Original Paper

Comparative study of the neuroprotective and nootropic activities of the carboxylate and amide forms of the HLDF-6 peptide in animal models of Alzheimer's disease

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Abstract

A comparative study of the neuroprotective and nootropic activities of two pharmaceutical substances, the HLDF-6 peptide (HLDF-6-OH) and its amide form (HLDF-6-NH_2), was conducted. The study was performed in male rats using two models of a neurodegenerative disorder. Cognitive deficit in rats was induced by injection of the beta-amyloid fragment 25–35 (βA 25–35) into the giant-cell nucleus basalis of Meynert or by coinjection of βA 25–35 and ibotenic acid into the hippocampus. To evaluate cognitive functions in animals, three tests were used: the novel object recognition test, the conditioned passive avoidance task and the Morris maze. Comparative analysis of the data demonstrated that the neuroprotective activity of HLDF-6-NH_2, evaluated by improvement of cognitive functions in animals, surpassed that of the native HLDF-6-OH peptide. The greater cognitive/ behavioral effects can be attributed to improved kinetic properties of the amide form of the peptide, such as the character of biodegradation and the half-life time. The effects of HLDF-6-NH_2 are comparable to, or exceed, those of the reference compounds. Importantly, HLDF-6-NH_2 exerts its effects at much lower doses than the reference compounds.

Keywords

Human leukemia differentiation factor (HDLF), HLDF-6 peptide, neuroprotection, nootropic activity, Alzheimer's disease (AD), beta-amyloid fragment 25–35 (βA 25–35)

Introduction

Cerebrovascular and neurodegenerative diseases are among the most urgent medical and social problems of the modern world, being the major cause of mortality and disablement of the population in developed countries. Worldwide, nearly 44 million people have Alzheimer's disease (AD) or a related dementia. (Alzheimer's Disease International, 2014).

Progression of AD is slow, but inevitable, leading to 'lockout' of the most important organ of the organism, the brain, and numerous functional disorders of the organism. According to World Health Organization data (2006), 0.38% of the world population suffered AD in 2005. It is estimated that in 2015, the value will reach 0.44%, while as many as 0.56% may be diseased by 2030. In recent decades, AD has been acknowledged as one of the four major medical and social problems of modern society. This is due to both a consistently growing population subjected to the risk of the disease, and the severe disablement of patients, requiring life-long hospitalization.

The development of brain tissue damage mechanisms in conditions such as AD necessitates the introduction of a special type of therapy, that is, neuroprotection. Neuroprotection is aimed at interrupting the delayed mechanisms of cell death—oxidative stress, cytokine imbalance, local inflammation, trophic dysfunction, and apoptosis. A tight interconnection between all mechanisms of nervous tissue death motivates exploitation of the modulating effect that could be achieved through stimulation of the regulatory systems. Peptides are among the key players in regulatory processes (Rybnikov and Zakutsky, 2000). An optimal scenario for therapy of AD and other cerebral disorders should comprise the application of agents simultaneously affecting several key components in the pathogenesis of the disease. Another important requirement for these compounds is the absence of toxic effects when used in prolonged preventive treatment. Being endogenous

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regulators, peptides can potentially exert such multidirectional effects with low toxicity. In recent years, peptide agents have undergone extensive development due to the high selectivity of their effects, in addition to their minimal adverse effects, solubility in aqueous media, low toxicity, lack of immunogenicity, and low molecular weight (Korolyova and Ashmarin, 2006).

In 1994, we discovered the human leukaemia differentiation factor (HLDF) and were the first to isolate it from the culture medium of retinoic acid-treated HL-60 cells (Kostanyan et al., 1994). The investigation of HLDF revealed a hexapeptide fragment, Thr-Gly-Glu-Asn-His-Arg (HLDF-6), that reproduced the differentiating effects of the full-size factor and exhibited a wide spectrum of nootropic and neuroprotective activities (Kostanyan et al., 2000). Direct evidence of the neuroprotective effects of HLDF-6 were obtained in experiments in a primary culture of neuronal cells of the hippocampus and cerebellum, as well as in immunocompetent cells. The HLDF-6 peptide exerts anti-apoptotic activity, protecting cells from the effects of amyloid beta peptide, sodium azide, ceramide, and ethanol, as well as cold stress and hypoxia (Kostanyan et al., 2006).The HLDF-6 peptide increased the viability of early mouse embryos in vitro (Sakharova et al., 2000).

Neuroprotection refers to the relative preservation of neuronal structure and/or function. Neuroprotection aims to prevent or slow disease progression and secondary injuries by halting, or at least slowing, the loss of neurons. Nootropics—also called *smart drugs, memory enhancers, neuro enhancers, cognitive enhancers* and *intelligence enhancers*—are drugs and supplements that improve one or more aspects of mental function. Specific effects can include improvements to working memory, motivation or attention (Lanni et al., 2008). Studies of HLDF-6 performed in various experimental models in different animal species have demonstrated that central and systemic administration of the peptide to intact animals results in the improved formation and maintenance of long-term memory (Kostanyan et al., 2006). In experimental models of clinical pathologies—Alzheimer's disease and ischemic stroke—the peptide was demonstrated to alleviate pronounced cognitive deficiency and promote memory retrieval (Sewell et al., 2005; Storozheva et al., 2006). Administration of the HLDF-6 peptide to animals with chronic brain ischemia provided a reliable neuroprotective effect, protecting brain neurons from death under the conditions of ischemia (Kostanyan et al., 2009).

In the years 2012–2014, we performed a full-scale preclinical study of a highly potent innovative therapeutic agent based on HLDF-6 exhibiting nootropic and neuroprotective effects, aimed at the development of a pharmaceutical product for the prevention and treatment of neurodegenerative (AD) and cerebrovascular (ischemic stroke) disorders.

In the course of studying the pharmacokinetics of HLDF-6 peptide, a biodegradation of the peptide radiolabelled with tritium in the rats' blood plasma was studied. Radioactively labelled peptides were obtained by the reaction of high temperature solid state catalytic isotope exchange (HSCIE), carried out without solvents, at elevated temperature under the action of spill-over hydrogen (Zolotarev et al., 2003, 2006). Application of the HSCIE reaction facilitates a high degree of tritium substitution for hydrogen. The isotopic label is distributed across the whole molecule, and the isotope exchange in peptides and proteins occurs with reduced physiological activity and configuration of the asymmetric carbon atom, which avoids racemization upon isotope exchange (Zolotarev et al., 2010, 2014a).

Analysis of biotransformation of the [^{3}H]TGENHR peptide in heparinized blood plasma was performed by HPLC. It was revealed that the key process during the degradation of HLDF-6 peptide was its cleavage by carboxydipeptidyl peptidase resulting in the formation of the TGEN tetrapeptide and HR dipeptide. To protect the peptide from the attack of the carboxydipeptidyl peptidase we decided to convert the C-terminal carboxyl group into an amide and to compare the properties of the two forms of the HLDF-6 peptide—that is, the native peptide (HLDF-6-OH) and its amide (HLDF-6-NH_2). We found that HLDF-6-NH_2 (H-TGENHR-NH_2) possessed high stability to hydrolysis in blood plasma. The half-life of HLDF-6-NH_2 in plasma is eight minutes, which is several times longer than the value for the carboxylate form of the peptide (H-TGENHR-OH) (two minutes). In the process of conducting preclinical trials, we performed an extensive comparative study of the neuroprotective and nootropic activities of the two pharmaceutical substances—HLDF-6-OH and HLDF-6-NH_2—to find the most efficient form of the HLDF-6 peptide. The investigation was performed in animal models of AD and ischemic stroke.

