Chapter

Glyproline Pro-Ampakine with Neuroprotective Activity

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Abstract

Previously it was shown that neuropeptide cyclo-L-prolylglycine (CPG) is a positive modulator of AMPA receptors, which increases BDNF level in neuronal cell cultures. The spectrum of CPG's pharmacological effects corresponds to that of BDNF. Dipeptide N-phenylacetyl-glycyl-L-proline ethyl ester (GZK-111) was designed and synthesized as a linear analog of CPG. The aim of the present work was to reveal the pharmacological profile of GZK-111. Dipeptide GZK-111 was shown to metabolize into CPG in vitro and increased cell survival by 28% at concentrations of 10-7–10-6 M in a Parkinson's disease cell model. In a model of cerebral ischemia, GZK-111, at a dose of 0.5 mg/kg, i.p., was found to have neuroprotective effects, reducing the cerebral infarct volume by 1.6 times. Similar to CPG, GZK-111, at the range 0.1–1.0 mg/kg, i.p., possessed a stereospecific antiamnesic activity. A significant anxiolytic effect was observed at a dose of 1.5 mg/kg. GZK-111, at the range 0.5–4.0 mg/kg, i.p., demonstrated analgesic activity. GZK-111, at a dose of 10 mg/kg/7 days, i.p., possessed antidepressant-like activity. So, the neuroprotective, nootropic, antihypoxic, anxiolytic, antidepressant-like, and analgesic effects of GZK-111 were revealed. Thus, GZK-111 can be considered as a pharmacologically active pro-ampakine with a BDNF-ergic mechanism of action.

Keywords: glyproline GZK-111, pro-ampakine, cyclo-prolylglycine, BDNF, neuroprotective activity

1. Introduction

Cyclo-*L*-prolylglycine (CPG) was designed as a potential peptide prototype of piracetam, the classic nootropic drug [1] and was subsequently discovered as endogenous compound in the brain of intact rats in micromolar concentration [2]. CPG is similar to piracetam both in structure and main pharmacological effects; it possesses nootropic [3], anxiolytic [4, 5], antihypoxic [6], neuroprotective [6, 7], analgesic [8], and antidepressant [9, 10] activities at central administration at doses 100–1000 times smaller than those for piracetam. Recently, we have demonstrated CPG to be ampakine, i.e., a positive modulator of AMPA receptors [11]. Like other ampakines, it increases brain-derived neurotrophic factor (BDNF) content in

neuronal cell cultures [12]. The range of CPG pharmacological effects corresponds both to that of piracetam and BDNF. In view of this, CPG can be regarded as a basis for creation of new group of drugs with neuroprotective properties.

CPG is a hydrophilic compound. The task to create an amphiphilic CPG prodrug with improved pharmacokinetic properties, converted to active molecule in the brain, was established to increase drug passage through biological membranes, including the blood-brain barrier. Two variants of substituted dipeptides, based on the Pro-Gly or Gly-Pro sequence, could be used for this purpose. We selected the second one (i.e., Gly-Pro), following the known information that an imide bond with proline in Gly-Pro dipeptide sequence increased the proportion of the cisoid peptide bond [13, 14], which, in turn, promoted cyclization of the dipeptide [15, 16].

In this work, we synthesized substituted glyproline *N*-phenylacetyl-glycyl-*L*-proline ethyl ester (GZK-111) to prove the hypothesis, confirmed the CPG formation during GZK-111 metabolism in the presence of blood plasma enzymes, and demonstrated the compound pharmacological effects typical for CPG and BDNF, namely, neuroprotective, nootropic, antihypoxic, anxiolytic, antidepressant, and analgesic activities.

2. Materials and methods

2.1 Chemical experimental part

The chemical reagents used in the synthesis were obtained from commercial suppliers and used without purification. All solvents were dried and purified by standard procedures if required. Melting points were measured in open capillary tubes using OptiMelt melting point apparatus (Stanford Research Systems, USA). The structures of the compounds were confirmed by elemental analysis and ^{1}H NMR spectroscopy. The NMR spectra were obtained on a Bruker Fourier 300 (Bruker, Germany) spectrometer using tetramethylsilane as an internal standard. The NMR peaks were designated as follows: s, singlet; d, doublet; t, triplet; and m, multiplet. Microanalyses for C, H, and N agreed with calculated values within 0.4%. Specific optical rotations were recorded by automatic polarimeter ADP 440 (Bellingham + Stanley Ltd., England). The TLC was carried out on Merck silica gel 60 F 254 plates with spot visualization by iodine vapor or UV light.

***N*-Phenylacetyl-glycine.** 13.24 ml (0.1 mol) of N-phenylacetyl chloride and 25 ml of 4 M NaOH were added dropwise successively to a solution of 7.5 g (0.1 mol) of glycine in 25 ml of 4 M NaOH under stirring at −10°C. The reaction mixture was stirred for 30 min on cooling, extracted by ethyl acetate (3 × 15 ml) to remove impurities and unreacted N-phenylacetyl chloride. Then the solution was acidified by 4 M HCl to pH 2–3; the resulting precipitate was filtered, washed with cooled distilled water, and dried in air. The yield of N-phenylacetyl-glycine was 5.83 g (30%); m.p. 143–144°C; R_f 0.8 (BuOH/AcOH/H_2O, 4:1:1). ^{1}H-NMR spectrum (DMSO-d_6 + CF_3COOD, d, ppm): 3.47 (s, 2H, CH_2Ar), 3.76 (d, *J* 6.0 Hz, 2H, CH_2 Gly), 7.27 (m, 5H, ArH), 8.40 (t, *J* 6.0 Hz, 1H, NH Gly). Lit. data [17]: m.p. 138–143°C.

H-*L*-Pro-OC$_2$H$_5$·HCl. 2.54 ml (35 mmol) of thionyl chloride was added dropwise to 60 ml of anhydrous ethanol cooled to −20°C. Then 2.0 g (17.5 mmol) of *L*-proline was added portionwise under vigorous stirring. The resulting mixture was stirred for 2 h at −5°C and then for 2 h at room temperature. The solvent was removed in vacuo. This operation was repeated twice, each time adding 30 ml of anhydrous ethyl alcohol to afford 2.4 g (77%) of *L*-proline ethyl ester hydrochloride

as an oil. $[\alpha]^{23}_D$ −43° (*c* 3, EtOH); R_f 0.75 (i-PrOH/NH_3, 7:3). ^{1}H-NMR spectrum (DMSO-d_6, d, ppm): 1.19 (m, 3H, $C\underline{H_3}CH_2O$), 1.8–2.1 (m, 4H, $C^{\gamma}H_2$ Pro, $C^{\beta}H_2$ Pro), 3.2 (m, 2H, $C^{\delta}H_2$ Pro), 4.2 (m, 2H, $CH_3C\underline{H_2}O$), 4.5 (m, 1H, $C^{\alpha}H$ Pro), 9.9 (br. s, 1H, NH). Lit. data [18]: oil; $[\alpha]^{23}_D$ −44.8° (*c* 3.03, EtOH).

