DOI 10.1007/s10517-020-04734-8

GK-2 Reduces Death of Cultured Granule Neurons in Cerebellum Induced by the Toxic Effects of Zinc Ions

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Translated from *Byulleten' Eksperimental'noi Biologii i Meditsiny*, Vol. 168, No. 4, pp. 458-463, October, 2019
Original article submitted May 28, 2019

Peptide mimetic of nerve growth factor GK-2 in a dose of 1-2 mg/liter improves survival of cultured rat cerebellar granule neurons exposed to the cytotoxic effect of zinc ions, but has no protective effect against copper ion cytotoxicity. Experiments on cultured rat hippocampal slices demonstrated that GK-2 did not affect reactivity of pyramidal neurons and long-term potentiation in the hippocampal field CA1 and the probability of glutamate release from presynaptic terminals in the synapses of the CA3-CA1 fields. The results suggest that GK-2 does not affect the functional properties of synaptic transmission under normal conditions, but protects neurons from the toxic effects of zinc, which creates prerequisites for GK-12 use in the treatment of neurodegenerative diseases.

Key Words: *GK-2 nerve growth factor mimetic; neuroprotection; neurons; zinc ions; long-term potentiation*

Nerve growth factor (NGF) is involved in the growth and plastic rearrangements of central and peripheral neurons during differentiation, as well as in the maintenance of their vital functions. Experimental and clinical studies revealed changes in the content of NGF in the brain in different pathologies, but the therapeutic use of this growth factor is limited by not only poor penetration through the blood—brain barrier, but also the risk of immune reaction and side effects associated with its pleiotropy [9]. Therefore, a promising approach to the use of the positive properties of neurotrophic factors in the treatment of different CNS pathologies is construction and synthesis of low-molecular-weight mimetic growth factors. One of these low-molecular-weight mimetics of NGF, peptide GK-2, was synthesized based on the structure of β-turn of NGF loop 4 at the V. V. Zakusov Research Institute of Pharmacology and represents a dimeric N-acyl substituted dipeptide hexamethylene diamide bis-(N-monosuccinyl-L-glutamyl-L-lysine) [4]. The neuroprotective properties of GK-2 were demonstrated in both immortalized and primary neuronal cultures subjected to oxidative stress and glutamate toxicity as well as in ischemia and traumatic brain injury modeled *in vivo* [2,5,6].

Here we studied the protective effect of GK-2 on the model of zinc neurocytotoxicity and evaluated the effect of GK-2 on synaptic transmission and its plastic properties in rat hippocampal slices.

MATERIALS AND METHODS

All experimental protocols were approved by the Ethics Committee of the Research Center of Neurology (protocol No. 2-5/16).

We used 7-8-day-old cultures of granule neurons of the cerebellum from 7-day-old rats obtained by enzymatic and mechanical dissociation: incubation with trypsin (0.05%) and EDTA (0.02%) on phosphate buffer for 15 min at 36.5°C followed by stepwise pipetting in medium [6]. The cells were transferred to 96-well polylysine-precoated plastic plates (0.1 ml

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0007-4888/20/1684-0474

cell suspension per well; final seeding density 3-5× 10^3 cells/mm^2) and grown in nutrient medium containing 90% minimum Eagle's medium with Earl salts, 10% fetal calf serum, 2 mM glutamine, 25 mM KCl, and 10 mM HEPES buffer (pH 7.2-7.4) in a CO_2 incubator at 36.5°C and a relative humidity of 98%.

For survival experiments, granule neurons were cultured in Eagle's medium with Earl salts containing 10 mM HEPES buffer and 0.5% B27-AO supplement for serum-free media; $ZnCl_2$ or $CuCl_2$ (40-50 μM), and GK-2 (1-2 mg/liter) were added for 24 h. After incubation, the cultures were fixed with ethanol:formaldehyde:acetic acid (7:2:1) mixture and stained with trypan blue. The proportion of live neurons was estimated by counting morphologically intact granule neurons in 5 consecutive fields of view at ×40 and expressed relative to the control (100%). All data on cultured neurons were obtained on 9-12 independent cultures in 3-4 experiments; the distribution of variables corresponded to the normal law.

The data were statistically processed by ANOVA with the Newman—Keuls post hoc test. The differences were significant at $p<0.05$. The results were expressed as *M*±*SEM*.