Neuroprotection refers to the relative preservation of neuronal structure and/or function. Neuroprotection aims to prevent or slow disease progression and secondary injuries by halting or at least slowing the loss of neurons.

The aim of the work was to choose the most efficient form of the HLDF-6 peptide. Here we report the results obtained from the comparative study of the neuroprotective and nootropic activities of HLDF-6-OH and HLDF-6-NH_2 in animal models of AD. Data obtained in the models of ischemic stroke will be reported in a subsequent paper (Storozheva et al., unpublished). On the basis of the results obtained we selected the amide form of the peptide (TGENHR-NH_2) as the pharmaceutical substance for the remedy to develop.

During the investigation of the mechanism of action of our preparation, a comparative analysis of density changes in the serotonin, $GABA_A$ and NMDA-glutamate receptors on the membranes of inbred C57B1/6 and Balb/c mouse prefrontal cortex and hippocampus under the action of subchronic peptide administration was performed. Participation of these receptors in different neurological disorders (ischemic stroke, craniocerebral injury) and in chronic neurodegenerative diseases (AD and Parkinson's disease) was shown (Break et al. 1993; Olney et al 1998). We showed that only the case of NMDA-glutamate receptors subchronic introduction of the peptide into the Balb/c mouse hippocampus resulted in an increase of the Bmax value, showing the density of the corresponding receptors. (Zolotarev et al., 2014b). Thus, under the action of the amide form of the HLDF-6 peptide, an increase in the amount of NMDA receptors occurred, which resulted in the improvement of cognitive behavior.

The pathogenetic mechanisms of AD are still unclear. It has been found that they involve selective loss of basal forebrain cholinergic neurons, particularly in the nucleus basalis of Meynert. This part of the brain is one of the main sources of cholinergic projections to the cerebral cortex (together with the substantia innominata, the diagonal band of Broca, etc. (Grothe et al., 2014)). The lesion of the cholinergic neurons of the nucleus basalis of Meynert in rodents is one of the most utilized animal models for the study of in vivo pathogenesis of cortical cholinergic involution.

In turn, the toxic effects of beta-amyloid, a major pathogenic molecule for AD, on the neurons of the nucleus basalis of Meynert in rodents, are a quite valid model of part of the verified chain of AD pathogenesis (Harkany, 1998, 2000). On the other hand, the impairment of glutamatergic neurotransmission plays an important role in the development of AD. The pathological process that involves the production of amyloid-β peptides spreads over neuroanatomical circuits, including the hippocampus, and leads to the development of excitotoxicity and neurodegeneration (Zadori et al., 2014). One of the widely used models of these pathological pathways in rodents is one based on co-injection of beta-amyloid fragments and an irreversible agonist of NMDA glutamate receptors, ibotenic acid, into hippocampus (Hruska and Dohanich, 2007; Morimoto et al., 1998). In the present study the two models of AD described above were used to compare the protective effects of HLDF-6-OH and HLDF-6-NH_2 on cognitive deficit caused by beta-amyloid. Respectively, the inhibitor of acetylcholinesterase, neuromidin, (Meuner et al., 2006) and the non-competitive antagonist of NMDA glutamate receptors, memantin, (Tucci et al., 2014) were used as reference drugs.

When selecting behavioral tests to assess cognitive deficits in the model with neurotoxic lesion of the nucleus basalis of Meynert, it should be noted that, in the cerebral cortex, volume cholinergic transmission coexists with phasic acetylcholine release (Sarter et al., 2009). Such organization provides for the important role of the basal forebrain cholinergic system in arousal and attentional functions that participate both in the processing of behaviorally relevant stimuli and in execution mechanisms (Parikh et al., 2007). Visual cue detection and visuospatial attention have been shown to become impaired after lesion of the nucleus basalis of Meynert (Berger-Sweeney et al., 1999; Ridley et al., 1999). To test these functions we selected the novel object recognition task and the Morris water maze task, featuring a cued platform. Passive avoidance has also been shown to be impaired after nucleus basalis lesion (Altman et al., 1985, Harkany et al., 1998, 2000), and this deficit may be due not only to cortical dysfunction, but also to the depletion of cholinergic projections to the amygdala as well (Holland, 2007). To test cognitive deficit caused by coinjection of βA 25–35 and ibotenic acid into the hippocampus we used well-known hippocampal-dependent tasks: working spatial memory in the non-cued version of Morris water maze (Spellman et al., 2015) and passive avoidance (Ambrogi Lorenzini et al., 1999).

Methods and materials

Synthesis of the HLDF-6-OH and HLDF-6-NH_2 peptides

Solid-phase synthesis of the acidic and amide forms of HLDF-6 was performed using the Boc/Bzl approach with minimal protection of the side-chain functionalities. The process of preparation of the HLDF-6 acidic form started with loading of Merrifield resin (chloromethylated copolymer of styrene with 1% divinylbenzene) using the zwitterionic Boc-arginine derivative directly as a source of reactive carboxylate anion (rather than the commonly employed caesium salts of amino acids) in a nucleophilic substitution of the polymer-bound chloride. Further elongation of the peptide chain was performed using a standard set of reagents and amino acid derivatives used in the Boc/Bzl approach, with the following exceptions: the hydroxyl of threonine was left unprotected (Fischer et al., 1991) and the N_α-Boc groups were removed with a sulphuric acid solution in dioxane (Houghten et al., 1986). TBTU was used as a coupling reagent (Reid and Simpson, 1992) in the syntheses of both peptides. Acylation reactions were monitored for completeness using Kaiser's ninhydrin test (Kaiser et al., 1970). Synthesis of the HLDF-6 amide was accomplished using a standard Boc/Bzl scheme with the modifications mentioned above. A copolymer of styrene with 1% divinylbenzene modified with the *p*-methylbenzhydrylamino functional group (MBHA-resin) (Matsueda and Stewart, 1981) was used as a starting material, to which the Boc-Arg(Tos)-OH was initially attached. Peptides were cleaved from the polymeric support using HF/*m*-cresol (9:1). The products were analyzed by HPLC on a mixed-phase column with a C18 stationary phase, partially modified with hydrophilic groups (Synergi Hydro-RP, Phenomenex) to suppress the phenomenon of phase collapse under the conditions of high (>95%) water content in the mobile phase (Przybyciel and Majors, 2002). Preparative HPLC purification of the target peptides was carried out using a Synergi Hydro-RP (250×50 mm; Phenomenex) column. Quality control of the preparations was performed using a combination of mass spectrometry and amino acid analysis.

Production of the tritium-labelled samples of the HLDF-6 peptide. Tritium-labelled HLDF-6-OH and HLDF-6-NH_2 peptides were obtained by the reaction of high temperature solid state catalytic isotope exchange (HSCIE) with gaseous tritium. The HSCIE reaction was carried out at 180°C in the solid mixture, formed by the peptide, coating aluminum oxide and finely dispersed heterogenic catalyst Pd/$BaSO_4$ in the presence of gaseous isotope of hydrogen.

