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Neuroprotective and Antiamnestic Effect of Nerve Growth Factor Dipeptide Mimetic GK-2 in Experimental Ischemic Infarction of Brain Cortex

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The neuroprotective and antiamnestic effects of GK-2 dipeptide (nerve growth factor mimetic) were studied on rats with photoinduced bilateral focal ischemia of the prefrontal cortex. Intraperitoneal injection of GK-2 in a dose of 1 mg/kg on days 1, 2, 4, and 6 postoperation led to a 62% reduction of cortical infarction volume on day 9 and completely preserved conditioned passive avoidance response trained before stroke.

Key Words: *nerve growth factor mimetic; photoinduced thrombosis; GK-2 dipeptide; neuroprotection; antiamnestic effect*

According to epidemiological data, stroke is the leading cause of mortality in countries with well-developed economy [1]. Neuroprotection is one of the most important steps in drug therapy for stroke [3], and hence, the search for drugs reducing the severity of neurodegeneration and improving mnestic functions in cerebral ischemia is an important problem of pharmacology. Many compounds with different mechanisms of action possess a neuroprotective effect. The pathogenetic mechanisms of stroke and the available data on nerve tissue maintenance and recovery of its viability suggest the focus on neurotrophins [7,9].

Many-year basic studies at V. V. Zakusov Institute of Pharmacology resulted in creation of an original nerve growth factor dipeptide mimetic (GK-2). Results of *in vitro* studies suggest that this compound exhibit neuroprotective activity [6].

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Here we studied the neuroprotective and antiamnestic effects of GK-2 *in vivo* on an experimental model of photoinduced thrombosis of the cerebrocortical blood vessels reproducing clinical picture of ischemic infarction of the brain [4,10]. This model was validated using drugs with neuroprotective and antiamnestic effects used for correction of brain diseases [4].

MATERIALS AND METHODS

Peptide GK-2 (bis-N-monosuccinyl-glutamyl-lysine hexamethylene diamide) was synthesized at Department of Chemistry (head Prof. T. A. Gudasheva) of V. V. Zakusov Institute of Pharmacology.

Experiments were carried out on outbred male rats (180-200 g) kept in vivarium with free access to water and food at natural illumination.

All experimental animals were divided into 3 groups. Group 1 were trained sham-operated rats, group 2 were trained rats subjected to photothrombosis of the prefrontal cortex, and group 3 were trained

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rats with photothrombosis treated with GK-2 dipeptide.

The experiment included the following steps: photothrombosis operation; study of motor activity (before and on day 9 after photothrombosis); passive avoidance training and testing on day 9 after photothrombosis; sacrifice, removal and fixation of the brain; morphological study and measurement of the ischemic focus volume.

The substance was injected intraperitoneally in a dose of 1 mg/kg 1 h after the operation, 24 h after the first injection, on days 4 and 6 after the first injection.

Passive avoidance conditioning was carried out in a rectangular box (45×23×25 cm) with acryl nontransparent walls and electrified metal floor. The box was divided with a wall with a 6×6 cm hole into two equal compartments. On day 1 of training, a rat was placed to the illuminated (100 W lamp) compartment. The rat examined the chamber and after some time passed into the dark compartment (latency of transition), after which the door between the compartments was closed and the rat was left in the dark compartment for 5 min. The procedure was repeated after 1 h and the rat was immediately removed from the dark compartment. On the next day, the procedure was repeated twice with 1-h interval. After the rat entered into the dark compartment, the door was closed and electric current (1.3 mA, 50 Hz, 5 sec) was delivered to the metal rods of the floor. The reflex was considered trained, if the latency of transition was no less than 300 sec. The animals with shorter latencies were excluded from the experiment.

TABLE 1. Passive Avoidance Latency (sec) on Day 8 after Photothrombosis in the Prefrontal Cortex

Rat No.	Sham-operated	Photothrombosis	
		saline	GK-2
1	300	20	300
2	300	30	300
3	300	103	300
4	300	38	300
5	300	105	300
6	300	99	300
7	300	29	300
8	300	21	300
9	300	42	300
Mean	300	59.2*	300^{+}

Note. $p<0.05$ compared to: *sham-operated animals, $^{+}$photothrombosis+saline.

Bilateral focal ischemic stroke of the prefrontal cortex (fields Fr1 and Fr2 according to Paxinos and Watson Atlas, 1986) was created by photoinduced thrombosis [10]. The animals were narcotized with chloral hydrate (300 mg/kg intraperitoneally). Photosensitizing Bengal Rose stain (Sigma Chem. Co.) was injected into the jugular vein (3% solution in water, 40 mg/kg). Animal head was fixed in stereotaxis, the skin was cut longitudinally, and the periosteum was removed.