H-*D*-Pro-OC_2H_5·HCl. Obtained similarly to H-*L*-Pro-OC_2H_5·HCl from *D*-proline. $[\alpha]^{23}_D$ + 42.0° (*c* 3, EtOH); R_f 0.75 (i-PrOH/NH_3, 7:3). ^{1}H-NMR spectrum (DMSO-d_6, d, ppm): 1.18 (m, 3H, $C\underline{H_3}CH_2O$), 1.8–2.1 (m, 4H, $C^{\gamma}H_2$ Pro, $C^{\beta}H_2$ Pro), 3.19 (m, 2H, $C^{\delta}H_2$ Pro), 4.2 (m, 2H, $CH_3C\underline{H_2}O$), 4.5 (m, 1H, $C^{\alpha}H$ Pro), 9.9 (br. s, 1H, NH). Lit. data [18]: oil; $[\alpha]^{23}_D$ +44.16° (*c* 3.03, EtOH).

***N*-Phenylacetyl-glycyl-*L*-proline ethyl ester.** 1.1 ml (10.0 mmol) of *N*-methylmorpholine and 1.35 ml (10.0 mmol) of isobutyl chloroformate were added simultaneously at −10°C to a vigorously stirred solution of 1.93 (10.0 mmol) *N*-phenylacetyl-glycine in dimethylformamide (10 mL). After 2 min a mixture of 1.79 g (10.0 mmol) of proline ethyl ester hydrochloride and 1.1 ml (10.0 mmol) of *N*-methylmorpholine in dimethylformamide (20 mL) was added dropwise. The reaction mixture was stirred at −10°C for 30 min and then at room temperature for 1 h. The precipitate was separated by filtration; the solvent was evaporated in vacuo; the residue was dissolved in chloroform (25 ml). The solution was washed with 3% solution of $NaHCO_3$ (3 × 7 ml), water (3 × 7 ml), and 1 N HCl (3 × 7 ml). The organic layer was dried with anhydrous Na_2SO_4, filtered, and evaporated to dryness. The yield of chromatographically homogeneous product was 2.87 g (90%), an orange oil, finally crystallized from ethyl acetate/hexane system. M.p. 111–112°C; $[a]^{23}_D$ −90.0° (*c* 1, water); R_f = 0.80 (dioxane/H_2O, 9:1). ^{1}H-NMR spectrum (DMSO-d_6, d, ppm): 1.2 (m, 3H, $C\underline{H_3}CH_2O$), 1.8–2.1 (m, 4H, $C^{\gamma}H_2$ Pro, $C^{\beta}H_2$ Pro), 3.5 (s, 2H, $C\underline{H_2}ArH$), 3.6 (m, 2H, $C^{\delta}H_2$ Pro), 3.85 and 4.0 (2 dd, *J* 17.6 Hz, *J* 5.6 Hz, 2H, $C^{\alpha}H_2$ Gly), 4.2 (m, 2H, $CH_3C\underline{H_2}O$), 4.5 (dd, *J* 4.0 Hz, *J* 8.5 Hz, 1H, $C^{\alpha}H$ Pro), 6.4 (br. t, *J* 5.6 Hz, 1H, NH), 7.3 (m, 5H, ArH). Calcd. for $C_{17}H_{22}N_2O_4$, %: C, 64.27; H, 7.05; N, 8.63. Found, %: C, 64.13; H, 6.97; N, 8.80.

C_6H_5-CH_2-C(O)-Gly-*D*-Pro-OEt (GZK-121). Obtained similarly to the *L*-enantiomer. Yield 64%. M.p. 112–113°C; $[a]^{23}_D$ +90.0° (*c* 1, water); R_f = 0.80 (dioxane/H_2O, 9:1). ^{1}H-NMR spectrum (DMSO-d_6, d, ppm): 1.2 (m, 3H, $C\underline{H_3}CH_2O$), 1.85–2.2 (m, 4H, $C^{\gamma}H_2$ Pro, $C^{\beta}H_2$ Pro), 3.5 (s, 2H, $C\underline{H_2}Ar$), 3.6 (m, 2H, $C^{\delta}H_2$ Pro), 3.86 and 4.0 (2 dd, *J* 17.6 Hz, *J* 5.6 Hz, 2H, $C^{\alpha}H_2$ Gly), 4.2 (m, 2H, $CH_3C\underline{H_2}O$), 4.5 (dd, *J* 4.0 Hz, *J* 8.5 Hz, 1H, $C^{\alpha}H$ Pro), 6.4 (br. t, *J* 5.6 Hz, 1H, NH), 7.3 (m, 5H, Ar). Calcd. for $C_{17}H_{22}N_2O_4$., %: C, 64.27; H, 7.05; N, 8.63. Found, %: C, 63.91; H, 6.90; N, 8.71.

2.2 Biological experimental

2.2.1 Study of GZK-111 metabolism

Blood plasma preparation. Blood plasma of outbred male rats weighing 250–280 g was used. Animals were decapitated, blood was collected in BD Vacutainer® tubes with EDTA, and plasma was obtained by centrifugation at 3000 rpm for 10 min.

Incubation and extraction. A solution of 3 mg of GZK-111 in 100 μl of saline was added to 900 μl of rat plasma. The mixture was incubated at 37°C in a water bath for 10 h. Then, an equal volume of acetonitrile was added to 100 μl of the incubated mixture, the samples were vigorously shaken for 10 min and centrifuged at 12,000 rpm for 10 min, and the supernatant was collected and then chromatographed.

Reverse-phase high-performance liquid chromatography (*RP HPLC*). HPLC was performed on a Gilson 41 chromatograph (Gilson, USA) with a Gilson 116 UV detector (Gilson, USA). Detection was carried out at 220 nm. A Nucleosil C18

column (4.6 × 150 mm, 5 μm) was used with a flow rate of 0.5 ml/min. The sample volume was 20 μl. The following eluents were used: (solution A) 3.7 mM sodium 1-hexanesulfonate/water (pH 3.5) and (solution B) 3.7 mM sodium 1-hexanesulfonate/40% acetonitrile. The elution was carried out in a gradient according to the following program: (1) 0% B for 3 min, (2) from 0 to 100% B in 20 min, and (3) 100% B for 6 min. The signal was recorded using the Multichrom 1.5 software (Ampersend, RF). Cyclo-*L*-prolylglycine and *N*-phenylacetyl-glycyl-*L*-proline, synthesized in the Medicinal Chemistry Department of the V.V. Zakusov Research Institute of Pharmacology, were used as standards for HPLC.

Calibration curve. Standard solutions of synthetic cyclo-L-prolylglycine were prepared in acetonitrile at the concentrations of 0, 0.1, 0.2, 0.4, 0.8, 1.6, and 3.2 mg/L. The 20 μl aliquot of the solutions were chromatographed under RP HPLC conditions as described above. The calibration curve was calculated based on the peak areas.