HLDF-6-OH or HLDF-6-NH_2 peptides (1.0 mg) were dissolved in 0.5 ml of water and mixed with 20 mg of aluminum oxide. The mixture was frozen, lyophilized to remove water and then 10 mg of catalyst 5% Pd/$BaSO_4$ (Fluka) was added. The obtained solid mixture was placed into a 10-ml ampule and vacuum-processed. Then, the ampule was filled with gaseous tritium up to a pressure of 30 kPa and exposed at 180°C for ten minutes. The ampule was cooled, vacuum-processed, and blown with hydrogen. The peptide was desorbed by 50% aqueous ethanol. To remove labile tritium the labeled peptide was further twice dissolved in 50% aqueous ethanol and evaporated to dryness. Purification of the obtained samples of the radiolabeled peptide was carried out by HPLC on the Kromasil 8×150 column in the gradient of concentration of aqueous acetonitrile in the presence of 0.1% of heptafluorobutyric acid. The peptides were dried and dissolved in ethanol. To obtain tritium labeled peptides we used the unique solid-brazed apparatus for tritium–hydrogen exchange OBT-1 (IMG RAS).

Study of biotransformation of the native peptide (HLDF-6-OH) and comparative study of life-time of HLDF-6-OH and HLDF-6-NH_2 peptides in rat blood plasma. Biotransformation of the HLDF-6-OH peptide was analyzed by HPLC of the products of interaction of the peptide with heparin-treated rat blood plasma. A series-connected UV-detector and a flowing radioactivity-detector were used for the analysis. Chromatography was performed in a Kromasil C18 column, 150×4.4 in

methanol gradient (0%–30%) in the presence of 0.1% mixture of trifluoroacetic and heptafluorobutyric acids (4:1). For identification of the peptide fragments, forming through biodegradation, 2-, 3-, 4- and 5-membered peptide fragments were synthesized, which could be formed by various pathways of biodegradation of the HLDF-6-OH peptide from its C- or N- termini. Comparative study of the life-time of HLDF-6-OH and HLDF-6-NH_2 peptides in heparin blood plasma was performed using tritium-labeled [$_3$H]TGENHR-OH and [$_3$H] TGENHR-NH_2 peptides.

Experimental animals: general conditions

Healthy adult male Wistar rats aged 180–200 days weighing 280–300 g were used in the study. Animals were kept according to the current hygienic rules for the arrangement, equipment, and maintenance of experimental biology clinics. The standard laboratory diet agreed with the current norms. Animals were kept four to a cage under conditions of free access to water and food at 21°C during a constant 14-h day (with the lights turned on at 08.00 h). Environmental factors (temperature, humidity, illumination, and bedding composition) met the requirements for laboratory animal care and use. Control and experimental animals were kept under the same conditions.

The study was performed according to the Rules of Laboratory Practice in the Russian Federation, as well as the recommendations of the Guidance on Preclinical Studies of Drug Substances (Mironov et al., 2012), and according to the requirements of the international FDA GLP and OECD GLP rules using specific pathogen-free (SPF) laboratory animals (experimental biomodels). The animals were produced at the Animal Breeding Facility (Branch of the Shemyakin and Ovchinnikov Institute of Bioorganic Chemistry, Russian Academy of Sciences, Pushchino, Moscow Region, Russia). The facility has full AAALAC (Association for Assessment and Accreditation of Laboratory Animal Care International) accreditation. The quality management system in the facility has been certified according to the requirements of the international ISO 9001:2008 standard.

All procedures were performed according to approved protocols. Each procedure involving animal treatment was reviewed and approved by the Committee on Humane Animal Treatment prior to the experiment.

AD modelling in rats by administration of the beta-amyloid 25–35 fragment (βA 25–35) into the nucleus basalis of Meynert (Protocol # 2/13)

Two models of Alzheimer's disease were used in the study. In the first model, βA 25–35 was administered into the giant-cell nucleus basalis of Meynert (*Nucleus basalis magnocellularis*) (Harkany et al., 1998, 2000). Together with the other forebrain structures (diagonal band nucleus, substantia innominata), the nucleus basalis of Meynert is a potent source of cholinergic innervations of the cerebral cortex.

To test cognitive functions in the animals, three tests were used: the novel object recognition test, the passive avoidance task, and the Morris maze (Giovannelli et al., 1995; Granic et al., 2010; Harkany et al., 1998, 2000). Neuromidin, which is known to be a potent inhibitor of acethylcholinesterase, to enhance cholinergic neurotransmission, and to improve cognitive functions in patients with AD, was used as a reference drug in this model.

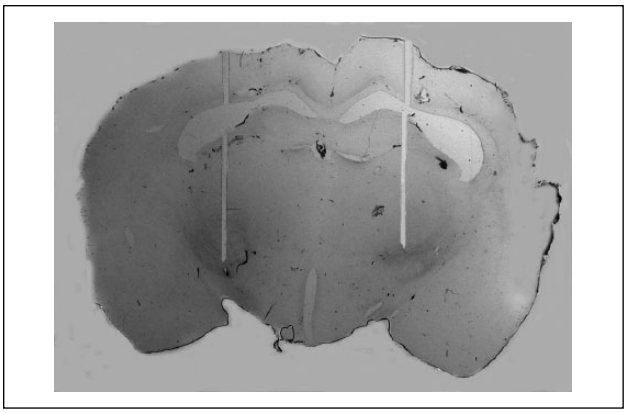

Figure 1. The sites of injection in the animal's brain.

Experimental animals. Rats were divided into nine groups (9–11 animals per group): group 1, sham-operated animals; group 2 (control), animals that were treated intranasally with saline; groups 3–5, animals that were treated intranasally with HLDF-6-NH_2 at a dose of 250 μg/kg, 50 μg/kg, and 10 μg/kg, respectively; groups 6–8, animals that were treated intranasally with HLDF-6-OH at a dose of 250 μg/kg, 50 μg/kg, and 10 μg/kg, respectively; and group 9, animals that were injected intraperitoneally with a reference drug, Neuromidin®, at a dose of 2 mg/kg. The doses of different HLDF6 forms were chosen taking into account our previous data indicating a neuroprotective effect of HLDF6-OH on rat hippocampal neurons treated with βA 25–35 and ibotenic acid (Kostanyan et al., 2006).

Injection of βA 25–35 into the rat nucleus basalis of Meynert. βA 25–35 was injected with a Hamilton microsyringe into rats under general anaesthesia (15 mg/kg zoletil + 2 μg/kg xylazine intraperitoneally) using a stereotaxic apparatus (TSE, Germany) at the coordinates AP –1.5, DL ±2.7, DV 8.1. Animals in groups 2–9 received βA 25–35 bilaterally at a dose of 2 μg in 1 μL of saline on each side. Animals of group 1 received saline bilaterally (1 μL on each side).

Overall, 101 animals were operated on, and mortality was 0%. After all experimental procedures finished, each rat was decapitated under general anesthesia, the brain was removed, fixed in 4% paraformaldehide in phosphate buffer saline, and sliced using a microtome to verify the site of injection (Figure 1). 9 animals were eliminated from the analysis because the site of injection was outside of the target area.

The protocols for the peptides injection and the assessment of cognitive functions. Solutions of the HLDF-6-NH_2 and HLDF-6-OH peptides were administered intranasally at a volume of 48 μL/kg divided between the two nostrils. This measure is a volume of injection which was calculated for each animal on the base of its weight. The reference drug, Neuromidin® (9-amino-2,3,5,6,7,8-hexahydro-1*H*-cyclopentaquinoline hydrochloride, monohydrate), was administered at a dose of 2 mg/kg intraperitoneally in 1 mL

saline per kg body weight. Animals of groups 1 and 2 were treated with 1 mL/kg saline. Injections were performed 1 h and 24 h after the surgery, as well as on days 3, 5, and 7 after the surgery. Cognitive functions were assessed according to the following scheme: days 14–16 after the surgery, a novel object recognition test; days 18–19, a conditioned passive avoidance task; and days 21–22, the Morris maze test. All behavioral tests were conducted between 11.00 h and 15.00 h.

