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Neuroprotective properties of GABA and its derivatives in diabetic encephalopathy in old animals

I.N. Tyurenkov[1], D.A. Bakulin[1], A.V. Smirnov[1,2], M.R. Ekova[1,2], A.I. Bisinbekova[1,2], G.L. Snigur[1,2], Yu.I. Velikorodnaya[1,2], E.I. Morkovin[1,2], D.V. Verkholyak[1], O.S. Vasilyeva[3]

[1] Volgograd State Medical University,
1, Pavshikh Bortsov Sq., Volgograd, Russia, 400131
[2] Volgograd Medical Research Center,
1, Pavshikh Bortsov Sq., Volgograd, Russia, 400131
[3] Herzen Russian State Pedagogical University,
48, Moika Embankment, St. Petersburg, Russia, 191186

E-mail: mbfdoc@gmail.com

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The aim of the work was to evaluate the GABA neuroprotective properties and its structural analogues in old animals after seven months of hyperglycemia.

Materials and methods. Diabetes mellitus was modeled in white outbred male rats (12 months old) by the administration of a streptozotocin (65 mg/kg) and nicotinamide (230 mg/kg) combination. After 6 months, the animals with a postprandial glycemia level between 11 and 18 mmol/l were selected for the study. After the groups had been formed, the animals were administrated with GABA and GABAergic compounds (Compositions MPBA and PPC), respectively, for 1 month, the control group animals were administrated with saline. After the treatment, an oral glucose tolerance test and a set of behavioral tests aimed at studying sensory-motor (Open Field, Adhesion test, Rotarod) and cognitive functions (New Object Recognition and Morris Water Maze), as well as the functional state evaluation of the endothelium were performed. Further on, sampling of blood and brain tissues for a biochemical and enzyme immunoassay (the level of glucagon-like peptide-1 (GLP-1) and TNF-α in serum and the level of Klotho protein, BDNF, Nrf2, NF-κB and malondialdehyd (MDA) in brain homogenates), as well as a morphological analysis of changes in CA1 and CA3 neurons of the hippocampus and somatosensory cortex, was carried out.

Results. GABA and compositions with its derivatives had a pronounced neuroprotective effect in old animals with prolonged hyperglycemia. The hypoglycemic effect of the studied compositions was accompanied by an increase in the production of GLP-1. In the animals with DM, after 6 weeks of the test substances administration, higher rates of sensory-motor and cognitive functions and a less structural damage to the sensory-motor cortex and the brain hippocampus were recorded. These effects may be due to higher levels of the Klotho proteins, Nrf2 and BDNF, as well as lower levels of NF-κB, which may underlie the suppression of the oxidative stress, the reduction of MDA and inflammation (TNF-α).

Conclusion. After 6 weeks of the administration, GABA and its compositions in old animals (19 months old) significantly improved sensory-motor and cognitive functions, reduced negative structural changes in the hippocampus and somatosensory cerebral cortex.

Keywords: GABA; Klotho protein; diabetes mellitus; streptozotocin; NF-kB; Nrf2; GLP-1; endothelium; hippocampus

Abbreviations: BDNF – Brain-Derived Neurotrophic Factor; eNOS – endothelial nitric oxide synthase; NF-κB – nuclear factor-kB; Nrf2 – nuclear factor erythroid 2-related factor 2; STZ-NA – the streptozotocin-nicotinamide diabetes model; TNF α – tumour necrosis factor alpha; vWF – von Willebrand's factor; KP – Klotho protein; GABA – gamma-amino-butyric acid; GLP-1 – glucagon-like peptide-1; DI – Discrimination index (New Object Recognition test); ELISA – enzyme-linked immunosorbent assay; CBF – cerebral blood flow; OF – Open Field; OGTT – oral glucose tolerance test; NOR – New Object Recognition; DM – diabetes mellitus; CEC – circulating endothelial cells.

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RESEARCH ARTICLE

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Нейропротективные свойства ГАМК и её производных при диабетической энцефалопатии у старых животных

И.Н. Тюренков[1], Д.А. Бакулин[1], А.В. Смирнов[1,2], М.Р. Экова[1,2], А.И. Бисинбекова[1,2], Г.Л. Снигур[1,2], Ю.И. Великородная[1,2], Е.И. Морковин[1,2], Д.В. Верхоляк[1], О.С. Васильева[3]

[1] Федеральное государственное бюджетное образовательное учреждение высшего образования «Волгоградский государственный медицинский университет» Министерства здравоохранения Российской Федерации,
400131, Россия, г. Волгоград, пл. Павших Борцов, д. 1
[2] Государственное бюджетное учреждение «Волгоградский медицинский научный центр»,
400131, Россия, г. Волгоград, пл. Павших Борцов, д. 1
[3] Федеральное государственное бюджетное образовательное учреждение высшего образования «Российский государственный педагогический университет им. А.И. Герцена»
191186, Россия, г. Санкт-Петербург, наб. р. Мойки, д. 48

E-mail: mbfdoc@gmail.com

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Цель. Оценить нейропротекторные свойства ГАМК и её структурных аналогов на старых животных после продолжительной гипергликемии (7 мес).

Материалы и методы. Сахарный диабет моделировали на белых аутбредных крысах-самцах в возрасте 12 мес посредством введения комбинации стрептозотоцина (65 мг/кг) и никотинамида (230 мг/кг). Через 6 мес для исследования были отобраны животные с уровнем постпрандиальной гликемии между 11 и 18 ммоль/л. После формирования групп, в течение 1 мес животные соответственно получали ГАМК и ГАМК-ергические соединения (композиция МФБА и ФПС), контрольная группа получала физиологический раствор. После лечения был выполнен пероральный тест на толерантность к глюкозе и комплекс поведенческих тестов, направленных на изучение сенсорно-моторных (Открытое поле, Адгезивный тест, Ротарод) и когнитивных функций (Распознавание нового объекта и Водный лабиринт Морриса), а также проведена оценка функционального состояния эндотелия. Далее был произведен забор образцов крови и тканей головного мозга (ГМ) для биохимического и иммуноферментного (уровень глюкагоноподобного пептида-1 (ГПП-1) и фактора некроза опухоли альфа (ФНО-α) в сыворотке и уровень белка Клото, BDNF, Nrf2, NF-κB и малонового диальдегида (МДА) в гомогенатах ГМ), а также для морфологического анализа изменений нейронов CA1 и CA3 зон гиппокампа и соматосенсорной коры.

Результаты. ГАМК и композиции с её производными оказали выраженное нейропротекторное действие у старых животных с продолжительной гипергликемией. Гипогликемическое действие исследуемых композиций сопровождалось повышением продукции ГПП-1. У животных с СД после 6 нед введения исследуемых веществ регистрировали более высокие показатели сенсорно-моторных и когнитивных функций и меньшие структурные повреждения сенсорно-моторной коры и гиппокампа ГМ. Эти эффекты могут быть обусловлены более высоким уровнем белка Клото, Nrf2 и BDNF, а также более низким уровнем NF-κB, что может лежать в основе подавления окислительного стресса, снижения МДА и воспаления (TNF-α).

Заключение. ГАМК и её композиции у старых животных (19 мес) после 6 нед введения значительно улучшали сенсорно-моторные и когнитивные функции, уменьшали негативные структурные изменения в гиппокампе и соматосенсорный коре ГМ.

Ключевые слова: ГАМК; белок Клото; сахарный диабет; стрептозотоцин; NF-kB; Nrf2; ГПП-1; эндотелий; гиппокамп

Список сокращений: BDNF – нейротрофический фактор мозга; eNOS – эндотелиальная синтаза оксида азота; ГМ – головной мозг; NF-κB – ядерный фактор каппа B; Nrf2 – ядерный фактор, родственный эритроидному фактору 2; STZ-NA – модель стрептозотоцин-никотинамидного диабета; ФНО-α – фактор некроза опухоли альфа; vWF – фактор Виллибранта; БК – белок Клото; ГАМК – гамма-аминомасляная кислота; ГПП-1 – глюкагоноподобный пептид-1; ИД – индекс дискриминации в тесте Распознавания нового объекта; ИФА – иммуноферментный анализ; МК – мозговой кровоток; ОП – открытое поле; ПТТГ – пероральный тест на толерантность к глюкозе; РНО – тест Распознавание нового объекта; СД – сахарный диабет; ЦЭК – циркулирующие эндотелиальные клетки.

INTRODUCTION

Diabetes mellitus (DM) is the most common and progressive endocrine pathology. According to the International Diabetes Federation (IDF), among the world's population aged 20–79 years, there are currently 537 million people with diabetes, and by 2030, the number will reach 643, and by 2045 – 783 million [1]. With an increase in the number of patients with diabetes, the prevalence of its complications, which lead to an early disability and premature death, is steadily increasing, as well as the cost of their treatment [2, 3].

