bursts we examined activation patterns (the first spike timing in the network burst). We have previously showed [17] that the time of appearance of the first spike in the network burst is less variable than the sequence of extracellular potentials in the structure of the network burst. Thus, this parameter allows quantitative evaluation of the individual structure of the network burst. Therefore, we analyzed activation patterns, first spike timing in small bursts. For each burst, a 64-mer activation pattern was determined, *i.e.* first spike timing on each electrode starting from T_x (beginning of the burst). Acute normobaric hypoxia induced restructuring of the neuronal network activity (Fig. 4). After preventive application of GDNF (1 ng/ml), changes in the activation pattern were recorded. However, these changes were not related to destruction of the functional network activity of cultures. On day 7 after hypoxia modeling, network burst structure remained stable. In the majority of cultures (68%) preincubated with GDNF, network activity was only slightly changed.

Initially, single calcium oscillations with a frequency of 6.83±1.78 per minute and duration of 3.7±0.2 sec were seen in dissociated hippocampal cultures. On day 7 after hypoxia modeling, the duration of calcium oscillations increased to 29.19±5.04 sec, while the number of cells exhibiting calcium activity decreased by more than 3 times (Fig. 5). Preventive administration of GDNF only partially preserved functional parameters of network calcium activity. The percentage of working cells was significantly ($p<0.05$) higher; duration and frequency of calcium oscillations did not differ from those in hypoxia.

Thus, modeled normobaric hypoxia induced irreversible changes in NN structure in the primary culture of hippocampal cells determined by death of functionally active neurons. Changes in spontaneous calcium activity during the posthypoxic period can be associ-

ated with increased intracellular calcium concentration mediated by excitotoxicity during reoxygenation. Against the background of increased duration and reduced frequency of oscillations, the number of cells exhibiting spontaneous calcium activity significantly decreased (Fig. 5). In 76.2% cultures exposed to hypoxia, spontaneous calcium activity was absent and in the rest 23.8% cells, spontaneous activity significantly differed from the control, the percentage of cells exhibiting spontaneous calcium activity significantly ($p<0.05$) decreased.

In our experiments, GDNF exhibited a pronounced anti-hypoxic effect. Preventive application of GDNF maintained viability and function of NN activity in cultures of dissociated hippocampal cells. However, mechanisms of protective effect of GDNF remain open. According to some reports, the effect of GDNF is mediated by activation of universal multicomponent receptor GFR1 (glycosyl-phosphatidylinositol-linked GDNF receptor family) [14,16]. Since this receptor has an intracellular domain, it acts as a signal transmitter to other proteins, *e.g.* receptor with tyrosine kinase activity Ret, that in turn activate several intracellular signaling cascades: RAS/MAPK, PI3K/Akt, phospholipase C-γ [8,19]. Possible mechanisms protecting the nervous system cells from hypoxic damage can be associated with activation of the RAS/MAPK, PI3K/Akt signaling pathways. It has been previously demonstrated that these intracellular signaling pathways improved survival of different neuronal populations exposed to damaging factors [10,12,19]. Moreover, GDNF acts not only at the site of its synthesis, but also remotely. Neurotrophin absorbed by the soma and proximal dendrites of neurons via retrograde transport is delivered into the soma and afferent synapses [18,21,23]. Thus, due to complexity and versatility of GDNF-associated signaling systems, this unique signaling neurotrophin not only provides viability of individual neurons, but also integrate metabolic reactions of individual neuron-glial network components into a functional structure.

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