Novel object recognition test. The novel object recognition test was performed under room lighting in a 45×45×40 cm grey plastic chamber. The test included three five-minute sessions at 24-h intervals: (1) without objects, for adaptation to the setup; (2) with two identical objects—metallic cylinders 6 cm in diameter and 8 cm high; and (3) with one cylinder replaced by a plastic cube with 6-cm sides. Animal behavior was recorded with a digital camera and analyzed using EthoVision XT (Noldus) software. The recognition index was calculated using the formula (Tn–Tf/Tn+Tf)×100%, where Tn is the duration of new object investigation, and Tf is the duration of familiar object investigation in the third session of the experiment (Nimmrich et al., 2008; Rutten et al., 2008).

Passive avoidance test. The passive avoidance test was conducted in an apparatus from Columbus Instruments (USA). The experimental box consisted of two identical 25×40×25 cm compartments with a metal grid floor. The compartments were separated by a guillotine door. One of the compartments was illuminated and white and the other one was dark and black. Passive avoidance was trained by putting an animal into the illuminated compartment and timing transitions—that is, the time that the animal took to move from the illuminated compartment to the dark one (which is naturally preferred, due to hole-exploratory behavior). Immediately after all four paws of the animal were in the dark sector, the guillotine door was closed and the rat received a mild inescapable electrical shock through the metal rod floor (0.6 mA, 3 s); then, the rat was taken out of the box and placed into the home cage. Forty-eight hours after the acquisition/conditioning session, passive avoidance was tested. The rat was again placed in the illuminated compartment, and the time of the animal's movement to the dark compartment was recorded (Harkany et al., 1998; Ma et al., 2001).

Morris maze. Morris maze experiments were performed in a grey circular pool 165 cm in diameter with 60-cm high walls filled with water to a depth of 40 cm. A round Plexiglas platform, 9 cm in diameter, was placed in the centre of one of the sectors, 2 cm below the water level. The training was performed for two days, four trials a day. During the training, animals were placed in the water at four different points, and the time to reach the platform was measured. Once the platform was reached, the animal was allowed to stay on it for 15 s, and then returned to the home cage for two minutes. As the cerebral cortex is mainly damaged in the AD model used, we used so-called cued spatial learning for testing. A black sphere (the key signal) was placed 20 cm above the platform, while the platform position was changed in each session. In this model, the animal learns to orient itself using the key signal, which depends not on the integrity of hippocampus functions, but on the functional state of the cerebral cortex (De Bruin et al., 1994; Le Pen et al., 2000; Save and Poucet, 2000).

Choline acetyltransferase assay. Measurement of choline acetyltransferase (ChAT) activity was performed in another group of rats which were not involved in the behavioral tests. Rats were divided into four groups: group 1, sham-operated animals (n=8); group 2 (control), animals which were treated intranasally with saline (n=7); group 3, animals which were treated intranasally with HLDF-6-NH2 at a dose of 250 μg/kg (n=8); and group 4, animals which were treated with HLDF-6-OH at a dose of 250 μg/kg. The animals were decapitated on the 15th day after surgery and the cerebral cortex was removed bilaterally on ice and then immediately transferred to liquid nitrogen. Cortical tissue samples were thinly sliced and thawed in radioimmunoprecipitation assay (RIPA) buffer (Pierce Biotechnology Inc., USA) containing 50 mM Tris-HCI, pH 7.4, 1% Nonidet NP-40, 0.5% Na-deoxycholate, 0.1% SDS, 150 mM NaCI, 2 mM EDTA, 50 mM NaF, 1 mM phenylmethylsulfonyl fluoride, at a ratio of 1.0 mg tissue/3.0 mL of precold RIPA buffer. Each sample was individually homogenized using a Sonopuls mini 20 sonicator (Bandelin Electronic GmbH, Germany) at +4°C and incubated on ice for 30 minutes. Homogenates were then centrifuged at 10,000 g for 10 minutes at +4°C (Eppendorf 5810R centrifuge, Germany). The supernatants were removed and centrifuged for a second time under the same conditions. The secondary supernatant fluids represented the total hippocampal cell lysates in which the protein concentration was determined using a Bradford protein assay kit (Pierce Biotechnology Inc., USA).

ChAT activity in cerebral cortex lysates was measured as described by Fonnum (1966). The reaction solution was prepared on the day of the experiment. The enzymatic reaction was started by mixing subfraction samples with the reaction solution. The reaction mixture contained a final concentration of 0.2 mM acetyl CoASA (Fluka) and [1-14C]-acetyl CoASA (Amersham Pharmacia Bioscience) with SPA 5 mCi/mmol, 300 mM NaCl, 3 mM MgCl2, 0.2 mM physostigmine salicylate (Sigma), 10 mM choline chloride (Serva), 0.5% Triton X-100 (Serva), 0.5 mg/ml bovine serum albumin Koch-Light), 10 mM sodium phosphate buffer/1 mM EDTA-Na2, pH 7.8, and the subfraction samples (about 3.5 mg of protein), at a volume of 0.05–0.1 ml. The reactive mixture was incubated in a water shaker at 37°C for 30–60 minutes. The reaction was stopped by adding 2 ml of ice-cold stop solution (0.2 mM acetylcholine in 10 mM sodium phosphate buffer/1 mM EDTA-Na2, pH 7.8) and placing the mixture in an ice bath. Then, the solution (1 ml) of sodium tetraphenylborate (Sigma) in butyl acetate (15 mg/ml) was added and quickly subjected to intensive mixing in a shaker (500 rpm, 4 min, room temperature). The organic phase was separated from the inorganic phase by centrifugation (1000 g for 15 minutes at 2–4°C). The organic phase with acetylcholine (0.5–0.7 ml) was transferred into scintillation liquid for organic solutions, and the radioactive-labeled acetylcholine (DPM) was quantified with a Beta counter.

AD modelling by coinjection of βA 25–35 and ibotenic acid into the rat hippocampus (Protocol # 3/13)

In the second model, βA 25–35 was coinjected with ibotenic acid into the rat hippocampus (Morimoto et al., 1995) The passive avoidance test (Li et al., 2005) and working memory in the Morris water maze (Hruska and Dohanich, 2007) were used for

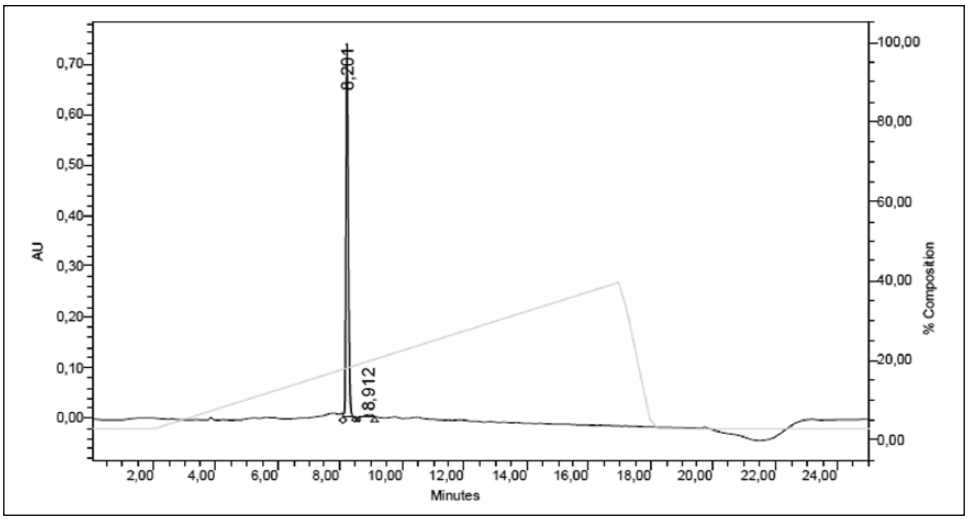

Figure 2. Chromatogram of the HLDF-6-NH_2 peptide after purification. Column Synergy™ 4µm Hydro-RP 80 Å. Gray line—gradient of acetonitrile concentration (4%–40%).

the assessment of hippocampal-dependent cognitive functions. This model was previously used in our investigation of HLDF-6 effects, and histological analysis revealed the neuroprotective action of native HLDF-6 peptide (Kostanyan et al., 2006).