2.2.2 In vitro pharmacological study

Cell culture. The studies were carried out on SH-SY5Y human neuroblastoma cells received from the cell bank of the N.N. Blokhin National Medical Research Center of Oncology of the Ministry of Health of the Russian Federation. Cells were grown at 37°C and 5% CO_2 in DMEM (HyClone, USA) with 10% FBS (Gibco, USA) and 2 mM L-glutamine (MP Biomedicals, Germany). After the monolayer formation, the cells were counted and placed into 96-well culture plates (Costar-Corning, USA) at a density of 3.5 thousand per well and into 6-well culture plates (Costar-Corning, USA) at a density of 280 thousand per well; both plates were pre-coated with a 0.1 mg/ml solution of poly-D-lysine (BD Bioscience, UK) for 1 h.

Parkinson's disease model in culture of SH-SY5Y human neuroblastoma cells. Neurotoxin 6-hydroxidopamine (6-OHDA) used in an experimental model of in vitro dopaminergic neuron degeneration according to [19] was applied to simulate Parkinson's disease in the culture of SH-SY5Y human neuroblastoma cells. 6-OHDA was added into the cell medium at a final concentration of 100 μM 24 h after cell seeding. Then the cells were incubated for 24 h. The neuroprotective effect was investigated by addition of compounds to the culture medium at final concentrations of 10^{-8}–10^{-5} M 24 h before 6-OHDA. Cell viability was determined after 24 h using the MTT test.

Neuronal viability assessment in culture using the MTT test. MTT (3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyltetrazolium bromide) is a yellow water-soluble tetrazolium salt that easily penetrates into cells. In living cells, MTT transforms into water-insoluble violet formazan crystals, which are dissolved in organic solvents (isopropanol, dimethylformamide, dimethyl sulfoxide) upon completion of the reaction followed by absorption measurements.

At the end of the experiment, the culture medium was replaced with MTT solution (0.5 mg/ml) and incubated for 30 min at 37°C. Then, a MTT solution was removed from the wells, and DMSO was added to dissolve the formazan. Absorbance was measured at 600 nm using a 96-well plate reader Multiscan (Thermo, USA).

2.2.3 In vivo pharmacological studies

In vivo studies were performed in outbred male rats weighing 200–270 g (nootropic and a anxiolytic activity), in outbred male mice weighing 25–28 g (antihypoxic and antidepressant-like activity), and in C57Bl/6 male mice weighing

25–29 g (analgesic activity), received from the Stolbovaya Branch of the Scientific Center of Biomedical Technologies of the Federal Medical Biological Agency (FMBA) of Russia, and in Wistar male rats weighing 200–250 g (neuroprotective activity) received from the Andreevka Branch of the Scientific Center of Biomedical Technologies of the FMBA of Russia. The animals were kept in vivarium under natural circadian light/dark cycles with free access to standard granular feed and water. The study complied with the requirements of Order of the Ministry of Health of the Russian Federation No. 199 "On Approval of the Rules of Good Laboratory Practice" and Decision of the Council of the Eurasian Economic Commission No. 81 "On Approval of the Rules of Good Laboratory Practice of the Eurasian Economic Union in the Area of Circulation of Medicines." All manipulations with animals were approved by the Bioethical Commission of the Zakusov Research Institute of Pharmacology. The experiments were carried out from 10 to 16 pm. The test substances were dissolved in saline or in distilled water and administered, i.p. The animals of the control groups were injected with saline or with distilled water, respectively.

2.2.4 Neuroprotective activity

A model of focal cerebral ischemia (ischemic stroke). Focal cerebral ischemia was induced by transient middle cerebral artery occlusion (MCAO) using a modification of the intraluminal filament model originally described in [20]. All surgical procedures were performed using titanium microsurgical instruments.

The rats were anesthetized with an i.p. injection of chloral hydrate (350 mg/kg) as a 5% solution in saline. The right common carotid artery, internal carotid artery, and external carotid artery were surgically exposed. A nylon suture (0.25 mm in diameter) with a silicon-coated tip was inserted from the external carotid artery into the internal carotid artery and then to the circle of Willis to occlude the origin of the middle cerebral artery. After 1 h of MCAO, the suture was carefully removed to induce reperfusion. Sham-operated rats (n = 6) underwent identical surgery except that the suture was not inserted. During the surgery the body temperature was maintained at 37.0 ± 0.5°C using a heating pad. Three rats received MCAO without any neurological deficits observed after awakening was excluded.

Design of the study. The rats with experimental ischemic stroke were randomly divided into two groups: a "stroke" group with water treatment (n = 10) and "stroke + GZK-111" group treated with GZK-111 (n = 7). Solution of GZK-111 in distilled water was administered, i.p., at a dose 0.5 mg/kg 6 h after surgery and then once a day for 6 days. Neurological functions were evaluated 3 and 6 days after surgery using the limb-placing test [21]. The infarct volume was evaluated according to [22] using 2,3,5-triphenyltetrazolium chloride (TTC) staining and computerized image analysis 7 days after surgery.

Limb-placing test. Neurological functions were evaluated using the limb-placing test [21], a modified version of the test described by De Ryck et al. [23]. This test assessed the forelimb and hind limb responses to tactile and proprioceptive stimulation and consisted of seven limb-placing tasks. The following scores were used to detect impairment of the forelimb and hind limb: 2 points, the rat performed normally; 1 point, the rat performed with a delay of more than 2 s and/or incompletely; and 0 point, the rat did not perform the task. The maximum possible score for the sham-operated rats for each side of the body was 14. Rats with experimental stroke exhibited decreased neurological score on the side of the body which is opposite to the ischemic lesion.

Evaluation of cerebral infarct volume. The cerebral infract volumes measured with TTC staining were used to describe the severity of cerebral ischemia. The animals

were deeply anesthetized with chloral hydrate (350 mg/kg, i.p.) and then decapitated. The brains were removed rapidly, frozen in −20°C for 10 min and then sectioned coronally into 2-mm-thick slices. The brain slices were incubated with 2% TTC at 37°C for 30 min. Stained slices were fixed in 10% formalin solution. The slices were digitalized on a flatbed scanner at 2400 dpi. The infarct volumes were measured using the ImageJ (National Institutes of Health, Bethesda, MD, USA) image analysis software program. The total infarct volume for each brain was calculated by summation of unstained areas of the subsequent slices and multiplying by the thickness (2 mm).

Nootropic antiamnesic activity. The antiamnesic effect of the substances was evaluated by their ability to prevent impaired reproduction of the conditioned passive avoidance reflex (passive avoidance reaction) caused by electroconvulsive shock (ECS). Compounds GZK-111 and GZK-121 were administered once, i.p., at the doses of 0.1, 0.5, and 1.0 mg/kg 40 min before training. Control animals were injected with the same volume of physiological saline.