Hyperglycemia provokes and exacerbates many pathological conditions in almost all organs and tissues. Prolonged hyperglycemia plays a significant role in the development of encephalopathy affecting psycho-

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emotional, sensory-motor and cognitive functions, especially in adults and the elderly [4–6]. It has been established that patients with diabetes are more prone to cognitive impairment [7–9]. Xue M. et al. (2019), based on a meta-analysis of 122 studies, showed that DM increases the risk of developing cognitive impairment and dementia by 1.25–1.9 times compared with people without DM [7]. Therefore, important tasks in the treatment of DM are not only the normalization of glycemia, but also the prevention and treatment of its complications.

Prolonged hyperglycemia in DM provokes not only gluco- and lipotoxicity, but also enhances an oxidative and nitrosative stress, inflammation and other pathological processes, which can lead to accelerated apoptosis and death of intensively functioning cells in various organs and tissues, as well as accelerate the aging process. Among all the organs, the most sensitive and vulnerable to these pathological factors is the brain, especially such structures as the hippocampus and cortex [10]. Based on the above, the authors concluded that it is necessary to investigate the encephalopathic effects associated with hyperglycemia, while focusing on the hippocampus and somatosensory cortex.

In recent years, understanding of the physiological role of the GABAergic system, which was originally considered as the main inhibitory system playing a decisive role in ensuring various brain functions, has radically changed. Currently, GABA receptors and the GABA-synthesizing enzyme (glutamate decarboxylase) are found in the tissues of the cardiovascular and respiratory systems, in immunocompetent cells, the gastrointestinal tract, etc. [11, 12]. In pancreatic β cells, the density of GABA receptors is comparable to that in the brain [13]. Numerous publications also reflect the effect of GABAergic substances on the pancreatic function [11, 12, 14]. GABA derivatives are widely used in the clinic as drugs with neuro- and psychotropic effects and continue to be actively studied in experimental pharmacology. Thus, since DM is associated with a decrease in the cognitive function and structural changes in the brain [4–6], it seems promising to search for agents for the correction of diabetic encephalopathy caused by long-term hyperglycemia in a number of substances with a GABAergic effect.

During the preliminary screening, among the derivatives of linear and cyclic GABA, the substances with a pancreatic protective effect were isolated (Fig. 1). The results obtained formed the basis for the selection of these compositions to study their effect on the function and structure of the brain in old animals with long-term hyperglycemia.

THE AIM of the work was to evaluate the GABA neuroprotective properties and its structural analogues in old animals after seven months of hyperglycemia.

MATERIALS AND METHODS

Model objects

All the experiments were performed in accordance with the legislation of the Russian Federation and the technical standards of the Eurasian Economic Union for good laboratory practice (GOST R 53434-2009, GOST R 51000.4-2011). The study design and protocol were approved by the local ethical committee of Volgograd State Medical University, Protocol No. 2022/116 dated March 4, 2022 (registration number IRB 00005839 IORG 0004900 [OHRP]).

The experimental study was carried out on 50 outbred laboratory rats obtained from the nursery of "Stolbovaya" (Moscow region, Russia) at the age of 4–5 months. Animals were kept in the vivarium of the Scientific Center for Innovative Medicines of Volgograd State Medical University at the temperature of 20–22°C, the air humidity of 40–60%, the light regime of 12/12 h and a free access to drinking water and food (GOST R 51849-2001) (LLC Laboratorkorm, Moscow, Russia). By the beginning of the experiment, at the time of the administration of streptozotocin, the animals had reached the age of 12 months.

Test compounds

Composition MPBA (a composition of a linear GABA derivative with L-arginine) exhibits pronounced endothelioprotective properties [15, 16]. This also indicates the expediency of its study as a means for the prevention of DM vascular complications CD (Fig. 1).

Composition PPC (a composition of a 2-pyrrolidone derivative with succinic acid) has a nootropic, neuro-, cardioprotective effect [17, 18].

The both compositions were selected among various GABA derivatives in preliminary screening studies to research their pancreoprotective effect on the alloxan model of DM in animal survival tests, a preservation of β-cell mass, an ability to stimulate the production of glucagon-like peptide-1 (GLP-1) and improve a glucose utilization.

GABA was chosen as a reference substance for comparison of pancreatic protective properties, which are well reflected in the literature [12, 14].

Pathology modeling

DM was modeled by an intraperitoneal (ip) administration of streptozotocin – 65 mg/kg with a preliminary (15 min before) administration of nicotinamide (230 mg/kg, i.p.). Streptozotocin was diluted in cold citrate buffer (1 mM, pH 4.5); nicotinamide was diluted in a NaCl solution (0.9%). After 3 days, blood glucose levels were determined in all animals, and 50 individuals with a postprandial glycemia level of more than 11 and less than 18 mmol/l were selected for the experiment. Glucose levels were measured using

a Contour TS glucometer and appropriate test strips (Bayer). The blood for measurements was taken by puncture of the hyoid vein.

Experiment design

Subsequently, 50 selected animals were under constant observation for 6 months (after modeling DM) in the vivarium of the Scientific Center for Innovative Medicines of Volgograd State Medical University. Every 4 weeks, the level of glycemia was measured. During this observation period, prolonged DM caused the death of 6 (12%) animals, and 4 (8%) had a significant increase in postprandial glycemia (>18 mmol/l) and they were excluded from the study before the distribution into groups. After 6 months, fasting glycemia (4 h after food intake) was evaluated in rats and an oral glucose tolerance test (OGTT) was performed, in which glucose was administered at the dose of 4 g/kg *(per os).* Next, the rats were randomly distributed into 4 equal groups (*n*=10) with an average comparable level of glycemia (from 7.5 to 9.5 mmol/l after 4 h of fasting). After that, GABA, MPBA, and PPC were administered daily and continuously at the dose of 1000, 20, and 50 mg/kg *(per os),* respectively, for 30 days and then for another 2 weeks, during which the condition of the animals was evaluated. The negative control group (DM+0.9% NaCl) was similarly treated with saline (0.9% NaCl) in the volume of 0.1 ml/100 g (*n*=10). For the positive control, the rats without DM (intact) of the same age from the same animal importation lot (*n*=10) were used.

After 30 days of the test substances administration, the following manipulations were performed in turn for 12 days: on the 1st day an OGTT with the determination of glucose and GLP-1 was carried out: on the 2nd–3rd days, a sensory-motor function was assessed in the Open Field (OP) tests, an Adhesion test, and a motor coordination in the Rotarod test. On the 4th day, a NOR test was performed; on the 5–9th days, a Morris water maze test took place; on the 10–12th day after an endothelial function testing, euthanasia was performed with blood and brain sampling for a morphological study and an enzyme immunoassay of homogenates.

Based on the studied parameters totality, a comprehensive assessment of the morphofunctional state of the brain in the old animals with prolonged hyperglycemia, which had received GABA derivatives for 6 weeks, was made.

The developed study design is shown in Fig. 2.

Assessment of psychoneurological deficit

The motor and exploratory activity of the animals was assessed in the OF test. The installation "Open Field", (Research and manufacturing complex "Open Science", Russia). The animal was placed in the center of the arena and, using a webcam, its behavior was observed for 3 min, fixing the number of crossed squares (the distance traveled) as an indicator of a motor activity (MA). Herewith, the sum of "racks" acts and the number of the examined holes – minks – were evaluated as an exploratory activity (EA) of the animals.

An Adhesion test was used to assess a sensory function and fine motor skills. As a foreign object, stickers were placed on the volar surface of the forepaws of the animal – square pieces of an adhesive tape on a tissue basis (6×6 mm, Veropharm, Russia), and then for 3 min., the detection time (a sensory function) and the removal time of the sticker (a motor function) were recorded.

Movement coordination was assessed in the Rotarod test (a device manufactured by Neurobotics LLC, Russia), in which the total duration of keeping the animal on a rotating rod (25 rpm) was recorded in 3 attempts.

The New Object Recognition Test (NOR) is based on the natural need of animals to explore new objects and does not require the presence of external motivation. It is used to assess a cognitive function (the ability to identify and compare the information stored in short-term memory) and was performed in 2 stages: the 1st stage was a familiarization in a home cage (545×395×200 mm) with 2 identical objects (A1 and A2) for 3 min. Then, 60 min after the familiarization, the 2nd stage was performed, when the animal was placed in the same cage for 3 min, but one of the old (studied) objects (A2) was replaced with a new one (object B). Based on the test results, the discrimination index (DI) – the time spent on the study of a new object (B) minus the time spent on the study of the old object (A) in the second landing, was calculated.

After the treatment course, learning and retention of long-term spatial memory were assessed in the Morris Water Maze. The installation of "Water maze (Morris test)" (RMC "Open Science", Russia). The animals were trained for 5 days (4 attempts per day) to search for a flooded platform using landmarks on the wall of the setup, placing the rat at different starting points relative to the platform. In this study, the duration of the search for a flooded platform during the first landing was recorded for 5 days of the experiment.

Evaluation of functional state of cerebral vessels endothelium

The functional state of the endothelium was tested to determine the endothelium-dependent vasodilation and an antithrombotic function, by the number of desquamated endotheliocytes, by the level of the von Willebrand factor (vWF) in blood serum.