Experimental animals. Five groups of rats were selected (10 animals each): group 1, sham-operated animals; group 2 (control), animals that were treated intranasally with saline; group 3, animals that were treated intranasally with HLDF-6-NH_2 peptide at a dose of 250 µg/kg; group 4, animals that were treated intranasally with HLDF-6-OH at a dose of 250 µg/kg; and group 5, animals that were treated with the reference drug, memantine hydrochloride, subcutaneously. Animals from groups 2–5 were injected with βA 25–35 together with ibotenic acid in the hippocampus.

Coinjection of βA 25–35 and ibotenic acid in the hippocampus. βA 25–35 and ibotenic acid were injected bilaterally at doses of 2 µg and 0.5 µg, respectively, on each side in a saline volume of 1 µL. The procedure was performed under general anaesthesia, similar to βA 25–35injection in the nucleus basalis of Meynert using a stereotaxic apparatus (TSE, Germany) at the coordinates AP -3,5, L ±2,0, DV 2,7 AP. Animals of group 1 received 1 µL saline on each side under the same conditions.

The protocols for PS injections and assessment of cognitive functions. Preparations of the HLDF-6-NH_2 and HLDF-6-OH peptides were administered intranasally in a volume of 48 µL/kg divided between two nostrils. This volume (48 µL) for injection contained the appropriate dose of the peptide. The reference drug memantine hydrochloride was administered at a dose of 10 mg/kg subcutaneously in saline (1 mL/kg). Animals of groups 1 and 2 received 1 mL/kg saline. Injections were performed 1 h and 24 h after the surgery, as well as on days 3, 5, and 7 after the surgery. Cognitive functions were assessed according to the following scheme: days 22–23 after the surgery—passive avoidance task; and, days 25–28—Morris maze test. The novel object recognition test was not used in this model because the data about the role of the hippocampus in this type of memory are controversial (Barker and Warburton, 2011).

Passive avoidance task. The passive avoidance task was performed under the same conditions as described in the first AD model.

Working memory assessment in the Morris maze. The pool (described in the first AD model) was kept in a room with many environmental stimuli (stands, calendar, drawers, etc.), the positions of which were constant on the experiment days. The key stimulus above the platform was absent. Therefore, the animal oriented itself using environmental stimuli (contextual spatial learning). The working memory model, where the platform position was changed between the sessions, was used (Hruska and Dohanich, 2007). Animals were trained over four days, with two sessions per day at ten-minute intervals. The session started by placing a rat with its nose facing the wall at a random point in the maze. The time to reach the platform was recorded. After the platform was reached, the animal was left on it for 15 s and then returned to the home cage for 10 minutes. If an animal could not find the platform within 60 s, it was gently directed towards the platform.

Data analysis. Statistical treatment of the results was performed using nonparametric methods: the Kruskal–Wallis criterion, median test, and Mann–Whitney–Wilcoxon parameters. The STATISTICA 6.0 (StatSoft, Inc. Tulsa, USA) software package was used.

Results

Pharmaceutical substance characteristics

Control of purification of the peptide was performed by analytical HPLC. The purity level of the peptide was 99.4% (see Figure 2).

The molecular mass of the synthesized peptide was controlled by the mass-spectrometry method. Determined molecular mass of the peptide (712 Da) complied with the calculated mass (see Figure 3).

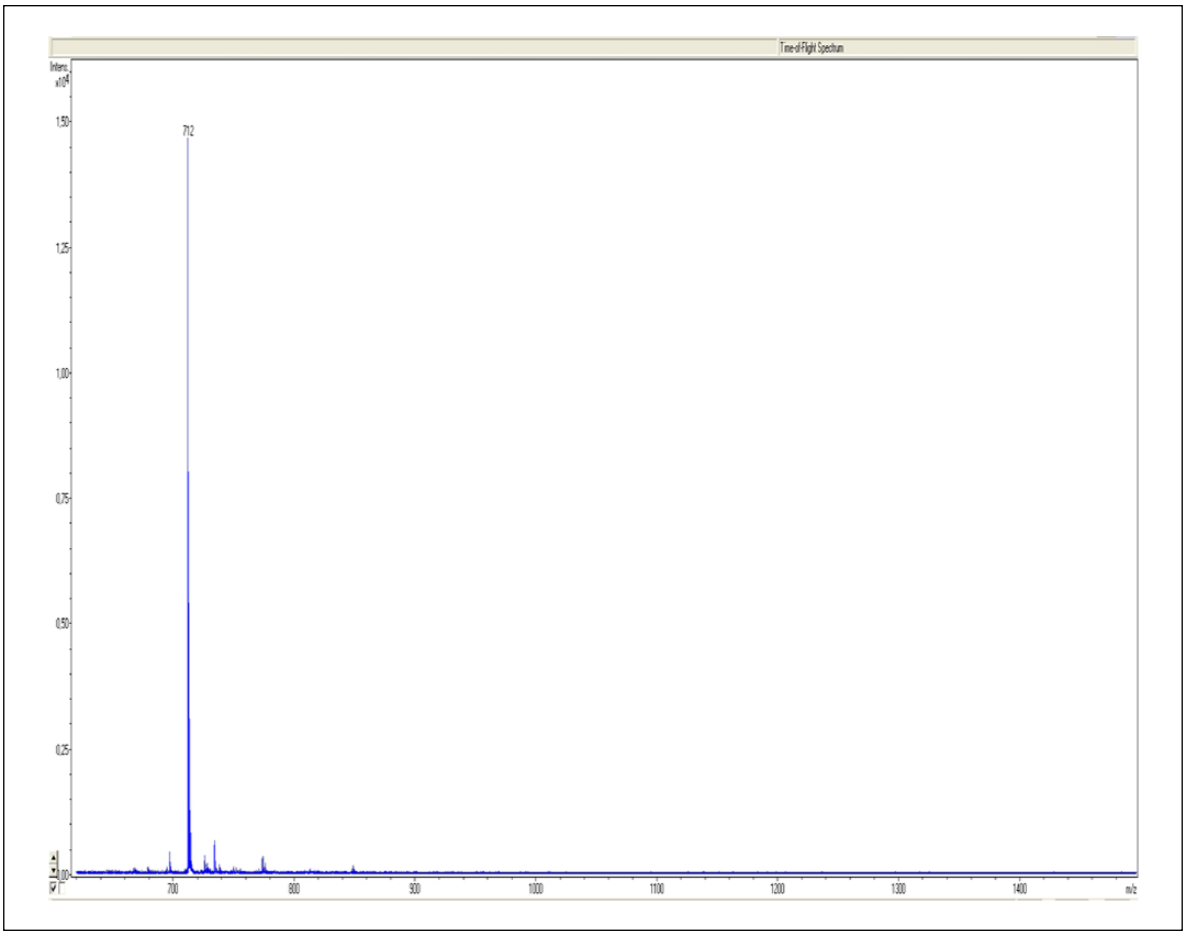

Figure 3. Mass-spectra of the HLDF-6-NH_2 peptide. Mass-spectrometer Ultraflex (Bruker Daltonics). Used materials: matrix-2,5-dihydrobenzoic acid, acetonitrile 50%, trifluoroacetic acid 0.1%, interval of 600–1400 m/z.