Transverse hippocampal slices (400 μ) were obtained from 4-6-week-old Wistar rats using a vibrating knife designed in the laboratory. The slices were incubated for 1.5 h at 25°C in a solution of the following composition (in mM): 124 NaCl, 3 KCl, 2.5 $CaCl_2$, 1.25 Na_2HPO_4, 2.5 $MgSO_4$, 26 $NaHCO_3$, and 10 D glucose (pH 7.4) with constant saturation with carbogen (95% O_2+5% CO_2). After that, the slices were placed in a superfusion chamber with the same solution (perfusion rate 1.5-2 ml/min, 30°C). Standard field excitatory postsynaptic potentials (fEPSP) and population spikes (PS) were recorded in *str. radiatum* and *str. pyramidale* of CA1 field, respectively, with glass microelectrode filled with 1.5 M NaCl (2-5 MΩ). The responses caused by orthodromic stimulation of Schaffer collaterals through a bipolar tungsten electrode placed in *str. radiatum* at the border of CA2-CA1 at 1 mm from the registration site were recorded. Stimulation was performed with paired pulses (interstimulus interval 50 msec, duration 0.1 msec) every 30 sec. The stimulus intensity used in the experiment was chosen to elicit a half-maximum response of the pyramidal neuron population (PS). Long-term potentiation was induced using a train of high-frequency stimulation (100 Hz, 1 sec) with pulses of the same current strength applied via the same stimulating electrodes. The signals were recorded with an A-M Systems 1800 amplifier using WinWCP v5.x.x software; the responses were filtered at 1 Hz and 1 kHz filters, sampled at 5 kHz, and analyzed using Clampfit 10.2 software (Axon Instruments).

RESULTS

We have previously demonstrated that methylene blue has a strong protective effect not only in zinc neurocytotoxicity *in vitro*, but also in traumatic brain injury [6]. As zinc ions are involved in the development of secondary brain damage in this pathological condition, we hypothesized that other neuroprotective agents that are effective in different models of traumatic brain injury can reduce neurocytotoxic action of zinc ions. The most promising drug with such properties is GK-2 that demonstrated a protective effect on the model of focal unilateral craniocerebral trauma [12]. We have previously shown that GK-2 in concentrations of 1-2 mg/liter effectively reduced the damaging effects of oxidative stress on cultured neurons [8]; therefore, in this study we used the same range of GK-2 concentrations.

It was found that the presence of 40-45 μM of zinc chloride in culture medium for 24 h dose-dependently reduced survival of cultured granule neurons; however, GK-2 (1-2 mg/liter) added simultaneously with $ZnCl_2$ to the culture medium for 24 h significantly improved neuronal survival (Fig. 1). In the absence of toxic salts, GK-2 in all concentrations had no effect on the viability of neurons (Fig. 1). However, GK-2 added for 24 h to the neuronal culture simultaneously with 40 μM $CuCl_2$ (instead of $ZnCl_2$) did not protect neurons and even potentiated the toxic effect of copper in a concentration of 2 mg/liter. It has been previously shown that GK-2 protected *Paramecium caudatum* cells from death caused by both zinc and copper ions. In this case, the protective effect of GK-2 was associated with its antioxidant properties [3]. However, in our experiments, the protective action of GK-2 is apparently not mediated by its antioxidant action, because this dipeptide did not protect neurons from the toxic action of copper ions, whose destructive action on this model is mediated by oxidative stress [11]. It can be hypothesized that the protective effect of GK-2 is associated with its antagonism with the TrkA-receptor complex [1], while the death of neurons mediated by zinc ions depends on activation of Trk receptors, as LIF (leukemia inhibitor factor), an agonist of this receptor complex, produces a protective effect in zinc toxicity [10]. It should be noted that NGF, unlike GK-2, potentiates the toxic effect of zinc ions [10].

Zinc is one of the essential elements involved in diverse biological processes and present in various brain structures (neocortex, amygdala, olfactory bulbs, and hypothalamus). The hippocampus contains the largest amount of vesicular zinc [7]. In the brain, zinc can be involved not only in the processes of neuronal death in ischemia and traumatic brain injury, but is also essential for memory and learning processes. Long-term potentiation of synaptic transmission in