An endothelium-dependent vasodilation was assessed in the anesthetized animals (chloral hydrate 400 mg/kg) by relative changes in the flow level of the cerebral blood in response to the intravenous

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administration of acetylcholine (0.01 mg/kg, Acros Organics, USA; eNOS activator). The administration of acetylcholine caused an increase in the NO production, the endothelium-dependent vasodilation of blood vessels and an increase in the cerebral blood flow, which was determined in the projection of the middle cerebral artery by Doppler ultrasound Minimax-Doppler_K (LLC SP MINIMAX, Russia).

An antithrombotic function was assessed by the time of a complete thrombosis development, i.e. the blood flow cessation through the carotid artery when applying an iron (III) chloride solution to its adventitia.

The number of descaminated (circulating) endothelial cells (CECs) in the blood was determined by the method of Hladovec J [19]. An increase in the number of CECs makes it possible to judge the severity of an endothelial dysfunction, the degree of damage to the endothelium and its reparative activity.

The Von Willebrand factor (vWF), which is produced by the endothelium, was determined in the blood serum by enzyme-linked immunosorbent assay (ELISA) as a factor, with an increase in which one can judge endothelial damage and an endothelial dysfunction. An elevated vWF level is observed in DM and is a predictor of mortality from cardiovascular diseases [20].

The brain samples were homogenized in the lysis buffer (1 ml lysis buffer per 50 mg tissue sample) in a glass homogenizer on ice. The resulting suspension had been sonicated with an ultrasonic disperser until it became clear, then the solutions were centrifuged for 5 min at 10000g. The resulting supernatant was used for an enzyme immunoassay and a biochemical analysis.

Carrying out biochemical and enzyme immunoassay

The concentration of malonic dialdehyde (MDA) in the homogenates was determined using the reaction with thiobarbituric acid.

An ELISA was performed using ready-made kits (Cloud-Clone Corp., USA) in accordance with the manufacturer's instructions. The supernatant of the brain homogenates and blood serum were used for the analysis. The optical density was measured using a microplate analyzer SPECTROstar Nano (BMGLabtech, Germany) at the wavelength of 450 nm.

Morphological study

For morphological studies, the brain was fixed for 24 h in 10% neutral buffered formalin (pH 7.4) at 22–24°C. Then the samples were dehydrated in a battery of ascending strength alcohols, clarified in chloroform using a Cytadel 2000 histoprocessor (Shendon, UK), and embedded in a Histomix paraffin medium (Biovitrum, Russia). Paraffin blocks were cut on a rotary microtome HM34OE (MICROM, Germany), the sections 5 μm thick were obtained and mounted on the glass treated with poly-L-lysine (Menzel, Germany). The staining was carried out with thionin according to the Nissl method.

The structural changes in the brain cortex, a specific number of hyperchromic neurons (a reversible damage) and hyperchromic shriveled neurons (an irreversible damage) were evaluated.

Histological sections were photographed with an AxioCam 305 color digital camera (Carl Zeiss Microscopy GmbH, Germany) based on an AxioImager A2 microscope (Carl Zeiss Microscopy GmbH, Germany).

Statistical processing

Statistical processing of the obtained results was carried out by methods of descriptive and analytical statistics using Prism 6 Software (GraphPad Software Inc., USA). The distribution of quantitative indicators was assessed using the Shapiro–Wilk test. The intergroup differences were assessed using the Kruskal–Wallis test and the Dunn's post hoc test. The numerical values were presented as the arithmetic mean and the standard error of the arithmetic mean (M±m). To express a specific quantity of neurons in the pyramidal layer of the hippocampus, the interquartile range Me (Q1; Q3) was indicated, where Me is the median, Q1 is a 25 percentile, Q3 is a 75 percentile. The differences were considered significant at $p < 0.05$.

RESULTS

Assessment of psychoneurological deficit

It is assumed that hyperglycemia in DM is the main pathogenetic factor contributing to the deterioration of cognitive functions, neurodegeneration, the progress of aging, brain atrophy, and dementia [21]. The test suite used was chosen to capture abnormalities consistent with the clinical consequences for the brain as viewed from long-term uncontrolled hyperglycemia.

In animals aged 19 months with prolonged hyperglycemia (7 months) in the RD (relative density) test, a lower motor activity and a significantly reduced exploratory behavior were recorded in intact animals of the same age. In all experimental groups receiving treatment, higher rates of exploratory activity were recorded. In the group that received the composition of 2-pyrrolidone derivative (PPC), a motor activity was also significantly higher (Fig. 3A).

In the Adhesion test, in the animals with chronic hyperglycemia without treatment, a sensory dysfunction and a pronounced deterioration in fine motor skills were observed compared with the animals of the intact group. In the group treated with PPC, the time of detection and disposal of a foreign object was significantly less than in the animals of the control group (Fig. 3B).

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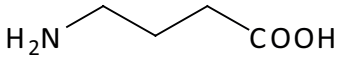
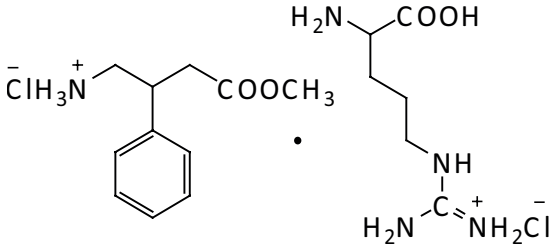


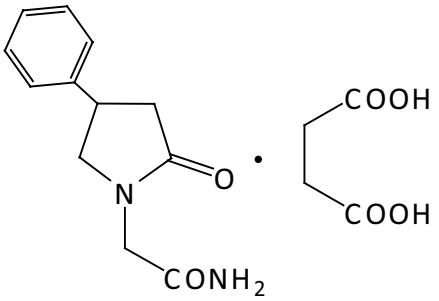
1

4-Aminobutanoic acid (GABA)
(Trivial nomenclature: γ-Aminobutyric acid or GABA)



2

DL-4-amino-3-phenylbutanoic acid methyl ester hydrochloride and L-2-amino-5-guanidinepentanoic acid monohydrochloride (L-arginine monohydrochloride) in a 1:1 ratio
(Substances code: MPBA)



3

2-(2-Oxo-4-phenylpyrrolidin-1-yl)acetamide and butanedioic (succinic) acid in ratio 2:1
(Substances code: PPC)

Figure 1 – Test compounds

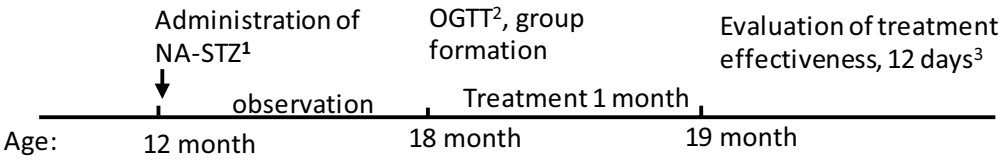


Figure 2 – Experiment design

Note: 1 – 3 days after the administration of streptozotocin with nicotinamide (NA-STZ), animals with postprandial glycemia levels >11 and <18 mmol/l were selected; 2 – after 6 months, an oral glucose tolerance test (OGTT) was performed and 4 groups were formed, comparable in terms of glycemia; 3 – after the treatment, the following indicators were assessed:
Day 1. Assessment of carbohydrate metabolism (OGTT) and determination of GLP-1.
Day 2. Motor and exploratory activity (Open field), sensory-motor disorders (Adhesion test).
Day 3. Coordination disorders (Rotarod).
Day 4. Assessment of short-term memory (a New Object Recognition).
Day 5–9. Long-term memory assessment (Morris Water Maze).
Day 10–12. Assessment of an endothelium-dependent vasodilation and an antithrombotic function of the endothelium, euthanasia, sampling of blood and brain for the morphological and enzyme immunoassay (ELISA).

