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Role of Neurotrophic Factors BDNF and GDNF in Nervous System Adaptation to the Influence of Ischemic Factors

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We developed a complex *in vitro* model of ischemic damage. Analysis of hippocampal cell viability in primary cultures after modeling of various stress factors revealed the features of action of the main pathological factors of ischemia. Neurotrophic factors BDNF and GDNF produced pronounced neuroprotective effect during modeling both the complex ischemic damage and its individual pathophysiological components. Neurotrophic factor GDNF produced the most pronounced protective effect.

Key Words: *ischemia; brain-derived neurotrophic factor; glial cell line-derived neurotrophic factor; primary nerve cell cultures; neuroprotection*

Ischemia is a complex multifactorial state and the key elements are oxygen and glucose starvation [5,7,12,14]. Ischemia increases the intensity of LPO and leads to oxidative stress. Free radical oxidation is known as a common component in many pathophysiological processes, including cerebral pathology, cardiovascular diseases, and cancer [2,3,10,17,19,21]. Studies of individual pathogenetic factors of ischemia can provide better understanding of the molecular mechanisms and metabolic reactions that occur during oxidative stress and oxygen and glucose deprivation. During the last decades, ample experimental data were accumulated about physiological and molecular mechanisms underlying the development of ischemic processes in the CNS. However, the described regularities cannot explain individual peculiarities of the resistance of a particular organism and its nervous system to ischemia and, consequently, individual adaptation capacities. Therefore, evaluation of the role of various endogenous regulatory proteins in leveling of post-ischemic CNS damage and disturbances in brain functioning is a promising trend. Among great diversity of endogenous regulatory molecules, brain-derived neurotrophic factor (BDNF) and glial cell line-derived neurotrophic factor (GDNF) are of particular interest. The role of these proteins in brain protection during the development of neurodegenerative diseases [9,15,18,20], and the possibility of using these proteins in the therapy of brain and spinal cord injuries [11,16,24,25] have been demonstrated.

Here we studied the neuroprotective effect of neurotrophic factors BDNF and GDNF in modeled ischemic damage *in vitro*.

MATERIALS AND METHODS

In vitro experiments were performed on primary hippocampal cell cultures obtained from C57Bl/6 mouse embryos (gestation day 18). The primary cultures were prepared according to the previously developed protocol [23] by mechanistic and enzymatic (0.25% trypsin solution) dissociation of the hippocampal tissue. The dissociated cells were seeded on coverslips (18×18 mm) at initial density ≥9000 cells/mm^2 and cultured for 21 days in Neurobasal Medium (Thermo Fisher) in a CO_2 incubator (35.5°C 5% CO_2). These conditions allow long-term culturing of primary cultures (1 month and longer). Primary morphological assessment of the state and development of dissociated cultures was carried out using wide-field light microscopy under an DMIL HC inverted fluorescent microscope (Leica). Previous studies have demonstrated that the

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cultures obtained by this protocol contain both neurons and astrocytes. Their proportion and spatial organization changes during culture growth and stabilizes by day 14 of *in vitro* development. From day 14 through 21, glial cells predominate in cultures, while neurons formed loose uniform monolayer [8]. Electron microscopy showed that mature asymmetric axo-dendritic and axo-spinous synapses constitute the main population of synapses in hippocampal cultures at this stage of culturing [8], which determined our choice of terms for ischemia modeling.

Primary cultures of hippocampal cells were divided into groups (9 cultures in each group). Group 1 included intact cultures exposed to ischemic factors or test substances. In groups 2-5, acute 10-min hypoxia (group 2; hypoxia), glucose deprivation (group 3; GD), oxidative stress (group 3; OS), or full complex of ischemia factors to create ischemia-like conditions (group 5; ischemia) were modeled. In groups 6-8, recombinant BDNF (1 ng/ml; GF029), GDNF (1 ng/ml; GF030) (Merk), or a combination of these factors were added 20 min before acute hypoxia modeling, to hypoxic culture medium, and after medium replacement to normoxic one. In groups 9-11, recombinant BDNF (1 ng/ml), GDNF (1 ng/ml), or a combination of these factors were added 20 min before acute GD modeling, to culture medium containing no energy substrates, and after its replacement to complete medium. In groups 12-14, recombinant BDNF (1 ng/ml), GDNF (1 ng/ml), or a combination of these factors were added 20 min before OS modeling. In groups 15-17, recombinant BDNF (1 ng/ml), GDNF (1 ng/ml), or a combination of these factors were added 20 min before modeling of ischemia-like conditions, to hypoxic culture medium, and after its replacement to normal medium.