Passive avoidance reaction was developed in rats in a certified Lafayette Instrument Co installation (USA) according to the method of [24] using a single training procedure. The illuminated start platform (25 × 7 cm) was connected to a dark 40 × 40 × 40 cm chamber equipped with an electrified floor through a square guillotine door. The animal was placed on the start platform with its tail to a dark chamber. When governed by the hole exploratory behavior, the rat found the entrance and passed into the dark compartment; the hole was closed. In the dark chamber, eight unavoidable electric pain stimuli were applied through the floor to the rat (the training current was 0.45 mA, the duration of each pulse was 1 s, and the interval between consecutive pulses was 2 s). Immediately after this, the rat was removed from the dark chamber and subjected to EKS (250 V, 120–122 mA, 0.1 s) applied transcorneally using a certified Harvard apparatus (Germany). After 24 h, the animal was again placed on the illuminated platform for learning test. The latent period of the first animal entry into the dark chamber was recorded. Antiamnesic activity (AA) was calculated as Eq. (1):

$$\mathrm{AA\%} = \frac{\mathrm{LPtest} - \mathrm{LPamn}}{\mathrm{LPcontrol} - \mathrm{LPamn}} \times 100\% \qquad (1)$$

where AA% is antiamnesic activity, LPtest is the average latent period of entry into the chamber in the animals administered with the test compound and subjected to amnesia, LPamn is the average period of entry into the chamber in the animals administered with 0.9% NaCl and subjected to amnesia, and LPcontrol is the average latent period of entry into the chamber in animals administered with 0.9% NaCl without amnesia.

Antihypoxic activity was studied in a model of normobaric hypoxia with hypercapnia ("canned" hypoxia), according to [25]. Tests were performed on animals of the same weight (scatter in groups of ≤2 g). Each group consisted of 10 animals. The substances were administered, i.p., in saline 1 h before the start of the experiment. Antihypoxic activity of the compounds was studied at doses of 0.1, 0.5, and 1.0 mg/kg. Control animals received an equivalent volume of saline. Animals were placed singly into 200 cm^3 containers that were hermetically sealed. Deaths of animals were recorded as the final agonal gasp.

Anxiolytic activity was studied in an elevated plus maze test according to Pellow [26]. Compounds were administered once, i.p., 15 min before the experiment. GZK-111 was administered at doses of 0.75, 1.5, and 3.0 mg/kg and GZK-121 at a dose of 1.5 mg/kg. The control animals were administered with the saline. The apparatus consisted of four arms elevated 60 cm above the floor, with each arm

positioned at 90° relative to the adjacent arms. Two opposite arms were enclosed with high opaque walls (50 × 15 × 30 cm), and the other arms were open (50 × 15 × 1 cm) connected via a central area (14 × 14 cm) to form a plus sign. At the beginning of the experiment, the rats were placed in the center of the maze, randomly orientated relative to the arm entrance. The behavior of the animals was evaluated within 5 min. The following indicators were recorded: the number of visits to all arms, the number of visits to open arms, the time spent in all arms, and the time spent in open arms. The anxiolytic effect of the compound was estimated by the increase in the number of visits to the light arms and in the time spent in the light arms and on the central platform.

Antidepressant-like activity was assessed in a forced swimming test [27]. Mice were placed into cylinders (30 cm high and 10 cm in diameter), filled in two-thirds with water at a temperature of 22°C for 5 min; the time of preservation of the characteristic immobilization posture (rejection of active defensive and research behavior) was estimated. The behavior of animals was recorded using a video camera. Video recording of the experiment was processed in semiautomatic mode using the RealTimer program (Open Joint-Stock Company Open Science LLC). A decrease in the immobilization duration was regarded as a manifestation of antidepressant activity.

Analgesic activity. The hot plate test was used to evaluate the nociceptive response. Latent reaction period (the time before the hind paw pulling and/or jumping) was recorded with a Ugo Basile analgesimeter (Italy). 1–2 hours before the experiment, animals were selected following the basic response in the experimental model conditions and excluding mice that remained on a plate heated to 55 ± 0.5°C for longer than 10 s. A latent period of 20 s (cutoff value) was regarded as 100% analgesia. Hot plate tests were performed before and 30, 60, 90, and 120 min after i.p. drug injection. If no response occurred within 20 s, the mice were removed from the hot plate to avoid tissue injury. The percentage of the maximum possible effect (% MPE) was calculated as follows: $(\text{postdrug latency} - \text{predrug latency}) \times 100/(\text{cutoff value} - \text{predrug latency})$.

Statistical analysis of the biological results was performed using the standard software package "Statistica 10.0" (StatSoft, Inc., USA). To evaluate statistically significant differences between the experimental and control groups of animals, the following tests were used: nonparametric Mann-Whitney U test for nootropic, anxiolytic, antihypoxic, and neuroprotective effects, the unpaired Student t-test for antidepressant effect, and Duncan criterion for analgesic effect. The means and standard errors of the mean (m ± SEM) were calculated. Kruskal-Wallis ANOVA followed by Dunn's posttest was used to compare three or more samples in in vitro experiments. The means and standard errors of the mean (m ± SD) were calculated. The results were evaluated as significant at $p \leq 0.05$.

3. Results and discussions

3.1 Synthesis of GZK-111

N-Phenylacetyl-glycyl-L-proline ethyl ester was prepared according to **Figure 1** by the mixed anhydride method under Anderson conditions [28] using isobutyl chloroformate. *N*-phenylacetylglycine obtained from glycine and phenylacetic acid chloride according to Schotten-Baumann procedure [29] was used as carboxylic component, and proline ethyl ester obtained with thionyl chloride in absolute ethanol according to Brenner method [30] was used as amine component. The D-enantiomer of GZK-111 (GZK-121) was prepared by the same procedure.

3.2 Biotransformation of GZK-111 to CPG

Synthesized GZK-111 was subjected to biotransformation in the presence of plasma enzymes to show the fundamental possibility of its conversion to CPG. The initial plasma contained endogenous CPG at a concentration of 1 μM, according to RP HPLC. Upon GZK-111 incubation with plasma at 37°C for 10 h, an increase of the CPG peak and appearance of a peak corresponding to the retention time of the compound with an open carboxyl group, N-phenylacetyl-glycyl-L-proline, were observed (see **Figure 2**). Thus, CPG is actually formed from GZK-111 in the presence of blood plasma enzymes. The scheme of GZK-111 metabolism is shown in **Figure 3**.

3.3 Pharmacological effects of GZK-111

Based on the previously established pharmacological effects of CPG, the pharmacological activity of GZK-111 (viz., neuroprotective, antiamnesic, antihypoxic, anxiolytic, antidepressant-like, and analgesic effects) was studied.

