Neuroprotective Properties of Nootropic Dipeptide GVS-111 in *in Vitro* Oxygen-Glucose Deprivation, Glutamate Toxicity and Oxidative Stress

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Argon anoxia and glucose deprivation were used for modeling of ischemic damage in the cultures of cerebellar granule cells. Protective effect of peptide piracetam analogue GVS-111 was demonstrated. GVS-111 prevented neurodegeneration induced by glutamate and oxidative stress. In contrast to GVS-111, piracetam did not attenuate neurocytotoxic effect of glutamate.

Key words: *cerebellar granule cells; ischemia; GVS-111; piracetam; glutamate*

Brain ischemia is one of the most prevalent neurological diseases accompanied by severe disorders of cognitive, motor, verbal, and other functions of the central nervous system. Therefore, the search for new ways of pharmacological correction and preparations attenuating neurodegeneration during ischemia is an actual problem of modern neurology. Nootropics are widely used for improvement of impaired cognitive functions [6]. At the same time, the data on the ability of piracetam to prevent neurodegeneration during ischemia are contradictory. It was shown that piracetam and its analogues activate AMPA-subtype of glutamate receptors in cultured cerebellar cells [5], thus potentiating glutamate-induced effects. Hyperstimulation of glutamate receptors during ischemia is known to be the key pathogenic factor of neurodegeneration. Previous studies on the model of focal ischemia showed that new dipeptide piracetam analogue, GVS-111 (N-phenylacethyl-L-prolylglycine ester, patent USA No. 5,439,930) diminished lesion focus induced by photothrombosis in the cerebral cortex and restored impaired cognitive functions in rats [9]. It was also shown that GVS-111 prevents accumulation of diene conjugates and Schiff bases in the brain and serum of immobilized rats, which points to its antioxidant activity [7].

We examined protective effects of GVS-111 on *in vitro* model of ischemia (oxygen-glucose deprivation, OGD), during neurocytotoxic action of glutamate, and oxidative stress.

MATERIALS AND METHODS

The study was conducted on 7-8-day cultures of cerebellar granule cells obtained from 8-day-old Wistar rats as described elsewhere [3]. The cells were cultured on glass coverslips (22×22; Sigma) coated with poly-L-lysine (M.W. 150,000-300,000; Sigma) and placed in 40-mm plastic Petri dishes (St.-Petersburg Polymer Plant). The medium contained 10% fetal calf serum, 90% Eagle's minimum essential medium (Gibco), 0.8% glucose, 2 mM glutamine (Gibco), 0.2 U/ml insulin, and 10 mM HEPES. KCl was added to a final concentration of 25 mM after 24 h culturing.

For ischemia modeling, the cultures were washed with a modified Lock solution containing (in mM): 154 NaCl, 25 KCl, 2.3 $CaCl_2$, 3.6 $NaHCO_3$, 0.35 Na_2HPO_4, and 5 HEPES (pH 7.6), placed in an airtight plastic chamber with pure argon, and incubated for 1-1.5 h at

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36°C. Antagonist of NMDA receptors, D,L-2-amino-5-phosphonovalerate (200 μM), and GVS-111 (10^{-6} M) were added 30 min before the end of incubation in argon. Then the cultures were transferred into glucose-containing Lock solution (0.8%) and incubated for 2 h in a CO_2-incubator (95% air, 5% CO_2). Neurodegeneration (formation of pyknotic nuclei) was monitored under a phase contrast inverted microscope. Control cultures were incubated in glucose-containing Lock solution in a CO_2-incubator. After the end of the experiment, the cultures were fixed with alcohol-formaldehyde-acetic acid mixture (7:2:1), stained with vanadium hematoxylin [2], and pyknotic nuclei and intact granule cells (Fig. 1) were counted [1]. The ratio between the number of pyknotic nuclei and total cell number was regarded as the degree of degeneration.