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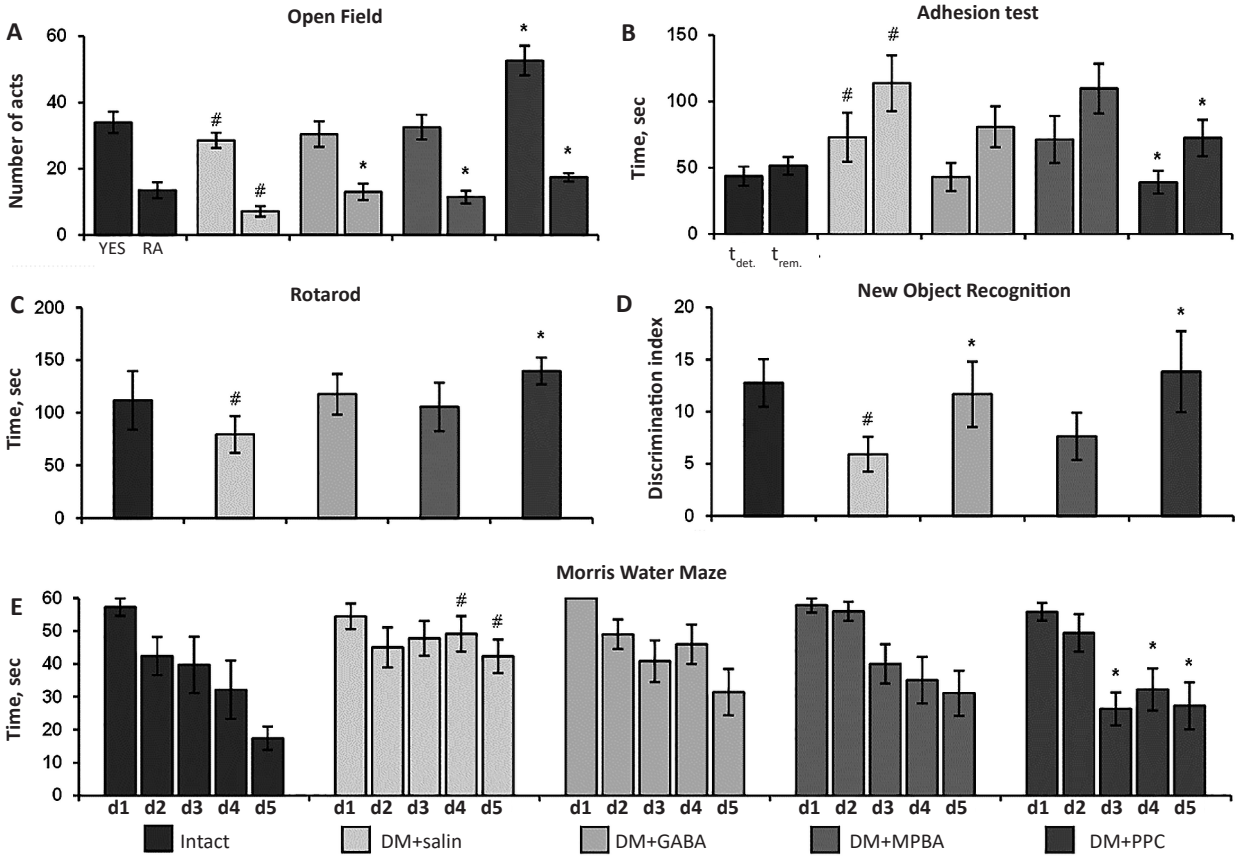

Figure 3 – Motor and exploratory activities in the Open Field test (A), average time of detection and removal of the sticker in the Adhesion test (B), retention time on a rotating rod in the Rotarod test (C), Discrimination index in the New Object Recognition test (D) and the duration of the search for a flooded platform in the Morris Water Maze test (E)

Note: # – differences are statistically significant in comparison with the animals of the "Intact" group (p <0.05); * – differences are statistically significant in comparison with the animals of the "DM+salin" group (p <0.05) (Kruskal–Wallis and Dunn test); the data are presented as M±m. In the Open field test: YES – motor activity (left columns), the number of sectors crossed in 3 minutes, RA – research activity (right columns), the sum of research acts. In the Adhesion test: $t_{det.}$ – average time of sticker detection on the palmar surface of the forepaws (left columns), $t_{rem.}$ – average sticker removal time (right columns); in the Morris Water Maze test, the duration of the search for a flooded platform during the first landing during 5 days of the experiment is shown.

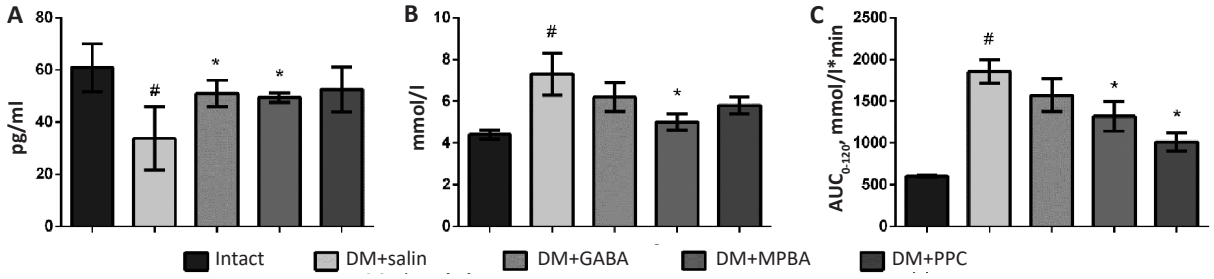

Figure 4 – Serum GLP-1 level (A), fasting blood glucose level (B) and area under glucose level–time curve (C)

Note: # – differences are statistically significant in comparison with the animals of the "Intact" group (p <0.05); * – differences are statistically significant in comparison with the animals of the "DM+salin" group (p <0.05; Kruskal–Wallis and Dunn test); data are presented as M±m.

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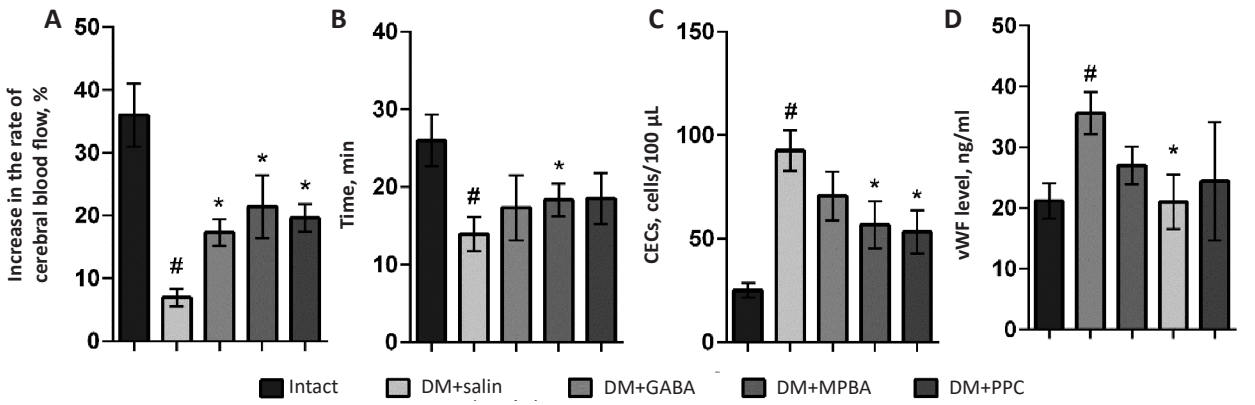

Figure 5 – Relative increase in the rate of cerebral blood flow after the administration of acetylcholine (A), the duration of thrombus formation in the carotid artery with the application of iron (III) chloride (B), the number of circulating endothelial cells (CECs) in plasma (C), the serum level of von Willebrand factor (vWF; D)

Note: # – the differences are statistically significant in comparison with the animals of the "Intact" group (p <0.05); * – the differences are statistically significant in comparison with the animals of the "DM+salin" group (p <0.05) (Kruskal–Wallis and Dunn test); the data are presented as M±m; MDA is malonic dialdehyde.

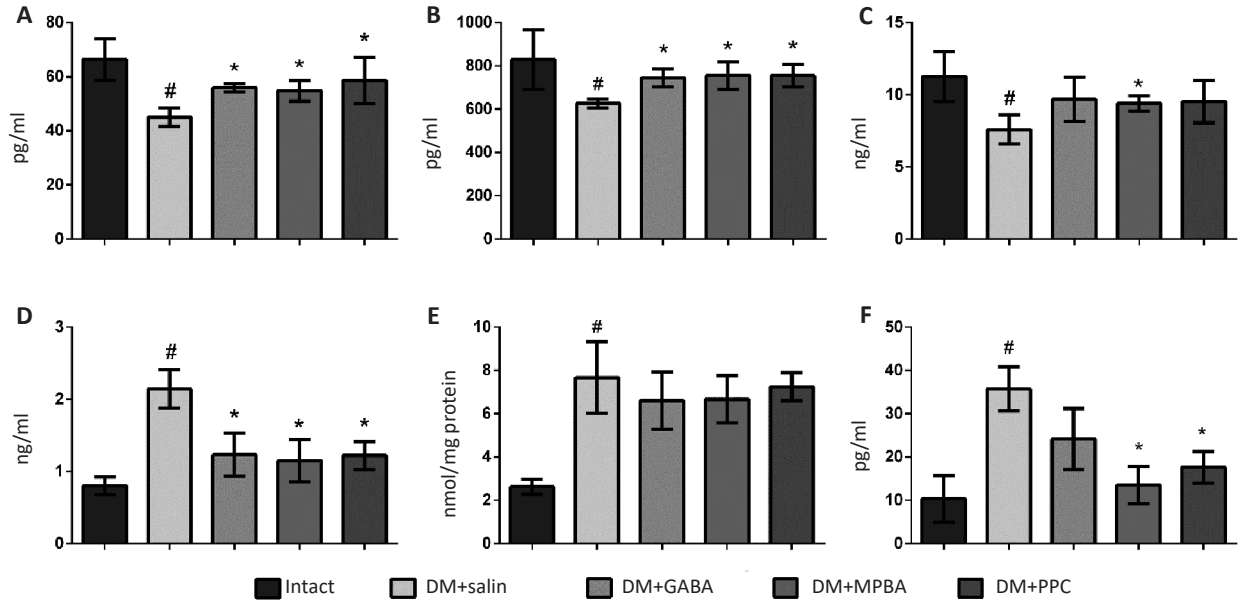

Figure 6 – The concentration of Klotho protein (A), BDNF (B), Nrf2 (C), Nf-kB (D), MDA (D) in brain tissues and TNF-α in blood serum (F) in animals with experimental DM

Note: # – the differences are statistically significant in comparison with the animals of the "Intact" group (p <0.05); * – the differences are statistically significant in comparison with the animals of the "DM+salin" group (p <0.05) (Kruskal–Wallis and Dunn test); the data are presented as M±m; MDA is malonic dialdehyde.