Exposure to ischemic factors (hypoxia, GD, OS, and their combination) was performed on day 14 of *in vitro* culture development according to the previously developed protocols [1,6,22]. Acute hypoxia was modeled by replacement of the normoxic culture medium with a medium with low oxygen content for 10 min. To this end, the medium was saturated with argon. The experiment was performed in a sealed chamber, where the air was replaced with argon. After 10-min treatment with argon, oxygen content decreased from 3.26 to 0.37 ml/liter. Oxygen concentration in the medium was measured by iodometric titration. For modeling GD, the culture medium was replaced with a special medium identical by its composition to Neurobasal Medium, but not containing nutrient substrates (glucose, lactate, and pyruvate) (specially manufactured by PanEco) for 1 h. Then, it was replaced normal medium. Chronic OS was modeled by adding glucose oxidase (5 ng/ml; Sigma) to the culture medium. Glucose oxidase catalyzes dehydration of glucose and hydrogen transfer to oxygen with the formation of hydrogen peroxide oxidation of glucose to gluconolactone (Fig. 1). Enzymatic destruction glucose leads to the formation of a reactive compound, namely hydrogen peroxide, and the reaction continues until medium has a sufficient amount of substrate (several hours and even days). The advantage of this method is that glucose oxidase produces systemic chronic effect and damages higher number of cells in comparison with single application of hydrogen peroxide that produces local short-term damaging effect [6]. Ischemia-like conditions were modeled by combination of all three damaging factors for 10 min. When modeling the effect of the complex of ischemic factors, the presence of glucose oxidase in glucose-free medium is justified, because even after complete replacement of the culture medium with a medium without energy substrates, residual concentration of glucose in the medium located in the intercellular space (0.56-0.76 mol/liter) maintains enzymatic reaction for several hours.

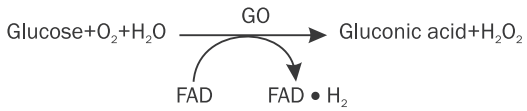

Fig. 1. Reaction catalyzed by glucose oxidase (GO) [6].

For evaluation of the damaging effect of modeled ischemic factors to the primary hippocampal cultures, cell viability was estimated by staining with fluorescent nuclear dyes PI (Sigma) and bis-benzimide (Sigma). The combination of these dyes allows evaluating the number of dead cells (PI^+ cells) and total number of cells (bis-benzimide$^+$ cells). Visualization was performed under a Leica DMIL HC inverted fluorescence microscope (Leica). In each culture, the cells were counted in 10 fields of view, the total number of cell nuclei in the field of view was 170-200. Cell viability index was calculated as the ratio of dead (PI^+) to total (bis-benzimide$^+$) cell count.

Significance of differences between the groups was evaluated by ANOVA using SigmaPlot 11.0 software (Systat Software, Inc). The obtained data are presented as the $M \pm SEM$. The differences between the groups were significant at $p<0.05$.

RESULTS

For evaluation of the role of neurotrophic factors in the realization of adaptive processes in the nervous system and their protective potential under conditions of ischemic damage, we studied the neuroprotective effects of BDNF, GDNF, and their combination on cell viability in primary dissociated hippocampal cultures exposed to individual factors of ischemic damage or