The light guide (output light beam 3 mm) was set up at a distance of 1 mm from the skull surface by coordinates: 2 mm rostrally from bregma and 2 mm laterally from the sagittal suture. Cold light exposure (xenon lamp, 25 V, 250 W) was carried out for 15 min on each side. Sham-operated animals were exposed to the same procedures except injection of Bengal Rose. In order to evaluate the functional status of animals, their motor activity (MA) was studied in a RODEO-1 automated device over 5 min. Motor activity was analyzed before and on day 9 after photothrombosis of the prefrontal cortex.

Morphometric measurements of the areas and volumes of the ischemic focus on serial sections were carried out on animal brain fixed by plunging into formalin–ethanol–acetic acid mixture (2:7:1). After fixation the material was transferred into 70^{o} ethanol for 24 h and sliced in distilled water on a vibrotome 1000 (Technical Product International Inc.) at a 100-μ step. Every other section was mounted one-by-one on gelatin-coated slides and stained with 0.2% methylene blue. The preparations were then processed by standard histological methods: dehydrated in ascending alcohols, clarified in xylene, and embedded in balm. Histological preparations were scanned using a slide attachment for V100 PHOTO scanner (Epson). Using this method, we obtained files with images of brain sections (light blue) with clearly seen foci of ischemic injury, dark at the periphery and clear in the center. Sometimes the necrotic tissue degraded; in these cases, the missing tissue fragment was considered as the focus. The area of an ischemic focus was evaluated using Image J software (Bethesda).

The volume of photoinduced thrombosis focus was evaluated by the formula:

$$V=\Sigma S_n \times d,$$

where d is the thickness of a pair of sections (200 μ); S_n is measured area of ischemic focus in a serial section in mm^2; and Σ is the sum of ischemic foci volumes in sections.

Protection efficiency coefficient (PEC), that is, the efficiency of treatment of animals with the studied substance, was calculated by the formula:

$$PEC=(V_{SAL}-V_{SUB})/V_{SAL}\times 100\%,$$

where V_{SAL} is the mean summary volume of the focus in animals injected with saline and V_{SUB} is the mean volume of the focus in animals injected with the substance. This parameter is used to compare the efficiencies of different substances on different ischemia models.

The data were statistically processed using Statistica 6.0 software. The normality of the sign distribution in the sample was evaluated using Shapiro–Wilk *W* test. The latencies in passive avoidance task were compared using Mann–Whitney *U* test for independent samples and Wilcoxon's test for paired comparisons for related samples. The statistical significance of differences in infarction volumes was evaluated by Student's *t* test.

RESULTS

No statistically significant differences in MA in all groups before and after focal cortical infarction were detected (the data are not presented).

Passive avoidance was trained in all experimental animals before the operation (latency >300 sec). After photothrombosis, the latency of entry into the dark compartment decreased from 300 to 59.2 sec. The reflex was completely retained in animals treated with GK-2: passive avoidance latency in these animals was equal to or longer than 300 sec (Table 1).

Hence, these findings attest to pronounced antiamnestic effect of GK-2 under conditions of local cerebral ischemia, manifesting in 100% retention of passive avoidance trained before photoinduced thrombosis of the prefrontal cortex.

Morphometry showed that the volume of right-hemispheric focus in animals treated with GK-2 was 3-fold less than in untreated animals. The volume of left-hemispheric focus in treated animals was 2-fold less than in the control. The mean volume of the foci in animals treated with GK-2 was 2.5 times less than in the controls (Fig. 1). The total volume of cerebral injury in the rats with photoinduced thrombosis was 29 mm^3, about 2% of brain volume. In treated animals,