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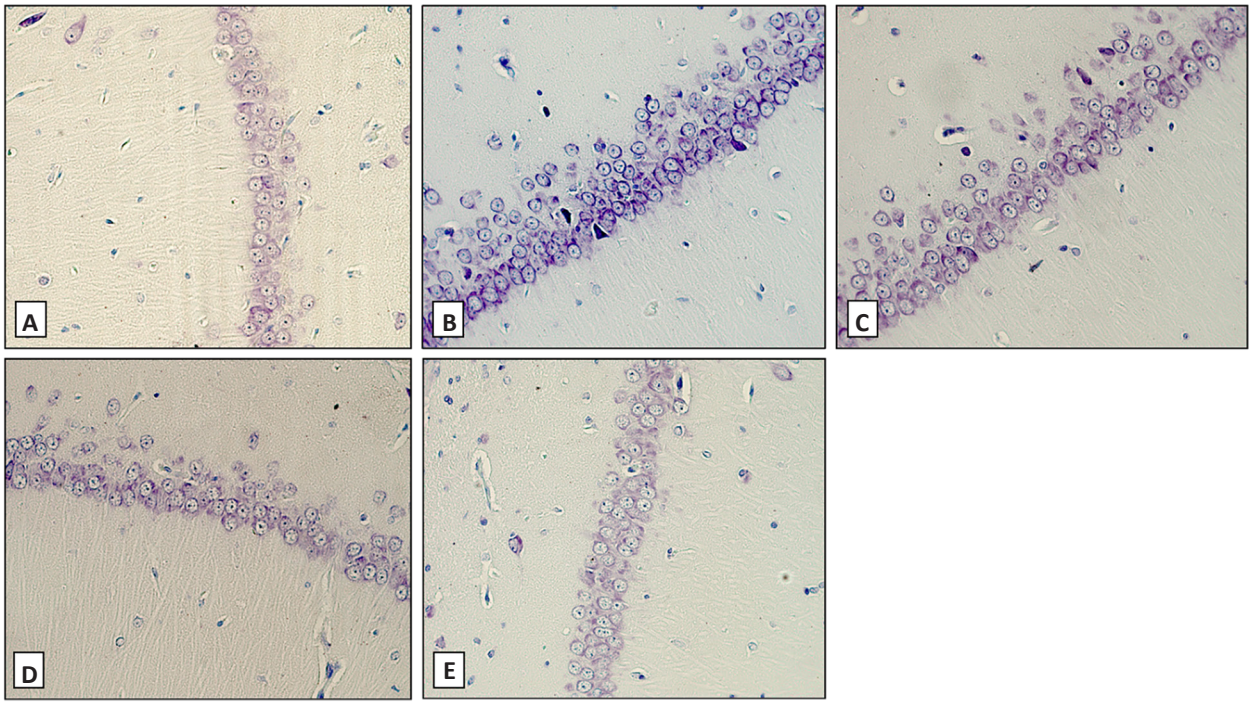

Figure 7 – Morphological changes in pyramidal layer CA1 of hippocampus

Note (here and in Fig. 8): A – Intact; B – DM+salin; C – DM+GABA; D – DM+MPBA; E – DM+PPC. Stained with thionine according to the Nissl method. Magn. ×400.

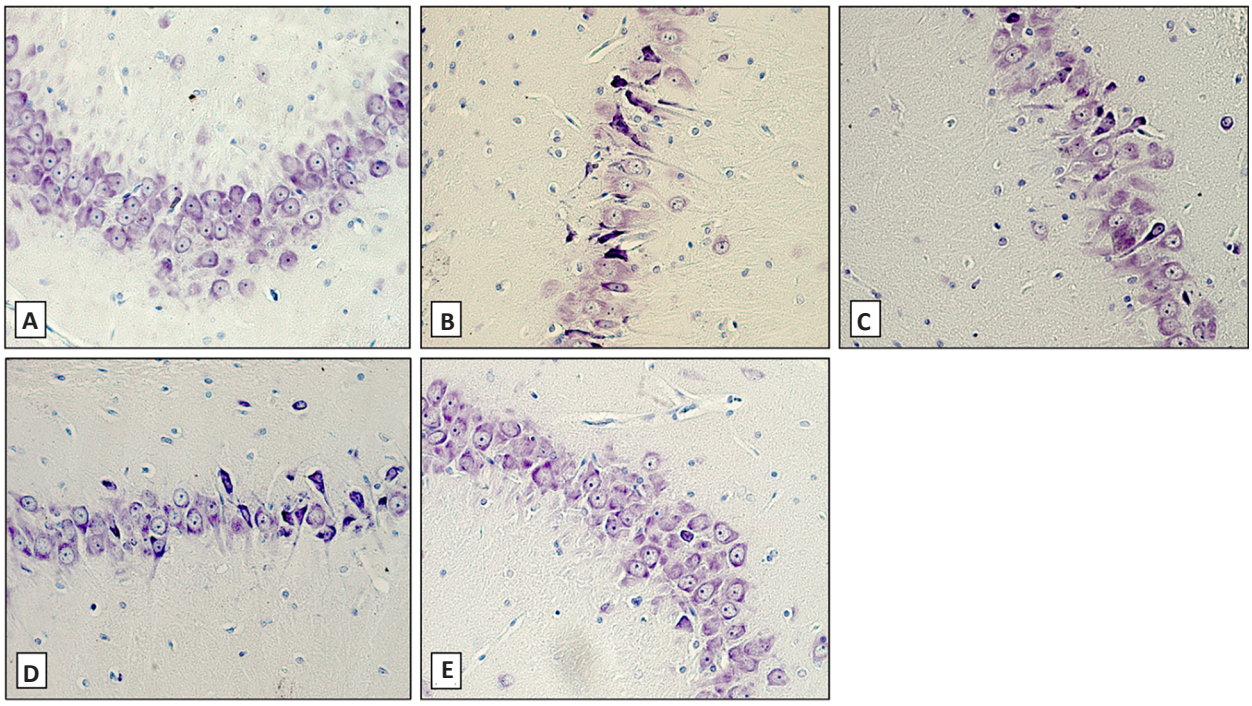

Figure 8 – Morphological changes in pyramidal layer CA3 of hippocampus

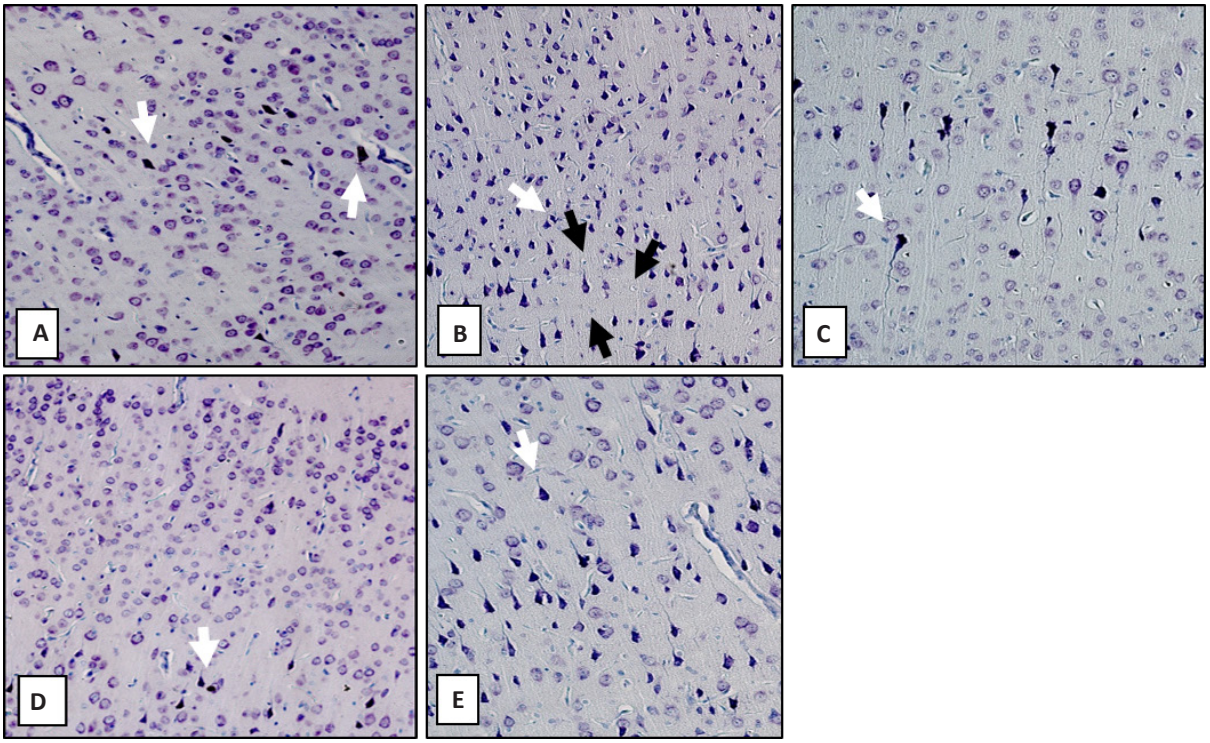

Figure 9 – Histological structure of the primary sensorimotor cortex of the studied animals