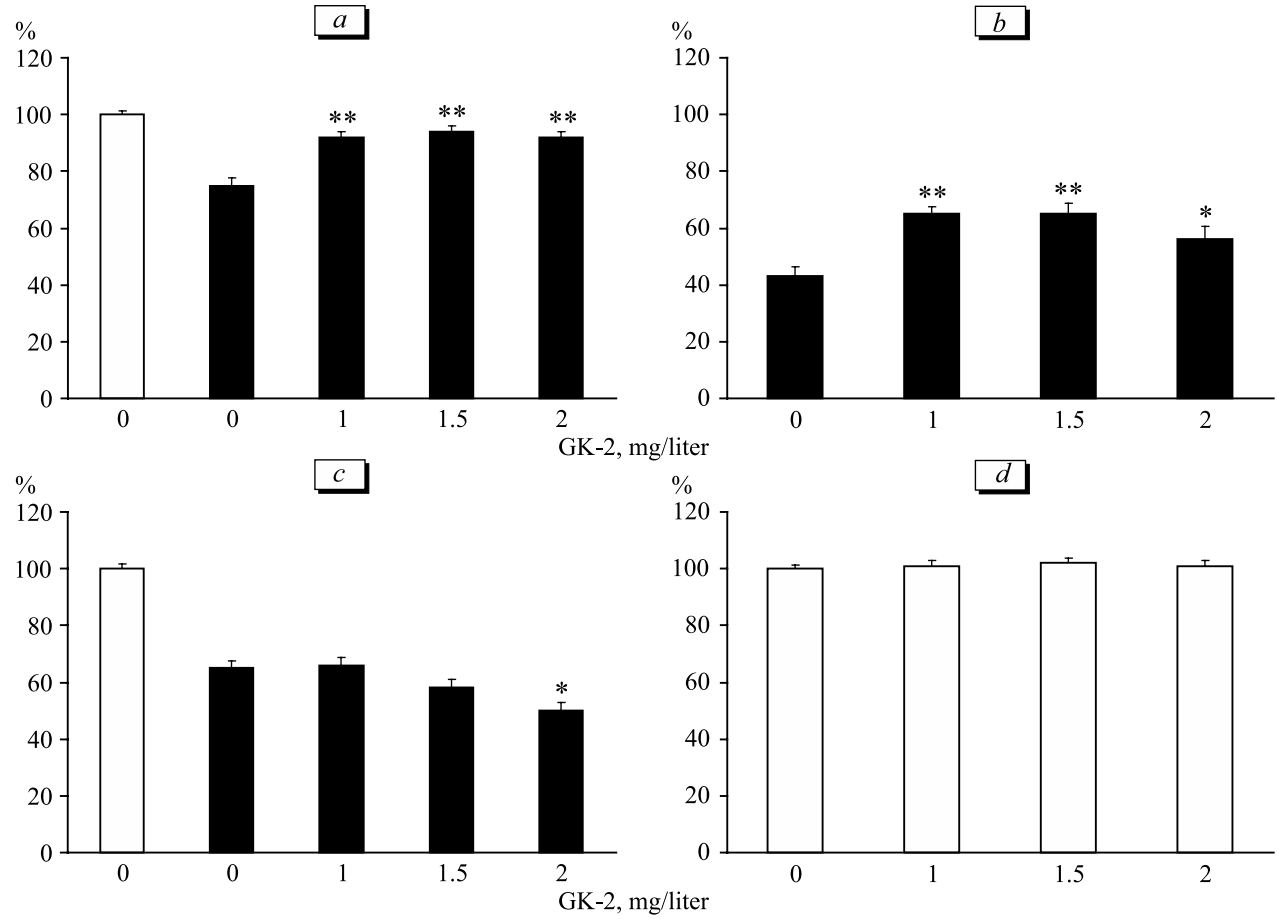

Fig. 1. The effect of GK-2 in different concentrations on survival of cultured neurons (%) in the control (100%; light bars) and in the presence of $ZnCl_2$ and $CuCl_2$ (dark bars). *a*) 40 μM $ZnCl_2$, *b*) 45 μM $ZnCl_2$, *c*) 40 μM $CuCl_2$, *d*) GK-2 alone. *p<0.05, **p<0.001 in comparison with the control.