Preparation of tritium-labeled samples of the HLDF-6 peptide

Under the conditions described above, tritium-labeled [^{3}H] TGENHR-OH and [^{3}H]TGENHR-NH_2 peptides were obtained by a procedure with gaseous tritium with molar radioactivity of 60 and 70 Ci/mol.

Biotransformation of the native peptide (HLDF-6-OH) and the life-time of HLDF-6-OH и HLDF-6-NH_2 peptides in rat blood plasma

Study of biotransformation of the tritium-labelled HLDF-6-OH peptide in rat blood plasma revealed, that the hydrolysis of the peptide occurred from the C-terminus of the peptide chain. The carboxydipeptidase mainly contributes to the process of biodegradation of the HLDF-6-OH peptide (Figure 4, peak 7). The TGEN tetrapeptide (peak 4) and the HR dipeptide (peak 1) are the main products of the hydrolysis.

Comparative study of the life-times of HLDF-6-OH and HLDF-6-NH_2 peptides in rat blood plasma showed that the HLDF-6-NH_2 peptide is highly resistant to hydrolysis in blood plasma (Figure 5). Its half-life time is eight minutes, which is several times higher than the half-life of HLDF-6-OH peptide, which does not have amide protection of the C-terminus of the peptide chain (Figure 6). The half-life of the HLDF-6-OH is two minutes, which is similar to the level of the neuroprotective peptides Semaks and Selank (Zolotarev et al., 2006).

A comparative study of the neuroprotective and nootropic activities of pharmaceutical substances based on the HLDF-6-OH and HLDF-6-NH_2 peptides was performed in healthy SPF male Wistar rats with induced Alzheimer's disease.

Alzheimer's disease is a neurodegenerative disorder characterized by the impairment of cognitive functions and dementia. Formation and accumulation of beta-amyloid are the major features of AD pathogenesis. The toxic effect of βA 25–35 upon injection into the brain of laboratory animals leads to the impairment of cognitive functions. In the present study, we used two animal models of AD: (1) injection of βA 25–35 into the giant-cell nucleus basalis of Meynert, which is one of the major sources of cholinergic projections in the cerebral cortex (Harkany et al., 1998, 2000; Szego et al., 2011), and (2) coinjection of βA 25–35 and ibotenic acid (an NMDA agonist of the glutamate receptor) into the hippocampus (Feng et al., 2012; Morimoto et al., 1998). Three tests were used to assess cognitive functions in the animals: the novel object recognition test, allowing for the evaluation of long-term memory; the passive avoidance task, characterizing the defensive long-term memory; and the Morris

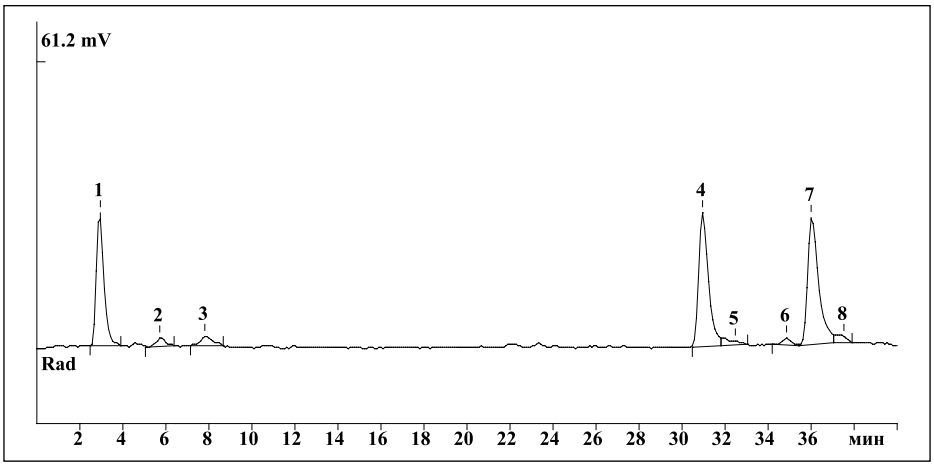

Figure 4. Biodegradation of the HLDF-6-OH peptide in rat blood plasma in 2.5 minutes. Peak 7—the peptide of interest; peak 4—TGEN tetrapeptide; and peak 1—HR dipeptide.

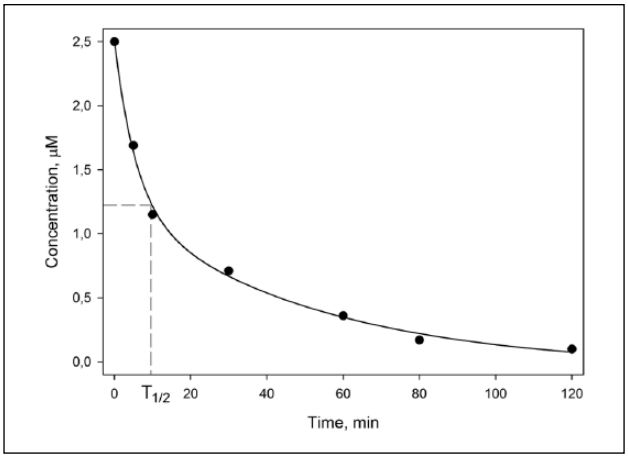

Figure 5. Kinetics of biodegradation of HLDF-6-NH_2 peptide in rat blood plasma.

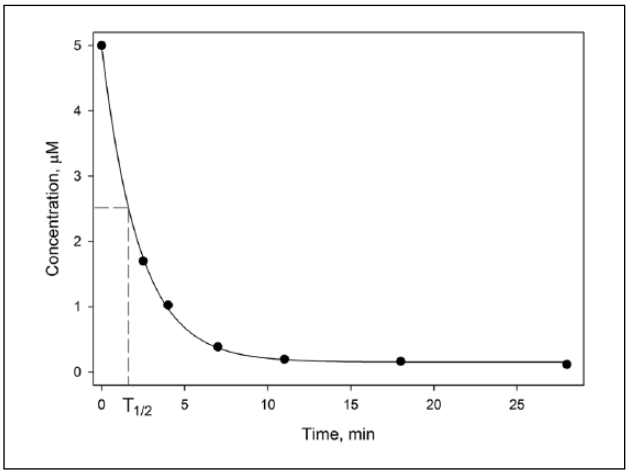

Figure 6. Kinetics of biodegradation of HLDF-6-OH peptide in the rat blood plasma.

maze, the protocols allowing the assessment of spatial contextual and cued memory.

AD modelling in rats by injection of βA 25–35 into the nucleus basalis of Meynert

Novel object recognition test. Analysis of the duration of object investigation per acquisition trial (Figure 7) did not reveal any statistically significant differences between the groups (Kruskal–Wallis test: H (10, N=92)=8.169765, p=0.5171)).

Therefore, none of the treatments affected locomotion or orientational or exploratory activity. At the same time, statistically important differences (Kruskal–Wallis test: H(10, N=92)=21.82703, p=0.0094) between the groups were revealed for the object recognition index, characterizing the declarative long-term memory associated with perirhinal cortex functions. A comparison of the individual groups using the Mann–Whitney test revealed a considerable decrease of the index in the control compared with sham-operated animals. At the same time, injection of both substances under study led to a significant increase in the index with respect to the control, up to the level of values for the sham-operated group. The most significant increase was observed in the groups treated either with the HLDF-6-NH_2 peptide at doses of 250 and 10 μg/kg and with the HLDF-6-OH peptide at doses of 250 μg/kg and 50 μg/kg, or with the reference drug. The effect of the HLDF-6-NH_2 peptide was the most pronounced. The data suggested a U-shaped dose–response curve for the HLDF-6-NH_2 peptide.