The **neuroprotective effect** of GZK-111 was studied **in vitro** in a 6-OHDA toxicity model in SH-SY5Y cells (cellular model of Parkinson's disease) [19, 31], which demonstrated the neuroprotective effect of CPG previously [6]. GZK-111 was introduced 24 h before 6-OHDA, because pharmacological effects of its metabolite (CPG) were probably caused by BDNF synthesis [12], which took no less than 6 h [32]. When introduced 24 h before the injury, GZK-111 was revealed to have neuroprotective activity at concentrations of 10^{-7} and 10^{-6} M (**Figure 4**). The range of active concentrations of CPG was 10^{-8}–10^{-5} M in this model [6].

The **neuroprotective activity** of GZK-111 was studied **in vivo** in a model of ischemic stroke caused by transient occlusion of the middle cerebral artery in rats, according to [20]. This model allows to simulate the most common cerebrovascular accident—an extensive ischemic stroke in the middle cerebral artery basin—and provides a reproducible volume of ischemic injury [33]. The compound was administered, i.p., at a dose of 0.5 mg/kg/day for 7 days; the first injection was given 6 h after surgery. The GZK-111 dose was taken accordingly to the results of other pharmacological studies (see **Tables 1** and **2**). The duration of substance introduction corresponds to the period of poststroke treatment in a hospital with consideration to differences in the metabolic rates of animals and humans [34]. The first administration of the compound 6 h after ischemia is explained by preservation of penumbra zone during this period [35], which makes it possible to decrease the volume of ischemic damage. Neurological functions were evaluated 3 and 6 days after surgery using the limb-placing test [21]. The infarct volume was evaluated 7 days after surgery according to [22] with 2,3,5-triphenyltetrazolium chloride staining and computerized image analysis. GZK-111 was found to have pronounced neuroprotective effects, reducing the volume of the infarct zone by 1.6 times (**Figure 5**) and improving the neurological status of animals by approximately 30% (**Figure 6**).

The neuroprotective activity of CPG (1.0 mg/kg, i.p., subchronic) was revealed previously in a model of incomplete global cerebral ischemia induced by permanent bilateral common carotid arteries occlusion in rats [7].

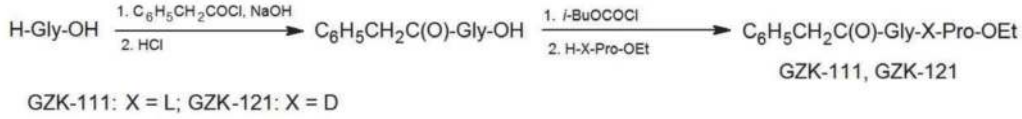

Figure 1.
The scheme of synthesis of N-phenylacetyl-glycyl-L-proline ethyl ester enantiomers.

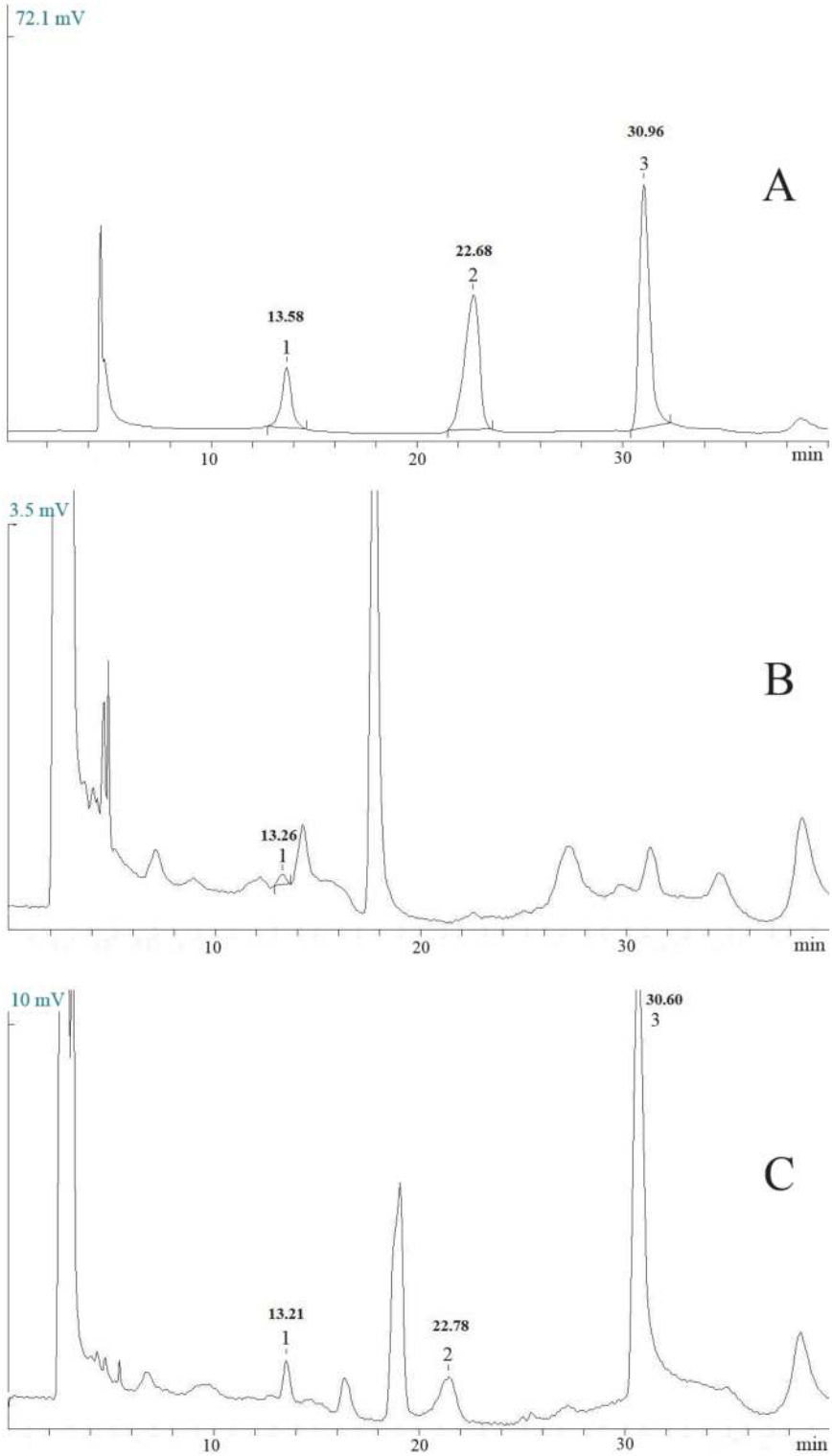

Figure 2.
The investigation of GZK metabolism in blood plasma by HPLC method. (A) Standards: (1) cyclo-L-prolylglycine, (2) N-phenylacetyl-glycyl-L-proline, and (3) N-phenylacetyl-glycyl-L-proline ethyl ester; (B) control blood plasma; (C) blood plasma + GZK-111, 10-h incubation.