the hippocampus is the model of synaptic changes underlying learning and memory formation. Therefore, the effect of GK-2 on synaptic transmission and its plastic properties in the synaptic system Schaffer collaterals—pyramidal CA1 neurons were evaluated in experiments on rat hippocampal slices. It was found that perfusion of slices with a solution containing 1.5 mg/liter GK-2 for 15 min did not significantly affect the reactivity of the pyramidal neurons assessed by PS amplitude, the slope of fEPSP rising phase (fEPSP slope), and the "input—output" curves. To evaluate the effect of GK-2 on the plastic properties of synaptic transmission, the slices before recording of electrical activity were incubated in a solution containing 1.5 mg/liter GK-2 for 1 h. In both control and experimental slices, high-frequency stimulation of the Schaffer collaterals induced potentiation of the amplitude of PS and fEPSP (assessed by changes in its slope persisting for at least 30 min) (Fig. 2). Slice preincubation with GK-2 did not cause significant changes in long-term potentiation assessed by the amplitude of PS or fEPSP slope. Under control conditions (without GK-2 pretreatment), the increment of PS amplitude was on average 209±18% and fEPSP slope 134±8% (n=6); for slices treated with a neuroprotector, these values were 189±12% and 127±4% (n=7), respectively (Fig. 2). As can be seen from the above data, PS potentiation after preincubation of slices with GK-2 was less than in the control, but this difference was insignificant (p=0.25, Mann—Whitney U test). It should be noted that PS and fEPSP reflect different processes involved in synaptic plasticity in the CA1 pyramids, therefore, potentiation estimated by these parameters of the evoked response can be different. Evoked fEPSP in this synaptic system reflects direct synaptic transmission, including the probability of neurotransmitter release from the presynaptic terminal and the response of the postsynaptic cell, while PS is affected by a greater number of processes accompanying synaptic transmission: E-S potentiation, intrinsic excitability, the ratio of inhibitory and excitatory inputs to the cell [13]. That could suggest that the trend to a decrease in PS potentiation under the action of GK-2 is associated with one of these mechanisms, however, this question requires further studies.

To identify the possible presynaptic effect of GK-2 on the same slices, we evaluated paired-pulse