Passive avoidance task. Values of the latency to enter the dark sector, characterizing locomotion, orientational, and exploratory activity and level of photophobia altogether, did not differ between animals from different groups (Kruskal–Wallis test: H(9, N=92)=10.81598, p=0.2885) (Figure 8).

While analyzing animal behavior on the testing day, significant differences were revealed in the values of increment of latency to enter the dark sector in animals of different groups (Kruskal–Wallis test: H(9, N=92)=29.96639, p=0.0004). Because the increment in the time in the well-illuminated compartment

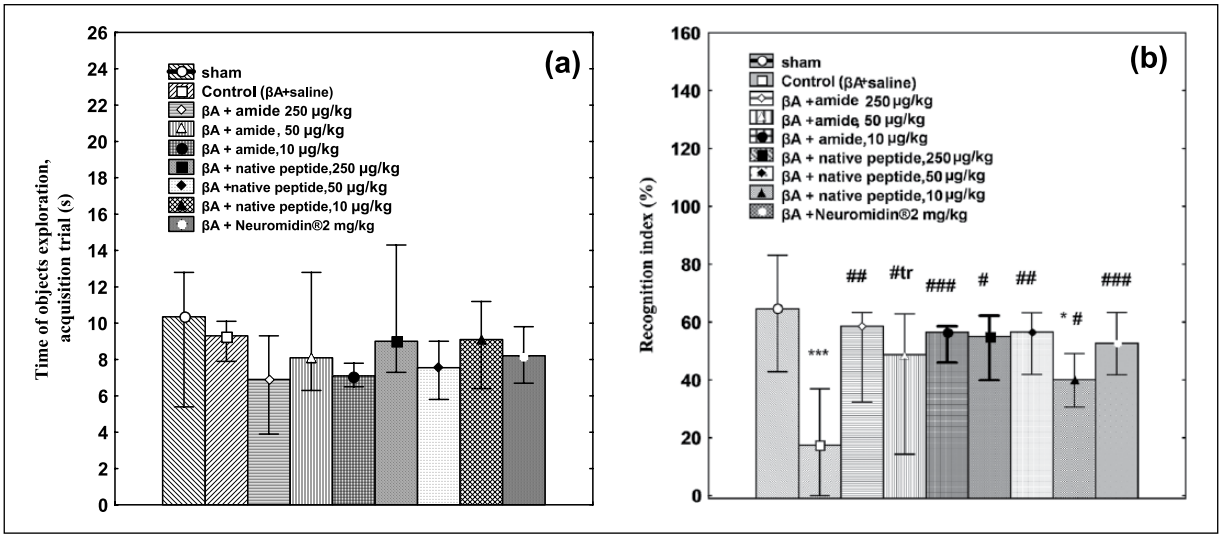

Figure 7. Effects of two forms of HLDF-6 peptide on cognitive deficit induced by βA 25–35 injection into the nucleus basalis of Meynert in the novel object recognition assay. Medians, upper, and lower percentiles are presented. Each group consists of 9–11 rats. *p<0.05 compared to sham operated group, ***p<0.001 compared to sham operated group, $^{\#tr}p$<0.1 compared to control group, $^{\#}p$<0.05 compared to control group, $^{\#\#}p$<0.01 compared to control group, $^{\#\#\#}p$<0.001 compared to control group, ^p<0.05 compared to group receiving 10 μg/kg of amide.

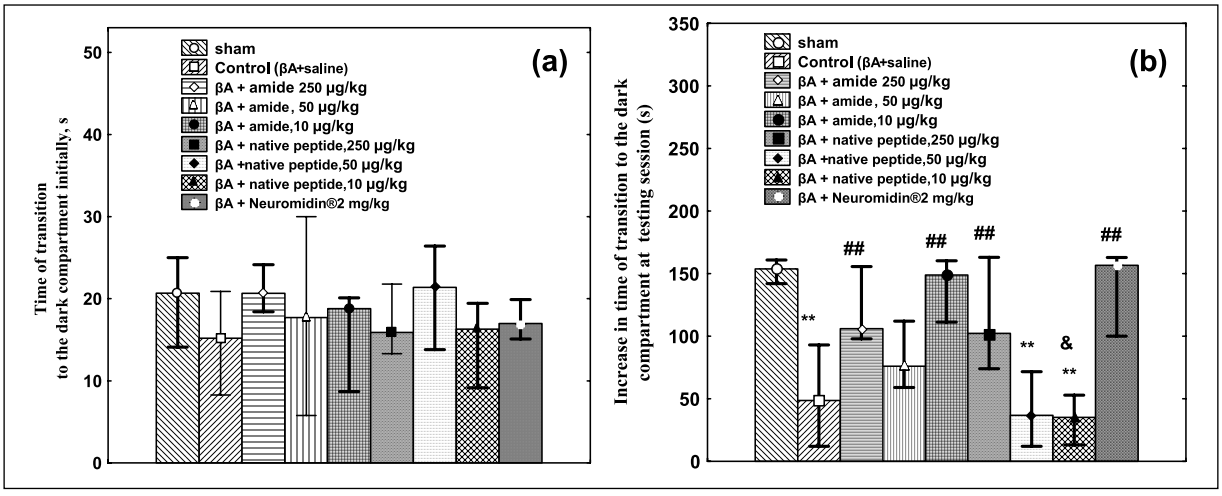

Figure 8. Effects of two forms of HLDF-6 peptide on leaning parameters in passive avoidance test in the rats with βA 25–35 injection into the nucleus basalis of Meynert. Each group consists of 9–11 rats. **p<0.01 compared to sham operated group, $^{\#\#}p$<0.01 compared to control group, $^{\&}p$<0.05 compared to group receiving 10 μg/kg of amide.

(ΔT) is caused by the formed association between entrance into the dark sector and foot stimulation, the value characterizes the long-term memory of defensive behavior. A paired comparison of the groups was performed using the Mann–Whitney test. Animals of the control group were found to learn much less well than the sham-operated animals. At the same time, animals from groups treated with 250 and 10 μg/kg of the HLDF-6-NH_2 peptide, 250 μg/kg of the HLDF-6-OH peptide and the reference drug did not demonstrate any significant differences from the sham-operated animals. Moreover, the increment in the latency to enter the dark sector in these groups considerably surpassed the control group value. In addition, the animals that received 10 μg/kg of the HLDF-6-NH_2, learned significantly better than the animals from the group treated with 10 μg/kg of the HLDF-6-OH.

Thus, the effect of the HLDF-6-NH_2 peptide was considerably more pronounced. It should be noted that, similar to the novel object recognition test, a U-shape of the dose–response curve was revealed, with the minimum at a dose of 50 μg/kg.