The **nootropic antiamnesic effect** of GZK-111 was evaluated by its ability to prevent impaired reproduction of the conditioned passive avoidance reflex (passive avoidance reaction) in rats caused by electroconvulsive shock (ECS) by the Ader method [24]. The test is based on the inborn hole exploratory behavior of rodents.

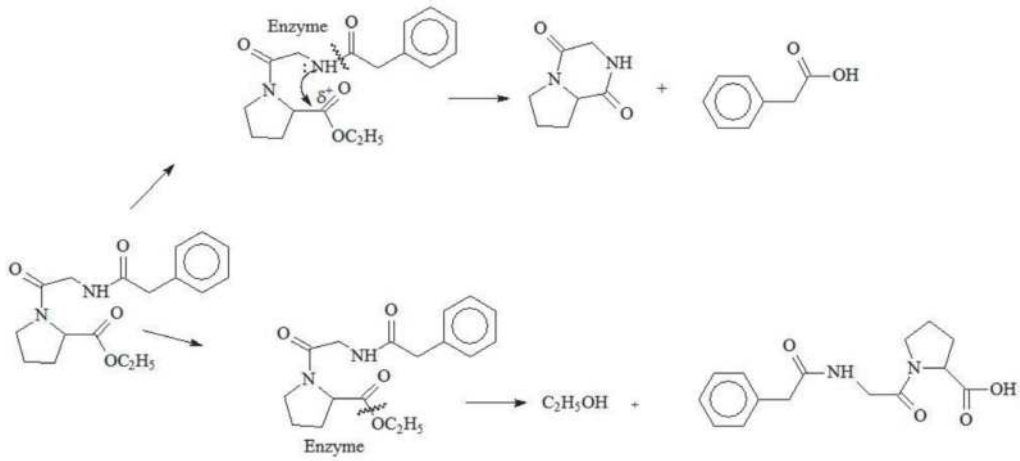

Figure 3.
The scheme of GZK-111 metabolism.

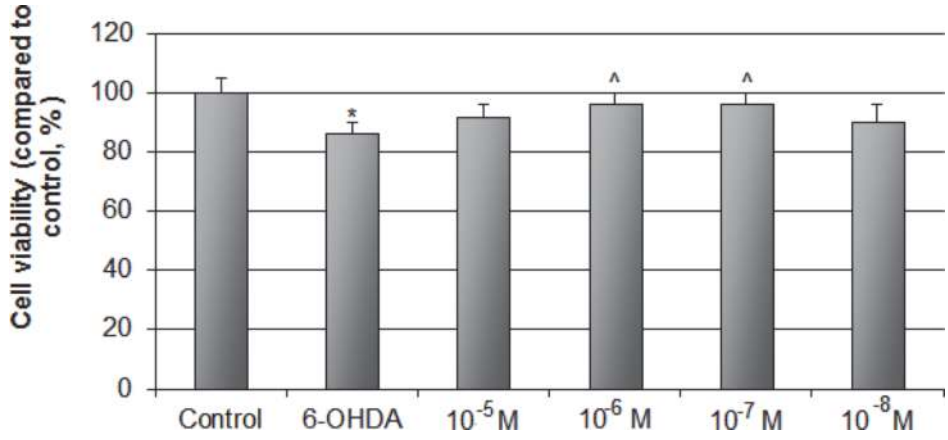

Figure 4.
*The neuroprotective effect of GZK-111 in a 6-OHDA toxicity model, SH-SY5Y cells. Notes: *p < 0.05 comparison with the control, ^p < 0.05 comparison with the damage group (Kruskal-Wallis ANOVA test with Dunn's post hoc).*

An animal learns not to enter the dark compartment using electric pain stimulation. Once the animal is subjected to EKS after training, the memorial trail is erased, and the animal again enters the dark chamber. Nootropic activity is defined as the ability to decrease the amnesic effect of ECS. GZK-111 was demonstrated to have antiamnesic activity at doses of 0.1, 0.5, and 1.0 mg/kg, i.p. (see **Table 1**), i.e., at least in the same dose range as CPG. Unlike the L-isomer, the D-stereoisomer of GZK-111, ethyl ester of *N*-phenylacetyl-glycyl-*D*-proline (GZK-121), had no antiamnesic activity.

Thus, GZK-111 possesses an antiamnesic activity similarly to CPG, and its effect is stereospecific, like that of CPG. However, if the *D*-enantiomer of CPG exhibits a pro-amnesic effect, the *D*-enantiomer of GZK-111 (GZK-121) has no activity. The possible explanation is that *D*-CPG blocks the receptor, while GZK-121 is not metabolized to CPG at all.

The **antihypoxic activity** of GZK-111 was studied in the normobaric hypoxia test with hypercapnia ("canned" hypoxia) in mice, which is the simplest method to assess antihypoxic activity [25]. GZK-111 was administered at doses of 0.1, 0.5, and 1.0 mg/kg, i.p., 60 min before test. Likewise CPG, the compound was found to

Compound	Dose, mg/kg, i.p. (n = 10)	Latent period, s			Effect, %
		Control	Amnesia	Amnesia + compound	
L-CPG [3]	0.05	91 ± 34	19 ± 8°	25 ± 7	+8
	0.1	91 ± 34	19 ± 8°	73 ± 26*	+75*
	1.0	91 ± 34	19 ± 8°	43 ± 19*	+33*
D-CPG [36]	0.1	113 ± 12	48 ± 11°	27 ± 8*	−32*
GZK-111	0.1	180 ± 0	129 ± 28°	169 ± 10*	+76*
	0.5	180 ± 0	100 ± 25	176 ± 6*	+95*
	1.0	180 ± 0	129 ± 28°	179 ± 2*	+98*
GZK-121	0.5	180 ± 0	95 ± 31°	133 ± 23	+44

Notes: n, number of animals;
°p < 0.05 comparison with the control;
**p < 0.05 comparison with the amnesia group (Mann-Whitney U test with a Bonferroni correction).*
Antiamnesic activity (AA) was calculated as follows: $AA\% = \frac{LPtest - LPamn}{LPcontrol - LPamn} \times 100\%$. *where AA% is antiamnesic activity, LPtest is the average latent period of entry into the chamber in the animals administered with the test compound and subjected to amnesia, LPamn is the average period of entry into the chamber in the animals administered with 0.9% NaCl and subjected to amnesia, and LPcontrol is the average latent period of entry into the chamber in animals administered with 0.9% NaCl without amnesia.*

Table 1.
The effects of GZK-111 and GZK-121 in passive avoidance reaction test, compared to CPG.