Note: A – Intact (the presence of single hyperchromic and hyperchromic shriveled neurons in all layers); B – DM+salin (pronounced hyperchromatosis in all layers, the presence of zones of "neurons loss"); C – DM+GABA (hyperchromatosis in the inner pyramidal layer); D – DM+MPBA (mild hyperchromatosis); E – DM+PPC (presence in the outer granular, outer pyramidal and inner pyramidal layers of a significant number of shriveled neurons with hyperchromatosis of the cytoplasm). White arrows are hyperchromic shriveled neurons. Black arrows are zones of neurons "loss". Stained with thionine according to the Nissl method. Magn.×200.

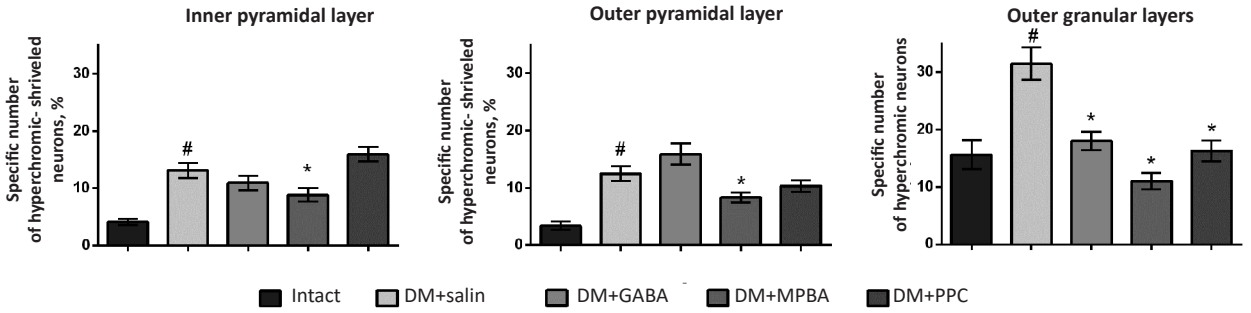

Figure 10 – Specific number of hyperchromic, hyperchromic-shriveled neurons in the primary somatosensory cortex of rats' brain

Note: # – the differences are statistically significant in comparison with the animals of the "Intact" group (p <0.05); * – the differences are statistically significant in comparison with the animals of the "DM+salin" group (p <0.05) (Kruskal-Wallis and Dunn test). The data are presented as M±m.

Table 1 – Specific number of neurons in pyramidal layer of hippocampus with hyperchromatosis and shriveled neurons with hyperchromatosis

Hyperchromatic neurons Me (Q1-Q3), %					
Hippocampus zone	Intact	DM+salin	DM+GABA	DM+MPBA	DM+PPC
CA1	0.8 (0–4.7)	11.6 (3.1–16.9) #	0 (0–0)*	0 (0–0)*	0 (0–0)*
CA3	0 (0–0)	17.5 (2.8–27.9) #	2.6 (0–18.8)	0 (0–5.9) *	0 (0–25.6)
Hyperchromic shriveled neurons Me (Q1-Q3), %					
Hippocampus zone	Intact	DM	DM+GABA	DM+PPC	DM+MPBA
CA1	1.8 (0–4.4)	3.6 (0–9.2)	3.1 (1.9–6.9)	5.5 (0–18.4)	2 (0–10.9)
CA3	4.4 (0–11.5)	2.3 (0–21.9)	0 (0–7.8)	2.4 (0–31)	0 (0–4.2)

Note: # – the differences are statistically significant in comparison with the animals of the "Intact" group (p <0.05); * – the differences are statistically significant in comparison with the animals of the "DM+ salin" group (p <0.05) (Kruskal–Wallis and Dunn test); GABA – gamma-aminobutyric acid; DM – diabetes mellitus.

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In the Rotarod test, in the animals of the negative control group, the retention time was statistically significantly less than in the intact animals, which indicated an impaired coordination of movements. The animals treated with GABA, MPBA, and especially PPC, stayed longer on the rotating rod (Fig. 3C).

In the New Object Recognition test, a significant decrease in the DI (impaired short-term memory) was noted in the group of the animals with DM. A significantly longer examination time of a new object was recorded compared to the old object, i.e. the animals were distinguished by a better ability to identify and compare the information stored (for 60 min) about a previously studied subject (Fig. 3E).

In the Morris Water Maze test, the preservation of long-term spatial memory was assessed by the time it took to search for a submerged platform for 5 days. In the group of the animals with chronic hyperglycemia without treatment, a violation of long-term memory was noted: every day of the experiment, on the first landing, they searched for a flooded platform longer than the intact ones (Fig. 3E). In the groups treated with GABA, MPBA, and especially PPC from the 3rd day in each first test session, the animals found the flooded platform faster than in the control group, i.e. the preservation of long-term spatial memory was better.

Thus, in the animals with DM, in comparison with the intact animals, there were especially pronounced impairments in a cognitive function (New Object Recognition, the Morris Water Maze, and an EA in the OF) and fine motor skills (Adhesion test). A decrease in the motor activity (OF), deterioration in coordination (Rotarod) and a sensory function of the forelimbs (Adhesion test) were also observed.

A course administration of GABA, its linear derivative (MPBA), and especially its cyclic derivative (PPC) contributed to the improvement of the functional state of the brain, reducing the severity of cognitive, sensory and motor impairments in the animals with prolonged hyperglycemia.

Effect of GABA derivatives on carbohydrate metabolism

At the time of assessing the functional state of the brain, in the animals with DM, the level of GLP-1 in the control group was significantly lower than in the intact animals of the same age. In the animals treated with the test substances, the level of GLP-1 in the blood serum was comparably on average by 50% higher than in the animals of the control group (Fig. 4A).

During the OGTT, against the background of the course administration of MPBA and PPC compositions, a normalization of carbohydrate metabolism was noted in the animals. It was manifested in a decrease in the level of glycemia (after a 4-hour fast) and the area under the glucose level-time curve (Fig. 4B and 4C).

Effect of test substances on endothelial dysfunction

DM significantly increases the risk of developing cardiovascular diseases, and the latter increase the likelihood of developing vascular dementia [6, 22, 23]. At the same time, an endothelial dysfunction plays a significant role in pathogenesis.

In the present work, after assessing the neurological deficit in these animals, a study of the functional state of the endothelium was carried out. In the animals with DM without treatment, a pronounced decrease in endothelium-dependent vasodilation was noted: a slight increase in the level of the cerebral blood flow in response to the administration of acetylcholine compared with the animals of the intact group (Fig. 5A). In the animals that were injected with GABA, PPC, and especially MPBA, a significantly more pronounced increase in the level of the cerebral blood flow than in the control group of animals, was observed in response to the administration of acetylcholine.

When evaluating the antithrombotic function of the endothelium, it was found that when iron (III) chloride was applied to the adventitia of the common carotid artery, the time to stop a blood flow in the animals of the control group was almost twice shorter than in the intact ones (Fig. 5B). In the animals treated with MPBA, the time of a thrombus formation was significantly longer than in the animals of the control group.

To assess an endothelial dysfunction in the DM animals, two more indicators were used: the number of CECs and the von Willebrand factor (vWF). The amount of CEC in control animals with DM was 4 times higher than in intact animals, and in animals treated with GABA derivatives (MPBA and PPC), the amount of CEC was significantly lower than in the control group of animals (Fig. 5C).

The level of vWF in the DM animals without treatment was 68% higher than in the intact animals (Fig. 5D). In the animals treated with the composition of MPBA, the content of vWF in the blood serum was significantly lower than in the animals of the negative control group.

Thus, against the background of prolonged hyperglycemia, a pronounced endothelial dysfunction is formed, which may underlie structural and functional changes in the brain. The studied GABA derivatives (MPBA and PPC) improved the vasodilating and antithrombotic function of the endothelium, which may play an important role in adaptation and ensuring an adequate blood flow in intensively functioning structures.