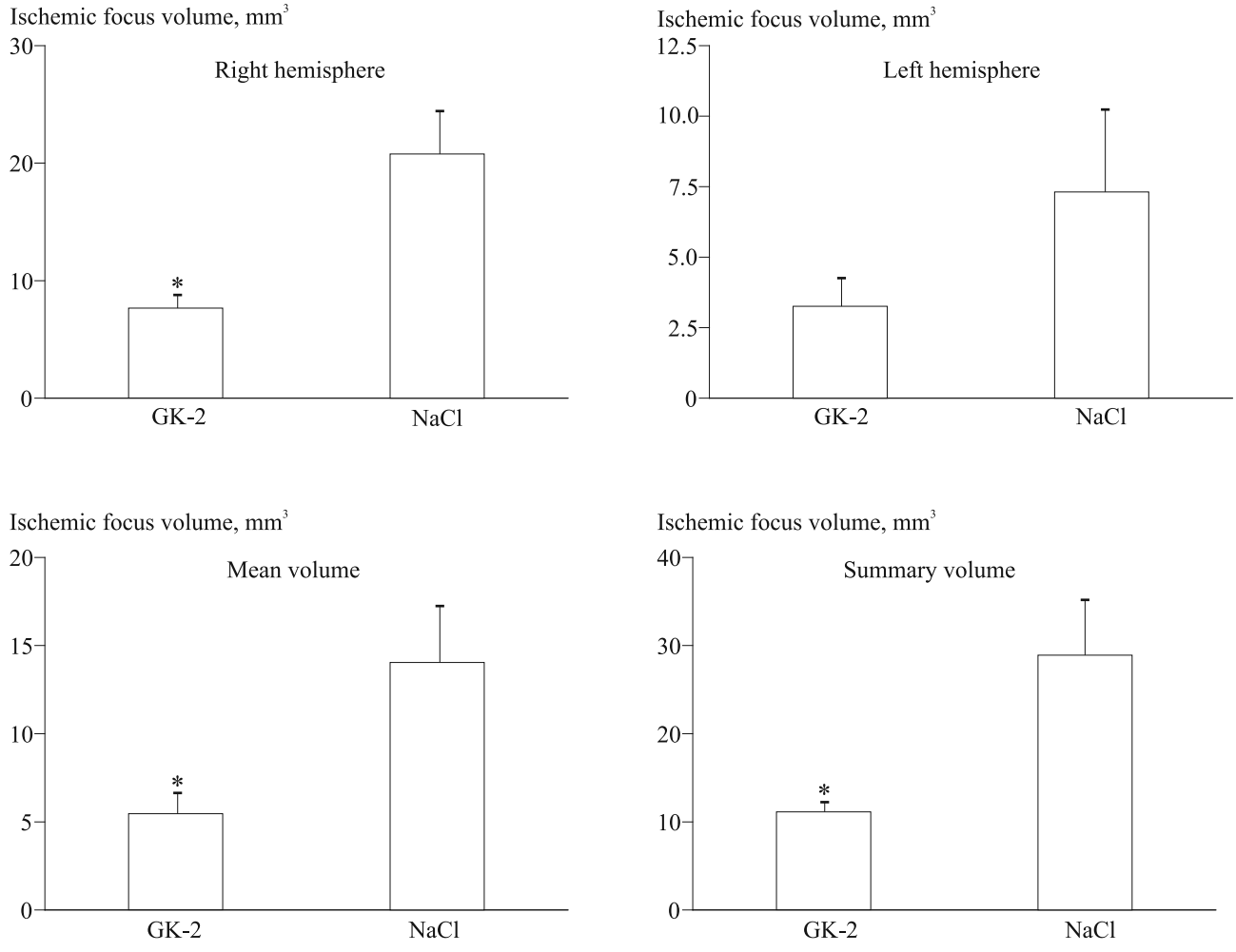

Fig. 1. Protective effect of GK-2 in photothrombosis of the rat brain prefrontal cortex. *p<0.05 compared to NaCl.

TABLE 2. Neuroprotective Effect of GK-2, Noopept [8], and Semax [5] in Photochemical Stroke

Focus volume	GK-2, 1 mg/kg intraperitoneally (*m*=4)	Noopept, 0.5 mg/kg intraperitoneally (*m*=9)	Semax, 0.25 mg/kg intranasally (*m*=7)
Thrombosis in control, V_{SAL}, mm^3	29	6.2	15
Thrombosis in experiment, V_{SUB}, mm^3	11*	3*	11*
Treatment efficiency, PEC	62	52	27

Note. *m*: number of days of drug injection. Treatment efficiency calculated by the formula $TEC=(V_{SAL}-V_{SUB})/V_{SAL}\times100\%$. $p<0.05$ compared to the control.

TABLE 3. Antiamnestic Effects of GK-2, Noopept [8], and Semax [5] in Photochemical Stroke

LP	GK-2, 1 mg/kg intraperitoneally (*m*=4)	Noopept, 0.5 mg/kg intraperitoneally (*m*=9)	Semax, 0.25 mg/kg intranasally (*m*=7)
Sham-operated animals, sec	300	300	300
Control animals with photothrombosis, sec	60	80	80
Experimental animals with photothrombosis and treatment, sec	300*	200*	240*
Treatment efficiency, A (%)	100	55	70

Note. *m*: number of days of drug injection. Treatment efficiency calculated by the formula A(%)=100×(LP (sham-operated)-LP (photothrombosis with treatment)/(LP (sham-operated)-LP (photothrombosis). *$p<0.05$ compared to photothrombosis+saline.

this volume decreased to 12 mm^3. Estimated protection efficiency coefficient was 62%.

Comparison of GK-2 efficiency with the efficiencies of noopept (dipeptide nootropic) and semax heptapeptide [2] revealed more pronounced neuroprotective and antiamnestic efficiency of GK-2 in this model in comparison with both drugs (Tables 2, 3).

The results confirm the neuroprotective effects of GK-2 detected *in vitro* and indicate good prospects of this substance as a potential anti-stroke agent.

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