Compound	Dose, mg/kg, i.p. (n = 10)			
	Control	0.1	0.5	1.0
	Life expectancy, min			
L-CPG [6]	20.9 ± 0.6	20.2 ± 0.8	24.0 ± 0.7*	25.0 ± 1.6**
D-CPG [37]	21.8 ± 1.7	23.3 ± 1.5	22.7 ± 0.5	22.3 ± 1.4
GZK-111	27.1 ± 0.9	25.2 ± 0.5	30.2 ± 0.7*	26.2 ± 0.3
GZK-121	25.8 ± 0.8	23.9 ± 0.7	26.0 ± 0.7	23.5 ± 0.8

Notes: The outbred white male mice were used in the experiments;
n, number of animals;
**p < 0.05;*
***p < 0.01 comparison with the control (Mann-Whitney U test).*

Table 2.
The effects of GZK-111 and GZK-121 in normobaric hypoxia test with hypercapnia in mice, compared to CPG.

exhibit an antihypoxic effect at a dose of 0.5 mg/kg, significantly increasing the life expectancy of animals (see **Table 2**). The stereoisomer GZK-121 at doses of 0.1, 0.5, and 1.0 mg/kg showed no antihypoxic activity in this test.

The **anxiolytic activity** of GZK-111 was studied in rats at doses of 0.75, 1.5, and 3.0 mg/kg, i.p., using the elevated plus maze test (EPM) according to Pellow [26]. This test is the primary model for anxiolytic activity detection; it is based on the conflict between rodents' inherent fear of open spaces and natural exploratory behavior. A significant anxiolytic effect was observed at a dose of 1.5 mg/kg. GZK-111 increased the time rats spent in the open arms by 12 times relative to the control, which is comparable with the effect of CPG. The enantiomer, GZK-121, was inactive at a dose of 1.5 mg/kg (see **Table 3**).

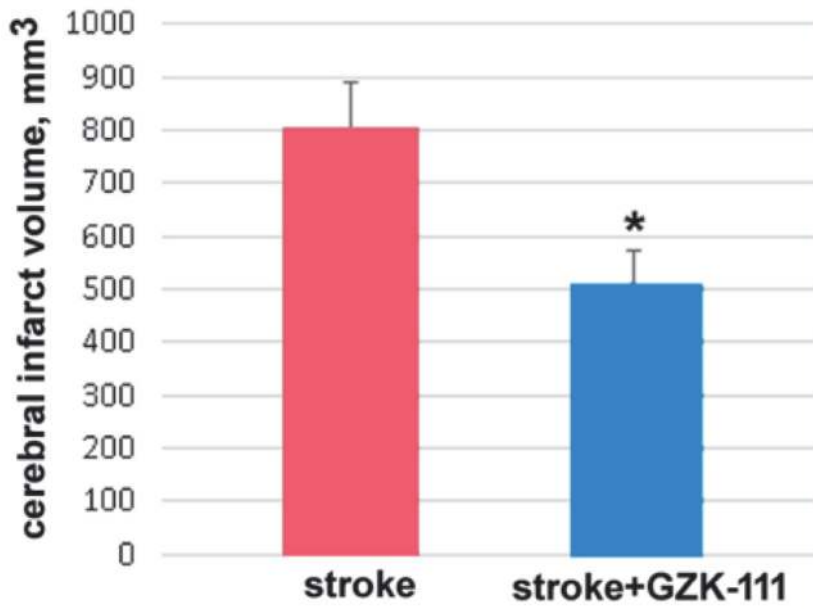

Figure 5.
*GZK-111 reduces the cerebral infarct volumes in rats with experimental stroke. Notes: Morphometric measurements of infarct volume were performed using TTC staining. The data are presented as mean ± SEM. *p = 0.05 compared to the "stroke" group (Mann-Whitney U test).*

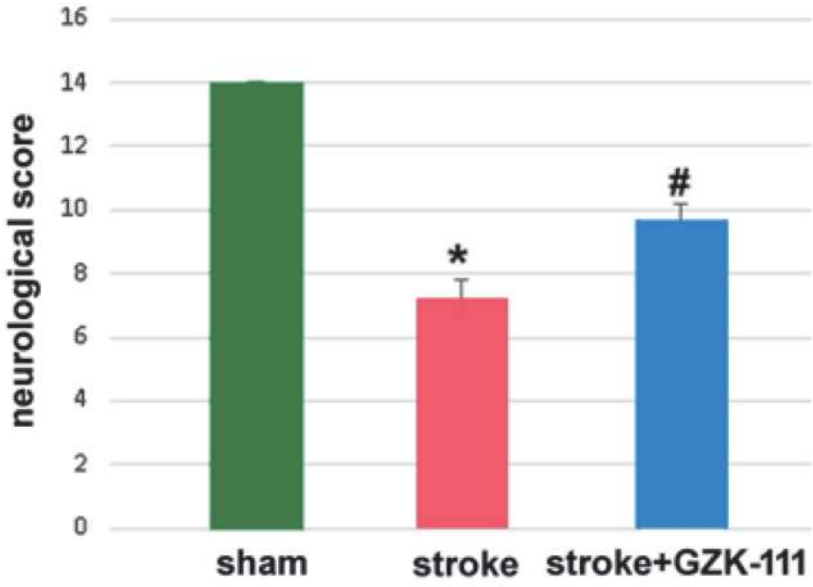

Figure 6.
*GZK-111 improves neurological status in rats with experimental stroke. Notes: Neurological status is given for the injured side of the body (which is the opposite to the ischemic lesion) in the limb-placing test. The data are presented as mean ± SEM. *p = 0.003 compared to the "sham" group, #p = 0.01 compared to the "stroke" group (Mann-Whitney U test).*

The **antidepressant-like effect** of GZK-111 was studied in the forced swimming test [27], one of the most widely used for this purpose [38]. The test is based on the ability of antidepressants to reduce the time of immobility in case of unavoidable swimming of the animal in a cylinder with water. The study was performed in mice at daily i.p. administration at a dose range of 0.01–20.0 mg/kg within 7 or 14 days.

Compound	Dose, mg/kg, i.p. (n = 10)	Number of entries into the open arms		Time spent in the open arms	
		m	%	s	%
L-CPG [4]	Control 1	0.20	100	2.15	100
	0.1	1.79*	895*	13.8*	641*
	1.0	0.25	125	1.46	68
	Control 2	0.58	100	6.38	100
	0.05	1.60**	276**	57.4**	900**
	0.2	1.50	259	19.3	303
D-CPG [4]	Control	0.20	100	2.15	100
	0.05	0.34	170	3.87	180
	0.1	0.26	132	2.58	120
GZK-111	Control	0.9 ± 0.6	100	4.8 ± 2.5	100
	0.75	1.3 ± 0.5	170	14.8 ± 7.0	180
	1.50	3.2 ± 1.1	355	58.2 ± 13.3**	1212**
	3.0	1.0 ± 0.5	132	6.4 ± 2.9	120
GZK-121	Control	0.2 ± 0.2	100	4.1 ± 1.5	100
	1.5	0.2 ± 0.2	100	4.7 ± 1.5	114

Notes: Number of entries into the open arms and time spent in the open arms were taken as 100% for control animals. n, number of animals; m, number of entries into the open arms of the maze;
**p < 0.05;*
***p < 0.001 comparison with the control (Mann-Whitney U test).*

Table 3.
The effects of GZK-111 and GZK-121 in EPM test in rats, compared to CPG.