Assessment of Klotho protein, BDNF, Nrf2, NF-κB, TNF-α and MDA levels

The literature data [12, 24] indicate that GABA increases the production of Klotho protein (KP),

which affects a cognitive function [25, 26]. It has been established that the main effects of KP are associated with its influence on the expression of nuclear transcription factors Nrf2 and NF-κB, which play an important role in the development of DM and its complications. In DM, along with a decrease in KP, there is also a decrease in the expression of a brain-derived neurotrophic factor (BDNF), which is expressed and synthesized not only in the brain but also in the pancreas, intestines, and other tissues, where it plays an important role in cytoprotection [27, 28].

Compared with the intact animals, the untreated DM animals had significantly lower levels of KP, BDNF, and transcription factor Nrf2, higher levels of transcription factor NF-κB, MDA, and higher serum levels of TNF-α (Fig. 6). Against the background of a course administration of GABA, PPC, and especially MPBA, there was a normalization of the content of the noted markers observed: KP, BDNF and transcription factors, as well as the pro-inflammatory cytokine TNF-α and the main product of lipid peroxidation, MDA.

Morphology of hippocampus

SD leads to the formation of structural and functional changes in the brain. Some areas of the brain, primarily the hippocampus, are particularly sensitive to prolonged hyperglycemia, which is one of the causes of a cognitive decline [29, 30]. At the same time, a relationship has been proven between the degree and duration of hyperglycemia and the risk of developing dementia in people with DM [4].

In the CA1 zone of the old intact rats' hippocampus, most neurons in the pyramidal layer were characterized by a close to rounded perikaryon with a centrally located rounded light nucleus and, as a rule, a well-defined nucleolus. There were sporadic areas of neuron loss, neurons with focal chromatolysis, and shriveled hyperchromic cells (Fig. 7A). In CA3, the pyramidal layer neurons were more dispersed than in CA1, and had a polygonal shape with a clearly visualized nucleus and one nucleolus; single hyperchromic neurons, shriveled neurons with hyperchromia, neurons with focal chromatolysis, and single areas of neuronal loss were found (Fig. 8A).

In the group of DM animals without treatment, in CA1 of the pyramidal layer compared with intact, some animals showed areas of neuronal loss, and in the terminal sections of the CA1 pyramidal layer, neurons with focal chromatolysis, hyperchromic and hyperchromic shriveled neurons were found, which were located in a group (Fig. 7B). In CA3 of the pyramidal layer, neurons were located more loosely than in CA1 (Fig. 8B). In all the animals there were areas of loss of neurons, focal chromatolysis. Most animals showed neurons with hyperchromic cytoplasm and hyperchromic neurons with shriveled perikaryons. There was a significant increase in the specific number of hyperchromic neurons by 10.8% in CA1 (p <0.05) and by 17.5% in CA3 of the hippocampus (p <0.05), compared with intact animals (Table 1). At the same time, there were no significant differences in the change in the specific number of hyperchromic shriveled neurons with CA1 and CA3 (p > 0.05) (Table 1).

In old DM rats, with a pharmacological correction of GABA, PPC, and MPBA, most neurons in the pyramidal layer were characterized by normochromic cytoplasm. In some animals, neurons with cytoplasmic hyperchromatosis and hyperchromic shriveled neurons were found; these neurons were located in a group in the terminal CA1 sections, and areas of neurocytes loss were observed (Fig. 7C–7E; Fig. 8–8E).

During statistical processing of the data in the CA1 zone of the hippocampus with the use of GABA, PPC and MPBA, a significant decrease in the specific number of hyperchromic neurons (p <0.05), compared with the DM group was found (Table 1). The use of MPBA also demonstrated statistically significant differences in CA3, a specific number of hyperchromic neurons decreased by 17.5% (p <0.05) (Table 1).

Morphology of somatosensory cortex

Histological examination of the intact rats' primary somatosensory cortex (Fig. 9) revealed single hyperchromic and hyperchromic-shriveled neurons in all layers. However, in some animals, loci of pronounced hyperchromatosis were found in layers 2nd, 3rd, and 5th, where, along with unchanged neurons, there were the neurons, the perikarya of which had an irregular shriveled, elongated or twisted shape, the nucleolus was not visualized. The pathological changes found in the intact rats' cortex, are associate with the age of the studied rats.

In experimental modeling of DM in aging rats (aged 12–19 months), the most pronounced signs of damage were found in neurons of layers 2nd, 3rd, and 5th of the primary somatosensory cortex. Herewith, an increase in the areas of neurons loss perikarya, revealed in layers 4th, 5th of the primary somatosensory cortex, indicates the development of atrophic changes in the cortex of the rats' brain, which is confirmed by a decrease in cognitive functions.

After treatment in aging rats (at the age of 12–19 months), the least pronounced pathological changes were found in neurons of layers 2nd, 3rd, 5th of the primary somatosensory cortex in the MPBA correction group; which indicates a protective effect of the composition on the structure of the cortex of the rats' brain.

A morphological study of the somatosensory cortex of DM rats' brain without treatment showed a significantly higher relative number of hyperchromic shriveled neurons compared to the animals without DM (Fig. 7).

In the MPBA pharmacocorrection group, a statistically significant decrease in the specific number of

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hyperchromic-shriveled neurons in the inner pyramidal, outer pyramidal, and outer granular layers was found compared to the DM group without treatment.

In DM rats treated with GABA and PPC, a significant decrease in the specific number of hyperchromic neurons in the outer granular layer was found compared to DM without treatment.

DISCUSSION

Under conditions of prolonged hyperglycemia, Nrf2 is considered as a key factor counteracting an oxidative stress, which activates the expression of genes responsible for the expression of antioxidant defense enzymes [31]. Therefore, the search for ways to activate the Nrf2 system is considered a potential cytoprotective strategy for the prevention and treatment of diseases with the pathogenesis based on an oxidative stress, including neurological pathologies [32–34].

The Nrf2 system and the NF-κB system are in an antagonistic relationship. For example, when Nrf2 is deficient, there is an increased NF-κB activity, and the Nrf2 activation has anti-inflammatory effects in many rodent models of inflammation. It has been shown that Nrf2 suppresses the expression of pro-inflammatory cytokine genes and has the ability to negatively affect NF-κB by inducing an antioxidant response [35].

In order to increase the translational potential, the present study was carried out on old animals with experimental DM, which already have pronounced functional and structural disorders in the brain. The vast majority of studies aimed at studying antidiabetogenic effects and diabetic complications, are performed on young animals at the age of 4–6 months, and the duration of modeled diabetes is limited to 12–16 weeks. Despite this fact, the development of age-related changes is significantly aggravated by prolonged hyperglycemia and an associated inflammation, glucose and lipotoxicity, oxidative and nitrosative stress.

The study was of a complex nature with the assessment of pathogenetic changes based on the morphofunctional approach. It showed that in old animals (19 months) with long-term (7 months) hyperglycemia, there were pronounced impairments of sensory and motor functions, coordination of movements, as well as a pronounced decrease in short-term, long-term and spatial memory. These cognitive impairments are of great importance in the clinic, as they reduce the quality of life of the patient, and in case of profound impairments, they become a burden for the family and society. The mitigation of cognitive impairments in patients with DM remains an unresolved task, even with the normalization of blood glucose levels.

Functional disorders are basically consistent with the structural disorders noted at the next stage. In old DM rats, the most pronounced signs of damage were found in pyramidal neurons in the CA1 and CA3 zones of the hippocampus, the areas of neuron loss, a significant increase in the specific number of neurons with signs of reversible changes (neurons with hyperchromia of the perikaryon cytoplasm without shriveling), an increase in the number of shriveled hyperchromic neurons. These are consistent with the data on progressive atrophic changes, ultrastructural damage to neurons and hippocampal synapses, which are accompanied by an increased oxidative stress, neuroinflammation, neuronal apoptosis, as well as cognitive deficits, learning and memory impairments in DM [21, 36, 37].

In the present article, the use of GABA derivatives, PPC and especially MFBA for pharmacological corrections, contributed to a significant decrease in signs of reversible disorders in pyramidal neurons CA1 and CA3 in the hippocampus zones, a decrease in the specific number of hyperchromic neurons compared to the DM group without treatment. At the same time, the drugs used did not have a significant effect on the level of neurons with signs of irreversible damage (hyperchromic shriveled neurons) in the pyramidal layer in the CA1 and CA3 zones of the hippocampus.

A comprehensive analysis of structural changes in the primary sensorimotor cortex and hippocampus of old rats with experimental DM showed the predominance of signs of irreversible and reversible neuronal damage. These changes were more pronounced in the outer granular, outer pyramidal and inner pyramidal layers. The damage to the neurons of the pyramidal layer of the CA1 and CA3 zones of the hippocampus was accompanied by the appearance of areas of neurons loss and a decrease in the absolute and relative areas of the perikarya of neurons in the primary sensorimotor cortex of the brain, which indicates the progression of atrophic processes.