For the study of neurotoxic action, the cultures were incubated in balanced saline (BS) containing (in mM): 135 NaCl, 5.0 KCl, 0.035 Na_2HPO_4, 12 $NaHCO_3$, 2.3 $CaCl_2$, and 11 glucose (pH 7.6-7.8) with 75 μM sodium glutamate (Serva) for 15 min at room temperature. GVS-111 (10^{-6} M) was added to the incubation medium 15 min before glutamate and was present in the medium throughout the entire postglutamate period. After washout the cultures were incubated in BS in a CO_2 incubator for 3-4 h until the formation of pyknotic nuclei, fixed, and analyzed quantitatively as described above.

Oxidative stress was modeled in the Fe^{2+}/ascorbate system. To this end, the cultures were washed and incubated in BS with 6 μM Fe_2SO_4 and 60 μM sodium ascorbate for 20-25 min. After washout, the cultures were incubated in the same solution in a CO_2-incubator for 2-3 h until the formation of pyknotic nuclei, fixed, and treated routinely. GVS-111 (10^{-6} M) was added to BS 15 min before Fe^{2+}/ascorbate treatment and left in the solution throughout the entire experiment. Control cultures were incubated in BS.

The results were processed statistically using Student's *t* test. In addition to the number of dead neurons, for each of the models coefficient of protection efficacy (CPE) was calculated by the formula:

$$CPE=(N_{DEG}-N_{GVS})/N_{DEG}\times 100\%,$$

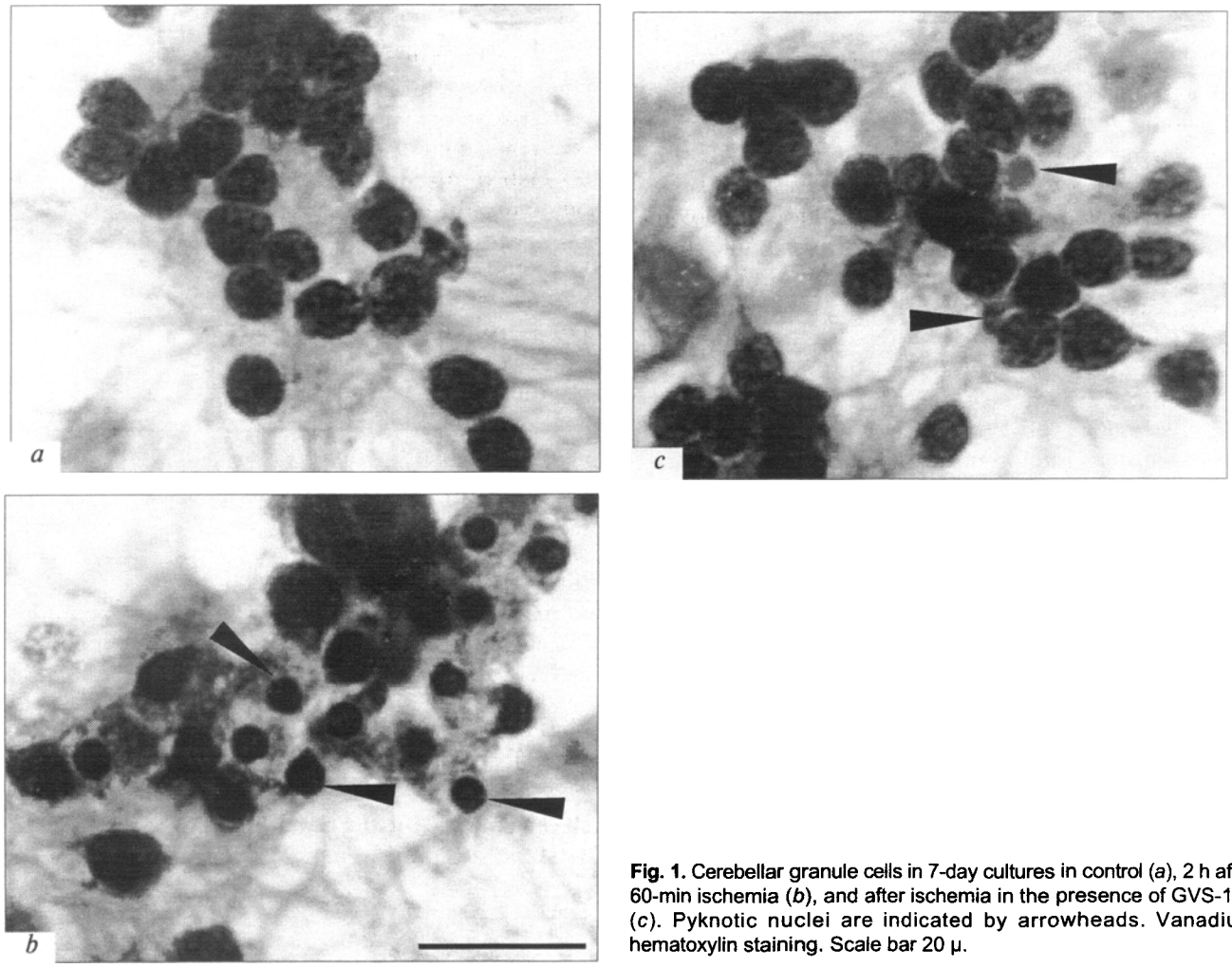

Fig. 1. Cerebellar granule cells in 7-day cultures in control (*a*), 2 h after 60-min ischemia (*b*), and after ischemia in the presence of GVS-111 (*c*). Pyknotic nuclei are indicated by arrowheads. Vanadium hematoxylin staining. Scale bar 20 μ.