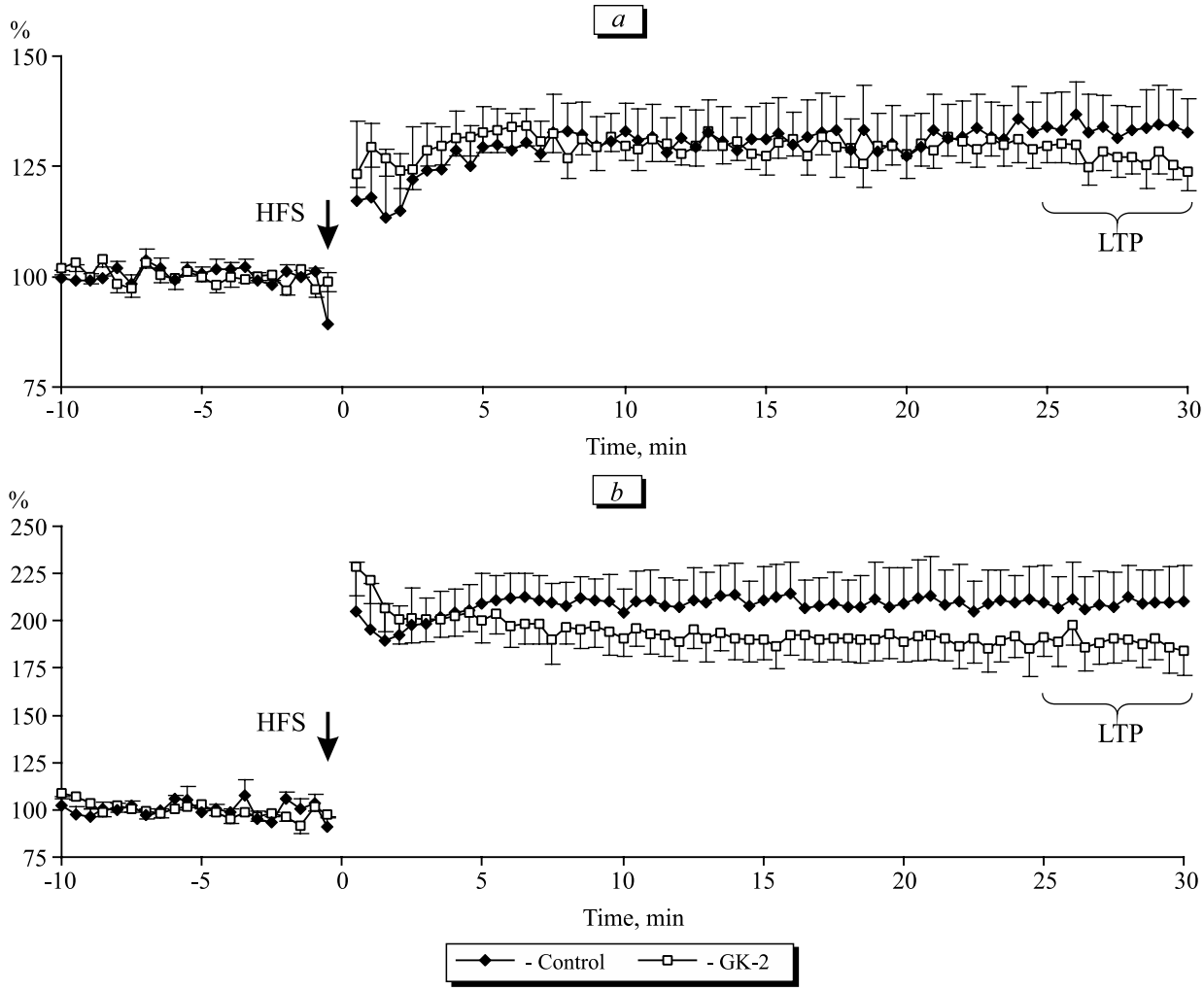

Fig. 2. The effect of pre-incubation with 1.5 mg/liter GK-2 on long-term potentiation in the CA1 field of the hippocampus. *a*) Dynamics of fEPSP slope; *b*) dynamics of PS amplitude. HFS: application of high-frequency stimulation (arrow), LTP: interval of values for calculation of the long-term potentiation.

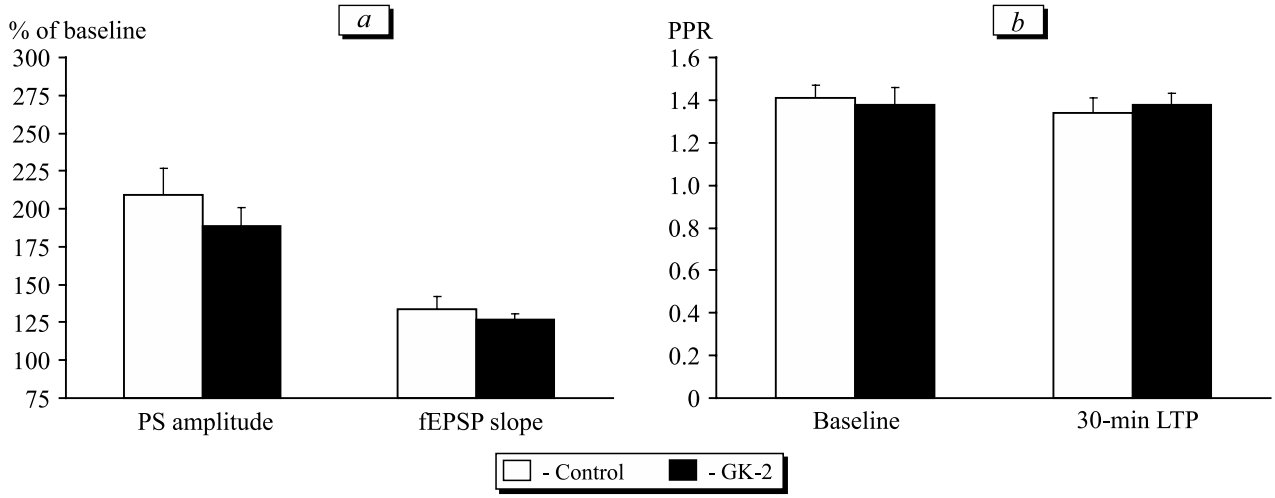

Fig. 3. The parameters of evoked responses of the Schaffer collaterals of the CA1 field in the hippocampus (*a*) and change of fEPSP slope in the PPR test in control and after incubation with GK-2 (*b*) averaged over 25-30 min after application of high-frequency stimulation.

ratio (PPR) measured as the slope ratio of the second and first fEPSP during baseline recording and after long-term potentiation induction. The change in PPR is a sensitive indicator of changes in the probability of glutamate release from the presynaptic terminal [8]. In paired stimulation with an interval of 50 msec, PPR in the control was 1.41±0.06 during baseline stimulation and 1.34±0.07 during the development of long-term potentiation. After incubation in GK-2, the corresponding values were 1.38±0.08 and 1.38±0.05, respectively (Fig. 3).

Thus, our results suggest that pretreatment with GK-2 affected neither long-term potentiation magnitude in rat hippocampal CA1 pyramids, nor the probability of glutamate release from presynaptic terminals in the CA3-CA1 synapses. However, in experiments with the neurocytotoxic effect of zinc ions, this NGF mimetic increases the survival of neurons. The protective effect of GK-2 shown by us indicates the prospects of using this agent for therapeutic purposes, because zinc imbalance in the brain is involved in a number of neurodegenerative diseases.

This work was supported by the Russian Science Foundation (grant No. 16-15-10108).

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