The tricyclic antidepressant amitriptyline (10.0 mg/kg, i.p.) was used as a comparison drug. GZK-111 administered at a dose of 10 mg/kg for 7 days was established to statistically significantly reduce the immobility time by 11% (**Table 4**), which is comparable to the effect of amitriptyline at a dose of 10 mg/kg.

With a 14-day administration, GZK-111 had a significant antidepressant effect at a dose of 10 mg/kg, i.p., and showed a tendency to decrease immobility time at a dose of 1.0 mg/kg, i.p. ($p = 0.08$) and 10 mg/kg p.o. ($p = 0.06$) (**Table 5**).

Recently, the antidepressant activity of started CPG was also discovered in the forced swimming test in mice (**Table 5**) [9] and in an experimental model of learned helplessness in rats [10] at i.p. administration for 14 days.

The **analgesic properties** of GZK-111 were determined in the hot plate test in inbred male C57Bl/6 mice [39] at acute i.p. administration at doses of 0.5, 1.0, 2.0, and 4.0 mg/kg. This test was widely used to study the pain sensitivity in rodents at supraspinal level, and the effectiveness of analgesics evaluates the pain response on paw pad contact with a hot surface; the time until the hind paw pulling and/or jumping is measured. The average baseline nociceptive response for C67Bl/6 mice was 8.2 ± 0.2 s. GZK-111 showed no analgesic effect ($F(4, 44) = 11{,}163$, $p = 0{,}36{,}098$) 30 min after administration at each of the doses studied. At a dose of 2.0 mg/kg, GZK-111 statistically significantly increased the latent period of the nociceptive reaction ($F(4, 44) = 3.1489$, $p = 0.02319$) 60 min ($p < 0.01$) and 120 min ($p < 0.05$) after administration, compared with the control group. At a

Experimental groups	Dose, mg/kg, i.p. (n = 10)	Immobilization time, s	Immobilization time compared to the control, %
Experiment 1			
Control	0.0	235.8 ± 11.8	100
GZK-111	0.1	240.2 ± 19.8	101
GZK-111	0.5	235.3 ± 17.7	100
GZK-111	1.0	240.0 ± 22.1	101
GZK-111	5.0	253.9 ± 9.2	108
Amitriptyline	10.0	218.5 ± 17.9*	93
Experiment 2			
Control		275.3 ± 8.7	100
GZK-111	0.01	260.1 ± 7.3	94
GZK-111	5.0	268.7 ± 10.2	98
GZK-111	10.0	245.5 ± 9.1*	89
GZK-111	20.0	259.9 ± 9.2	94

Notes: Immobilization time was taken as 100% for control animals.
n, number of animals;
**p < 0.05, the statistical significance of the differences relative to control group by the unpaired t-Student criterion.*

Table 4.
The effect of GZK-111 on immobilization time in the Porsolt forced swimming test in mice after 7-day administration.

Compound	Dose, mg/kg, i.p. (n = 10)	Immobilization time, s	Immobilization time compared to the control, %
GZK-111	Control	180.1 ± 10.7	100
	0.1	188.4 ± 9.3	105
	1.0	163.2 ± 11.2 (p = 0.08)	91
	10.0	157.7 ± 9.2*	88
	10.0 (p.o.)	160.3 ± 10.4 (p = 0.06)	89
CPG [9]	Control	139 ± 8	100
	1.0	97 ± 6#	70
	2.0	101 ± 8#	73
Fluoxetine [9]	10.0	87 ± 9#	63

Notes: Immobilization time was taken as 100% for control animals.
n, number of animals;
**p < 0.05, the statistical significance of the differences relative to control group by the unpaired t-Student criterion;*
#p < 0.05;

Table 5.
The effect of GZK-111 on immobilization time in the Porsolt forced swimming test in mice after 14-day administration, compared to CPG.

dose of 4 mg/kg, an increase of the reaction thresholds induced by GZK-111 was observed after 90 min ($F(4, 44) = 3.8743$, $p = 0.00881$) that persisted for up to 2 h ($F(4, 44) = 3.2141$, $p = 0.02124$), compared with the control group ($p < 0.05$ and $p < 0.05$, respectively) (**Figure 7**). The data obtained indicate that GZK-111 exhibits dose-dependent analgesic activity, like CPG in rats [40].

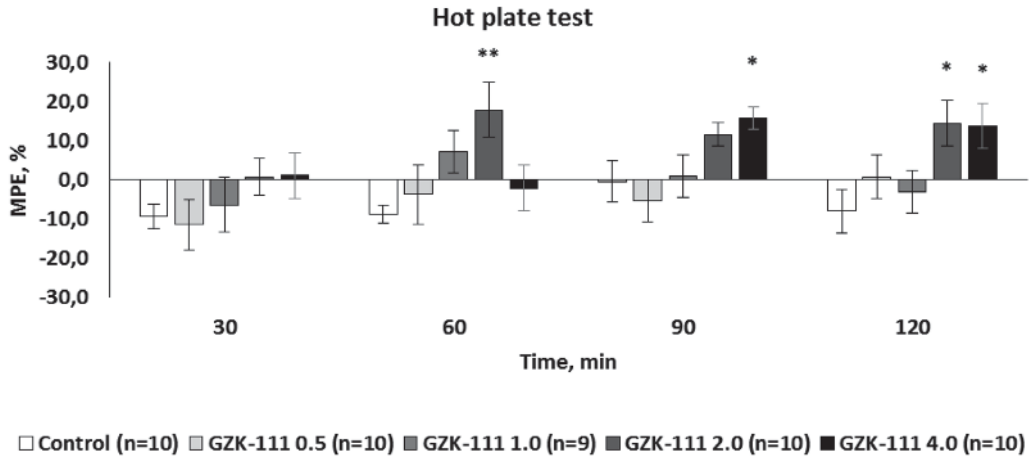

Figure 7.
*GZK-111 influences the pain response threshold during thermal stimulation in C57BL/6 mice. X-axis, time of the effect development after substance administration (min); y-axis, maximum possible effect (MPE), %. *$p < 0.05$; ** $p < 0.01$, statistically significant in relation to the corresponding control; according to Duncan criterion, the number of animals in groups n = 10; data are presented as mean values.*

4. Conclusions

Thus, GZK-111 exhibits nootropic, anxiolytic, antihypoxic, antidepressant, neuroprotective, and analgesic effects being characteristic to CPG. The compound is similar to CPG both in the spectrum of activities and in the stereospecificity and its nature. The ampakine CPG identical to endogenous one was proven to form during the fermentolysis of GZK-111. Therefore, GZK-111 can be considered as a "prodrug" of CPG and a pharmacologically active pro-ampakine with a BDNF-ergic mechanism of action.

Conflict of interest

The authors declare no conflict of interest, financial or otherwise.

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