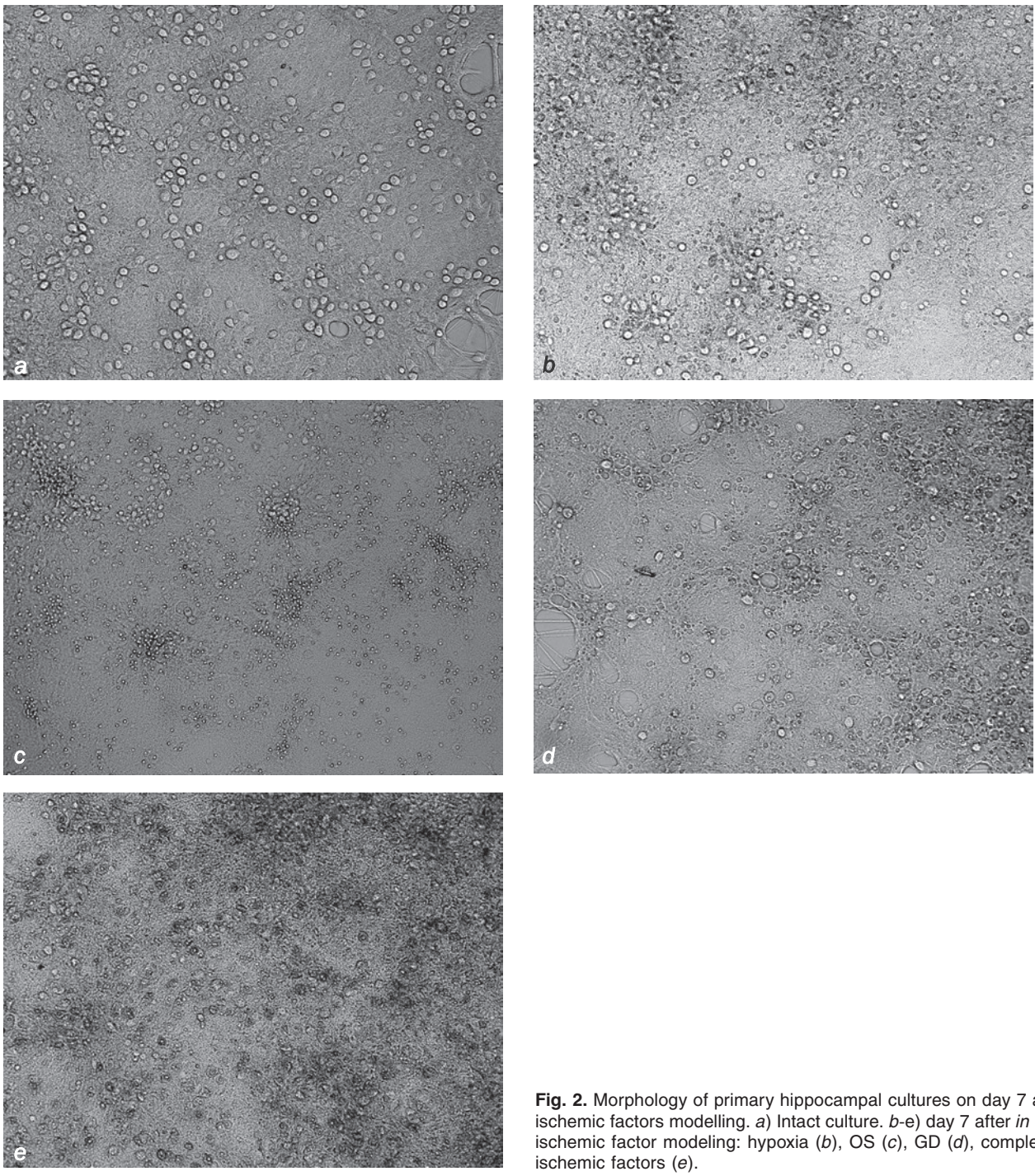

Fig. 2. Morphology of primary hippocampal cultures on day 7 after ischemic factors modelling. *a*) Intact culture. *b*-e) day 7 after *in vitro* ischemic factor modeling: hypoxia (*b*), OS (*c*), GD (*d*), complex of ischemic factors (*e*).

ischemia-like conditions. To this end, optimal parameters of individual ischemic factors (hypoxia, glucose deprivation, oxidative stress) were chosen on the basis of previous experiments [1,6,22] to create a model of complex ischemia *in vitro*. Complex ischemia modeling was performed by combined 10-min exposure to all three pathogenic factors.

Modeling of stress conditions was performed on day 14 in culture. During this period, the cultures are characterized by predominance of chemical synapses and spontaneous neural network activity [8]. Different stress-factors have different effects on viability of the primary cell cultures. Morphological changes caused by modeled factors were similar, but had different temporal patterns. The results of morphological analysis suggested that GD primarily induced necrotic processes in the culture, whereas hypoxia and OS lead to neuronal apoptosis associated with the formation of apoptotic bodies and their subsequent engulfment by astrocytes (Fig. 2).