Impairments of cognitive functions (an exploratory activity, short-term and long-term spatial memory) in rats with diabetes compared to intact animals of the same age (19 months) were accompanied by the development of morphological signs of neuronal damage and atrophic changes in the primary sensorimotor cortex and hippocampus. The use of GABA derivatives as a pharmacological correction for 1 month contributed to a decrease in the severity of sensory-motor and cognitive impairments, a decrease in morphological signs of neuronal damage and atrophic changes, which may be based on the normalization of glucose levels, improvement of the vasodilating and antithrombotic functions of the endothelium. They are considered leading pathogenetic factors in the development of diabetic encephalopathy and angiopathy [21].

Damage to neurons and glia contributes to the

combined effect of an increased oxidative stress, neuroinflammation, neurotransmitter abnormalities, respectively, the use of drugs that normalize the above processes, promotes neuroprotection, reducing neurodegenerative changes in the hippocampus and cerebral cortex during the progression of diabetic encephalopathy [38]. Therefore, close attention to GABA, which stimulates the production of PK, is paid. In this study, it was noted that the level of Klotho protein in the brain increased under the influence of both GABA and its derivatives, which had been used in much lower doses. At the same time, KP is considered a reasonable therapeutic target due to its ability to increase the activity of various body defense systems under the influence of a number of damaging factors: oxidative and nitrosative stress, inflammation, mitochondrial dysfunction, apoptosis and cell death, and to prevent early aging processes [12, 39].

Particularly noteworthy is the fact that the detected multiple heterogeneous signs of damage in the cerebral cortex and hippocampus arose with a combination of the implementation of age-dependent factors in conditions of hyperglycemia in DM. Accordingly, the use of drugs that have multimodal neuro-, geroprotective, hypoglycemic effects is becoming the preferred therapy strategy. Thus, it has been shown that aging and DM are accompanied by a decrease in the production of KP, expressed in the kidneys, brain, pancreatic beta cells and other tissues [24, 40, 41], and the use of GABA leads to pancreatic protective effects, increases the level of circulating Klotho protein. GABA and KP inhibit the activation of the NF-κB protein, which promotes the stimulation of inflammatory reactions that trigger beta-cell apoptosis [12, 39], which makes it possible to consider GABA derivatives as a promising group for studying their effects in the treatment of diabetic encephalopathy in the elderly.

What underlies the neuroprotective action of GABA and its derivatives (MPBA and PPC)? The answer to this question is of fundamental importance. All of them had a unidirectional effect, improving sensory-motor, cognitive, and endothelial function in DM rats. This may indicate a similar mechanism of action. Given the analogy of the action of GABA and KP on pancreatic β-cells. It has been shown that the cytoprotective effect of GABA is associated with an increase in the production and level of KP [12]. In animals with KP knockout, the pancreatic protective effect of GABA was significantly reduced. Therefore, in this work, after a 30-day administration of GABA, the content of KP in the brain was determined. In DM animals that did not receive the test substances, the content of KP in the brain was significantly lower than in intact animals. In the animals treated with GABA derivatives, the level of KP was statistically significantly higher than in the animals of the control group. This action of the studied substances deserves special attention, because its content in the brain, fluid media, blood serum, urine, etc. can serve as a marker of diabetic complications, aging, prognosis of dementia and cardiovascular risks, as well as the assessment of the severity course of various diseases, when its level decreases [42]. KP deficiency plays an important role in the pathogenesis of cognitive impairments [26] and cardiovascular diseases [43].

At present, a wide spectrum of biological activity of the anti-aging Klotho protein is presented in the literature [12, 43, 44]. It has been established that in many pathologies (cardiovascular diseases, DM, kidney pathology, aging), its level decreases. Therefore, the search and development of substances that stimulate the production of KP is an important task. In relation to the interpretation of the results obtained, its effect on the expression of nuclear factors Nrf2 and NF-κB, which play a key role in the development of complications of DM, should be noted.

CONCLUSION

In old animals (aged 19 months) with long-term (7-month) hyperglycemia, disturbances in sensory-motor functions, coordination of movements, a pronounced decrease in short-term and long-term memory, and a significant deterioration in the vasodilating function of the cerebral vascular endothelium were found. These functional disorders were accompanied by the development of morphological signs of neuronal damage and atrophic changes in the primary sensorimotor cortex and hippocampus. Compared with intact animals, untreated DM animals had significantly lower levels of Klotho protein, BDNF, and transcription factor Nrf2, as well as higher levels of transcription factor Nf-kB, MDA, and TNF-α in brain homogenates.

A course GABA administration, its linear and cyclic derivatives (MPBA and PPC) contributed to an increase in the GLP-1 production and normalization of carbohydrate metabolism, a decrease in the severity of cognitive and sensory-motor disorders, an improvement in the vasodilating and antithrombotic function of the endothelium, which was accompanied by a decrease in morphological signs of neuronal damage.

The neuroprotective properties of GABA and its derivatives in diabetic encephalopathy in old animals can be explained by an increase in the levels of the anti-aging PK, brain-derived neurotrophic factor (BDNF), the level of the transcription factor Nrf2, which regulates the activity of antioxidant defense enzymes, as well as a decrease in the level of nuclear transcription factor NfkB and TNF-α, which are responsible for the formation and maintenance of inflammatory processes that underlie the development of diabetes complications.

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CONFLICT OF INTEREST

The authors declare no conflict of interest.

AUTHORS' CONTRIBUTION

Ivan N. Tyurenkov – idea and planning of the study, writing a draft of the article, approval of the final version of the manuscript; Dmitry A. Bakulin – modeling and control of pathology, design of the final version of the manuscript; Aleksey V. Smirnov, Maria R. Ekova, Aislu I. Bisinbekova, Grigory L. Snigur, Yulia I. Velikorodnaya – histochemical staining and assessment of morphological changes in the hippocampus and sensorimotor cortex of the brain, analysis and description of the results; Evgeniy I. Morkovin – assessment of neuropsychiatric deficit; Dmitry V. Verkholyak – assessment of cerebral blood flow and the functional state of the endothelium of cerebral vessels; Olga S. Vasilyeva – development of the studied compounds. All authors made a substantial contribution to the conception of the work, acquisition, analysis, interpretation of data for the work, drafting and revising the work, final approval of the version to be published and agree to be accountable for all aspects of the work.

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AUTHORS

Ivan N. Tyurenkov – Doctor of Sciences (Medicine), Professor, Head of the Laboratory of Pharmacology of Cardiovascular Drugs, Scientific Center for Innovative Medicines of Volgograd State Medical University; Corresponding Member of RAS. ORCID ID: 0000-0001-7574-3923. E-mail: fibfuv@mail.ru

Dmitry A. Bakulin – Candidate of Sciences (Medicine), Senior Researcher, Laboratory of Pharmacology of Cardiovascular Drugs of Scientific Center for Innovative Medicines of Volgograd State Medical University. ORCID ID: 0000-0003-4694-3066. E-mail: mbfdoc@gmail.com

Aleksey V. Smirnov – Doctor of Sciences (Medicine), Professor, Head of the Department of Pathological Anatomy of Volgograd State Medical University. ORCID ID: 0000-0001-5351-6105. E-mail: alexeysmirnov.volggmu@gmail.com

Maria R. Ekova – Candidate of Sciences (Medicine), Associate Professor of the Department of Pathological Anatomy of Volgograd State Medical University; employee of the laboratory of morphopharmacology of Volgograd Medical Research Center. ORCID ID: 0000-0001-8655-1441. Email: maria.ekova@mail.ru

Aislu I. Bisinbekova – resident in the specialty "Pathological Anatomy" of Volgograd State Medical University; Junior Researcher of Volgograd Medical Research Center. E-mail: aandm08@mail.ru

Grigory L. Snigur – Doctor of Sciences (Medicine), Associate Professor, Head of the Department of Biology of Volgograd State Medical University; Head of Volgograd Medical Research Center. ORCID ID: 0000-0002-8612-6186. E-mail: sgrigoryl@mail.ru

Yulia I. Velikorodnaya – correspondence graduate student of the Department of Pharmacology and Pharmacy of Volgograd State Medical University; researcher of Laboratory of Pathomorphology of Volgograd Medical Research Center. ORCID ID: 0000-0003-2976-6352. E-mail: alta-u@mail.ru

Evgeniy I. Morkovin – Candidate of Sciences (Medicine), Associate Professor of Department of Fundamental Medicine and Biology of Scientific Center for Innovative Medicines of Volgograd State Medical University; Head of the Laboratory of Neuropsychopharmacology of Volgograd State Medical University. ORCID ID: 0000-0002-7119-3546. E-mail: e.i.morkovin@gmail.com

Dmitry V. Verkholyak – Assistant of the Department of Chemistry of Volgograd State Medical University. ORCID ID: 0000-0002-6783-1867. E-mail: verholyakdima@mail.ru

Olga S. Vasilyeva – Candidate of Sciences (Chemistry), Senior Researcher, Laboratory of Nitro Compounds of Herzen Russian State Pedagogical University. ORCID ID: 0000-0002-0309-1580. E-mail: ovasja@gmail.com