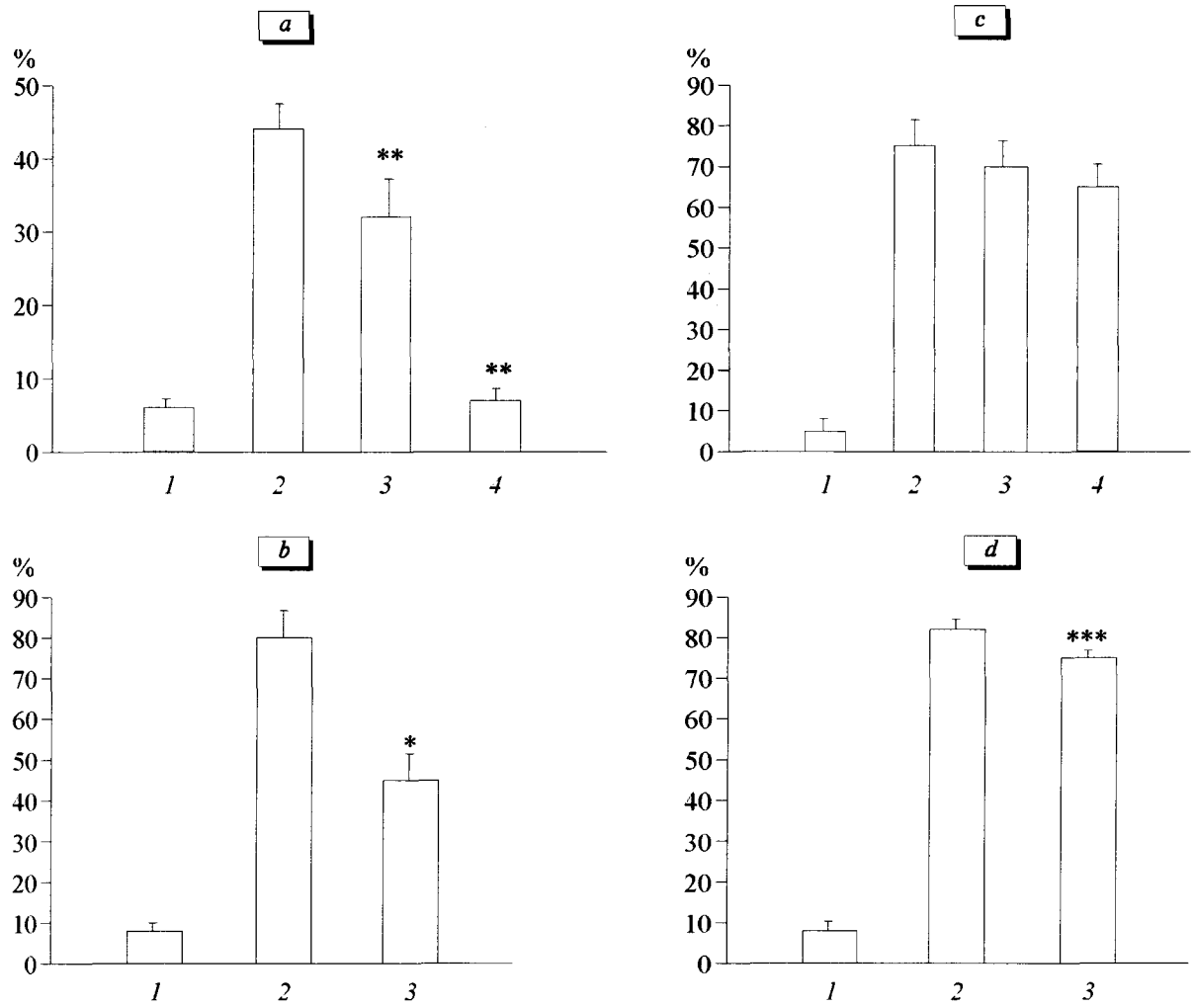

Fig. 2. Effects of GVS-111 and piracetam on the number of damaged neurons in cerebellar granule cell cultures under conditions of ischemia (*a*), neurocytotoxic exposure (glutamate 75 μM, *b, c*), and oxidative stress (Fe^{2+}/ascorbate, *d*). *1*) control; *2*) treatment; *3*) treatment in the presence of 1 μM GVS-111 (*a, b, d*) or 1 mM piracetam (*c*); *4*) treatment in the presence of 200 μM D,L-2-amino-5-phosphonovalerate (*a*) or 1 μM piracetam (*c*). *$p<0.001$, **$p<0.01$, ***$p<0.05$ compared to the experiment without correction.

where N_{DEG} and N_{GVS} are the numbers of dead neurons in cultures subjected to damaging treatment without or with GVS-11, respectively.

RESULTS

Incubation for 1.5 h under ischemic conditions induced death of 43.2±2.5% granule cells in cultures. GVS-111 decreased the number of damaged neurons by 12.3% (CPE=28%; Fig. 2, *a*), thus producing a pronounced protective effect during ischemic exposure.

In our experiments D,L-2-amino-5-phosphonovalerate completely inhibited granule cell damage induced by OGD: the number of pyknotic nuclei in these cultures did not differ from that in intact cultures (Fig. 2, *a*). This suggests that glutamate released from terminals of granule cells and activating NMDA-receptors is the main factor of neuronal damage in this model of ischemia.

Experiments with glutamate neurocytotoxicity showed that GVS-111 added to cultured cells during glutamate treatment and in the postglutamate period reduced the number of damaged cells by 36.6% (CPE= 46%; Fig. 2, *b*). Classical nootropic piracetam in equimolar (10^{-6} M) and higher (10^{-3} M) concentrations added according to the same scheme did not protect cerebellar neurons against glutamate toxicity (Fig. 2, *c*).

Ischemic neuronal damage is known to be associated with activation of lipid peroxidation (LPO) [10]. Our previous studies demonstrating that standard antioxidant α-tocopherol inhibits kainate neurotoxicity also confirm LPO activation associated with cytotoxic damage to neurons caused by excitatory amino acids [1]. In experiments with Fe^{2+}/ascorbate-induced oxidative stress, GVS-111 added during and after Fe^{2+}/ascorbate treatment moderately, but significantly decreased (by 8%) the number of damaged neurons (CPE=10%, Fig. 2, *d*).

Thus, nootropic dipeptide GVS-111 produced a pronounced protective effect on the original model of ischemic damage of cerebellar granule cells. This protective effect of GVS-111 depends on its antioxidant properties and its ability to inhibit the action of excitatory amino acids. The relatively weak protective effect of GVS-111 (CPE=10%) against oxidative stress is probably due to aggressive Fe^{2+}/ascorbate treatment causing death of more than 80% neurons.

GVS-111 proved to be more effective than piracetam, which showed no significant neuroprotective effect against glutamate-induced damage to cerebellar neurons. This can be explained by the ability of piracetam to potentiate the effect of excitatory amino acids shown in electrophysiological experiments [5,8], as well as by the absence of its antioxidant properties [4]. These data point to significant advantages of GVS-111 over piracetam and show the perspective of using this compound as a pharmacological corrector during brain ischemia.

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