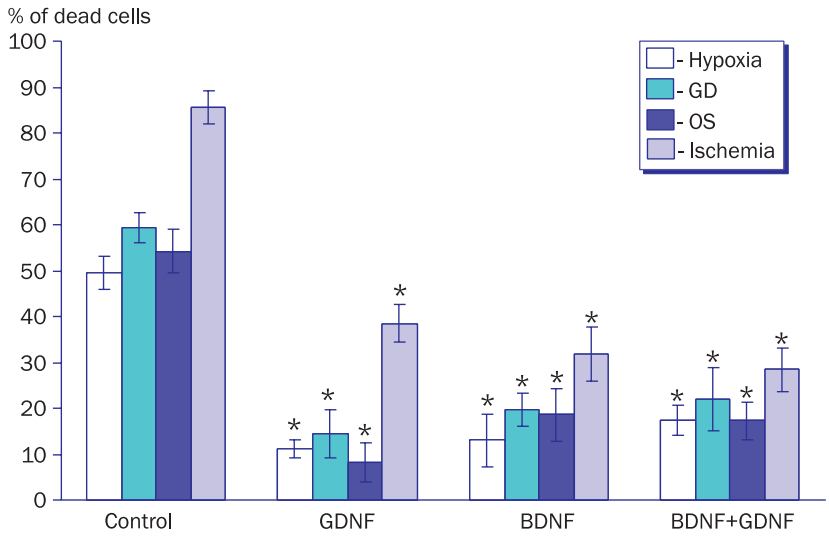

Fig. 3. Effect of preventive application of BDNF (1ng/ml), GDNF (1 ng/ml) and their combination on cell viability in primary hippocampal cultures on day 7 after in vitro ischemic factor modeling. *p<0.05 in comparison with the corresponding control group (hypoxia, GD, OS, or ischemia).

Analysis of viability in cell cultures after modeling of various stress factors revealed some features in the action of the main pathological factors of ischemia. In intact cultures (group 1), cell viability over 7 days after exposure did not significantly change: 4.95±0.83 and 6.32±2.26% on days 15 and 21, respectively. After hypoxia modeling (group 2), the percentage of dead cells sharply increased on the first day after exposure (24.72±1.99%); by day 3, this parameter little changed in comparison with the previous term, while by day 7, the relative content of dead cells significantly increased in comparison with that on day 1 and 3 (to 49.55±3.72%; p<0.05). Thus, days 1 and 7 of the post-hypoxic period are critical for neuronal cell survival. In contrast, in the GD and OS groups, a gradual cell death in the cultures was observed. The relative content of dead cells on day 1 was 15.93±2.12 and 12.95±1.18%, respectively; by day 3 their content increased by 1.75-3.3 times, and by day 7, their content increased to 59.36±1.94% (GD) and 54.26±5.61% (OS). In comparison with each examined ischemic factor, the negative effects of complex ischemic exposure were more pronounced (p<0.05): the content of dead cells was 45.08±3.41% on day 1, 61.24±4.65% on day 3, and 85.48±3.24% on day 7 (Table 1).

Despite all ischemic factors have a pronounced negative effect and significantly reduced cell viability in the primary hippocampal culture as soon as on day 1 after exposure (p<0.01), peculiarities of the effect of each factor and their combination suggest that they trigger different molecular mechanisms leading to cell death. The similarity of the effects caused by modeled hypoxia and OS can be explained by the fact that hypoxia inevitably activates free-radical processes. The decrease in oxygen concentration in the intercellular space leads to uncoupling of oxidative phosphorylation and activation of free radical processes [4,12]. Exhaustion of energy reserves in the cell leads to activation of necrosis; moreover, apoptosis is an energy-dependent process [12,13].

Comparative analysis of the effects of neurotrophic factors showed that BDNF, GDNF, and their combination significant decreased the percentage of dead cells in the primary cultures exposed to individual ischemic factors and to their combination.

It was shown that on day 1 after exposure to individual ischemic factors in the presence of neurotrophic factors, the content of dead cells in the experimental groups did not differ from that in intact cultures and was significantly lower than in the corresponding control groups (hypoxia, GD, and OS). This attests to neuroprotective effect of BDNF and GDNF on cells exposed to all pathophysiological factors of ischemic damage. It should be noted that in the groups ischemia+BDNF, ischemia+GDNF, and ischemia+BDNF+GDNF, cell viability on day 1 after treatment significantly differed from the intact group and the corresponding control groups: 14.57±2.85% in ischemia+BDNF, 18.29±4.39% ischemia+GDNF, and 12.56±4.66% in ischemia+BDNF+GDNF groups. These results were not surprising and showed that complex ischemia causes the most detrimental effect on the primary hippocampal cells.

Preventive application of GDNF (1 ng/ml) produced the most pronounced neuroprotective effect (Fig. 3). The maximum cytoprotective effect of GDNF was observed during oxidative stress. On day 7 after OS modeling, the percentage of dead cells in the

TABLE 1. Relative Content of Dead Cells in Primary Hippocampal Cultures after Exposure to Ischemic Factors (%; *M*±*SEM*)

Group	Day after exposure		
	1	3	7
Intact	4.95±0.83	5.65±1.16	22.82±2.05
Hypoxia	24.72±1.99*	28.78±3.14*	49.55±3.72*
GD	15.93±2.12*	26.04±3.13*	59.36±1.94*
OS	12.95±1.18*	38.39±2.09*	54.26±5.61*
Ischemia	45.08±3.1*	61.24±4.65*	85.48±3.24*
Hypoxia+BDNF (1 mg/ml)	6.78±2.7+	10.86±3.01+	12.98±4.8+
Hypoxia+GDNF (1 ng/ml)	7.07±1.39+	9.43±2.1+	11.12±3.21+
Hypoxia+BDNF+GDNF	7.93±1.34+	13.57±2.83*+	17.41±3.58*+
GD+BDNF (1 mg/ml)	5.72±2.66+	10.28±4.75+	19.78±4.39*+
GD+GDNF (1 ng/ml)	6.51±0.62+	8.56±0.48+	14.56±5.27+
GD+BDNF+GDNF	7.67±2.19+	13.88±2.53*+	22.06±4.12*+
OS+BDNF (1 mg/ml)	8.42±2.25+	16.29±4.46*+	18.54±5.73*+
OS+GDNF (1 ng/ml)	4.56±1.41+	5.84±0.61+	8.21±3.61+
OS+BDNF+GDNF	7.32±2.60+	17.48±2.94*+	17.23±5.88*+
Ischemia+BDNF (1 mg/ml)	14.57±2.85*+	22.44±4.36*+	31.77±4.24*+
Ischemia+GDNF (1 ng/ml)	18.29±4.39*+	26.95±4.14*+	38.51±6.83*+
Ischemia+BDNF+GDNF	12.56±4.66*+	21.38±2.87*+	28.48±4.73*+

Note. $p<0.05$ in comparison with *intact group, +corresponding control group (hypoxia, GD, OS, or ischemia).

GDNF+OS group did not differ from the level of intact cultures and was 8.21±3.61%, whereas cell viability in BDNF+OS and BDNF+GDNF+OS groups was significantly higher ($p<0.01$) and was 18.54±5.73% and 17.23±5.88% respectively.

The neuroprotective effect of BDNF (1 ng/ml) was more pronounced in cultures exposed to normobaric hypoxia and complex ischemia and was comparable with that of GDNF (12.98±4.8% in hypoxia+BDNF group, 11.12±3.21% in hypoxia+GDNF group, and 17.41±3.58% in hypoxia+BDNF+GDNF group on day 7 after exposure).

Individual and combined application of neurotrophic factors (BDNF, GDNF) exhibited a neuroprotective effect and prevented cell death in primary hippocampal cultures exposed to all ischemic factors. However, it should be noted that the effect of combined application of neurotrophic factors was less pronounced than their individual effects. After modeling hypoxia, GD, and OS in the BDNF+GDNF group, the number of dead cells on day 7 significantly increased in comparison with that in intact group ($p<0.05$) and sometimes in comparison with application of one neurotrophic factor (in OS and hypoxia relative to GDNF group). In modeled complex ischemia, the neuroprotective effect of combined application of the neurotrophic factors did not differ from their individual applications.

Thus, our experiments showed that exposure to different ischemic produced peculiar effects apparently associated with activation of different pathological molecular mechanisms leading to death of neurons and astrocytes in primary hippocampal cultures. Hypoxia is the main pathological factor in the modeled short-term ischemia (10 min) followed by reperfusion. This is confirmed by temporal characteristics of cell death in culture and the action of GDNF (the most potent antihypoxant agent). However, GD and OS produced their negative effects even within this short time interval leading to a significantly increase in the number of dead cells relative to the groups with individual effects of ischemic factors.

Neurotrophic factors BDNF and GDNF produced pronounced neuroprotective effect during modeling of complex ischemic damage and its individual pathophysiological components. Neurotrophic factor GDNF produced the most pronounced protective effect.

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