Morris maze. Analysis of the time to reach the platform using the Kruskal–Wallis test revealed statistically important differences between the groups on the first day of training (Kruskal–Wallis test: H(9, N=92)=20.82006, p=0.0135) (Figure 9). A comparison of the groups using the Mann–Whitney test revealed

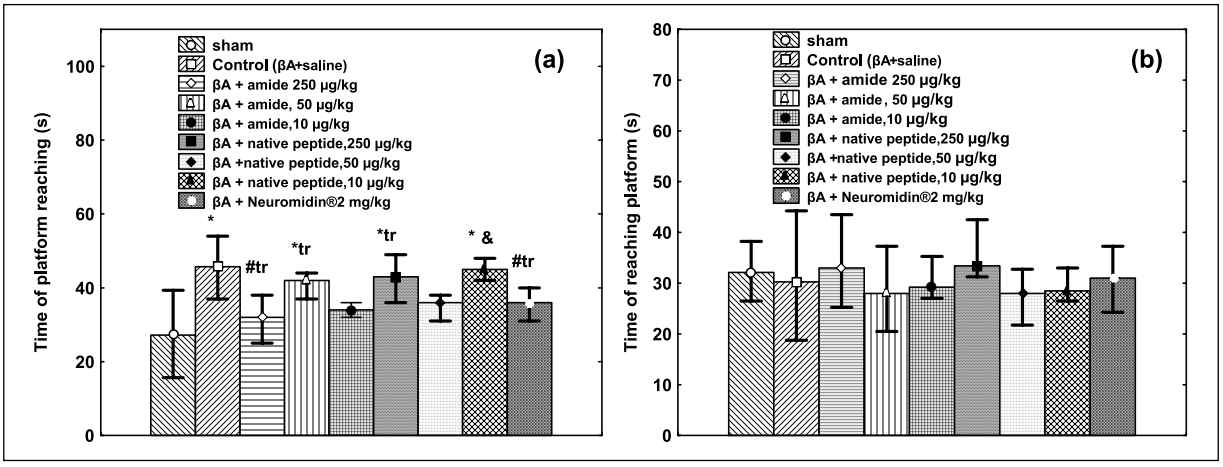

Figure 9. Morris maze test study of investigated substances effects on leaning parameters of the rats with βA 25–35 injection into the nucleus basalis of Meynert. Medians, upper, and lower percentiles are presented; (a) the first training day; (b) the second training day. Each group consists of 9–11 rats. *p<0.05 compared to sham operated group, *^{tr}p<0.1 compared to sham operated group, $^{\#}p$<0.05 compared to control group, $^{\#tr}p$<0.1 compared to control group, $^{\&}p$<0.05 compared to group receiving 10 μg/kg of amide.

Table 1. Effects of two forms of HLDF-6 peptide on ChAT activity in the rats injected with βA 25–35 into the nucleus basalis of Meynert.

Animal groups	ChAT activity, dpm/mg of tissue
Sham operation	6.85±0.46
Control (βa + saline)	4.28±0.29[a]
(βA + HLDF-6-NH_2, 250 μg/kg)	5.61±0.40[b]
(βA + HLDF-6-OH, 250 μg/kg)	5.29±0.34[a,c]

[a]p<0,05 compared to Sham operated group, [b]p<0,05 compared to control group, [c]p<0,1 compared to control group.

a statistically important increase in the time to perform the task in animals of the control group when compared to those of the sham-operated group (Figure 9(a)). No differences were observed between the groups on day two of training (Kruskal–Wallis test: H(9, N=92)=5.818653, p=0.7579) (Figure 9(b)).

Therefore, injection of βA 25–35 into the nucleus basalis of Meynert induced selective impairment of short-term spatial key memory. Values for the group receiving the HLDF-6-NH_2 peptide at a dose of 250 μg/kg, as well as for those treated with Neuromidin®, did not demonstrate statistically significant differences compared to the sham-operated animals. In these groups, a tendency (0.05<p<0.1) towards an increased rate of platform search compared with the controls was observed. Groups treated with 10 μg/kg of HLDF-6-NH_2 peptide and with 50 μg/kg of HLDF-6-OH peptide displayed intermediate performance rate, and did not differ either from sham or from control groups. The worst performance was observed in animals treated with 50 μg/kg of HLDF-6-NH_2 and with HLDF-6-OH at doses of 250 μg/kg and 10 μg/kg. Also, animals that received 10 μg/kg of the HLDF-6-NH_2 learned significantly better than animals from group treated with 10 μg/kg of the HLDF-6-OH.

Thus, substantial retrieval of learning capacity in the model occurred only upon treatment with the HLDF-6-NH_2 peptide at a dose of 250 μg/kg, and the strength of the effect was comparable to that of Neuromidin® at a dose of 2 mg/kg.

Choline acetyltransferase activity

Cortex ChAT activity was significantly lower in the control compared to the sham group (Table 1). In the animals that received HLDF-6-NH_2 peptide ChAT activity was significantly higher than in the control animals, and did not differ from sham group. In the animals that received HLDF-6-OH peptide, ChAT activity was lower than in the sham group and tended to be higher than in the control group

AD modelling by coinjection of βA 25–35 and ibotenic acid into the hippocampus

Passive avoidance test. No statistically significant differences between the groups by indication of the dark compartment transit time was observed on the training day (initially): Kruskal–Wallis test: H(4, N=50)=3.16, p=0.53 (Figure10(a)). Considerable differences in the value of the increment in latency to enter the dark compartment in the testing session when compared to the training session (ΔT) were observed between the groups (Kruskal–Wallis test: H(4, N=49)=13.16, p=0.0105) (Figure 10(b)).

When the groups were compared using the Mann–Whitney criterion, the value of the increment in latency to enter the dark compartment in the control group was found to be statistically lower than that in the sham-operated group, which demonstrates a pronounced impairment of the long-term memory of defensive behavior induced by injection of βA 25–35 + ibotenic acid. In rats treated with the HLDF-6-NH_2 peptide, the increment in latency to enter the dark compartment was significantly higher than in the control group, and tended to be higher than in those treated with the HLDF-6-OH form of the peptide or reference

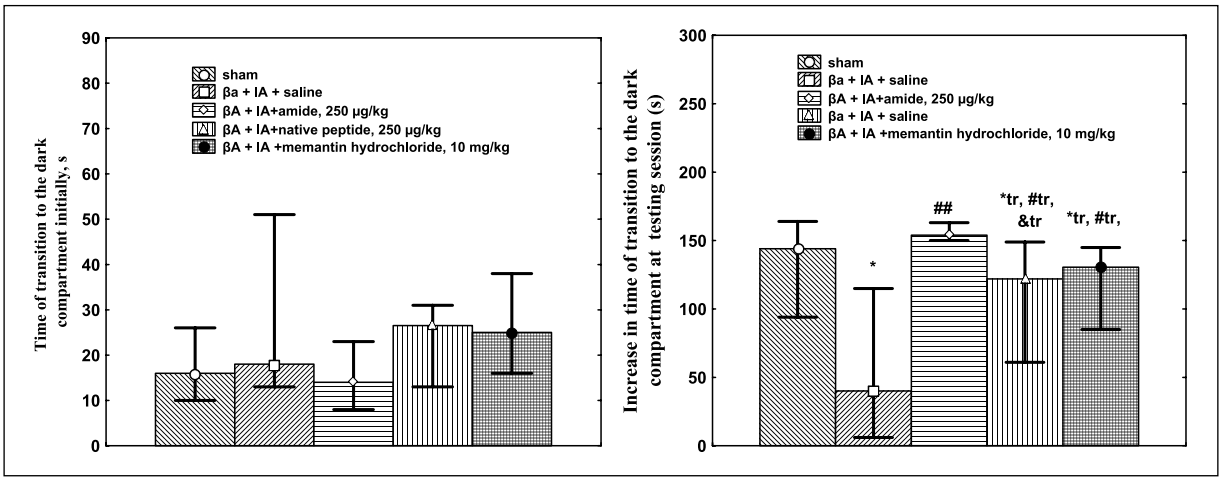

Figure 10. Effects of two forms of HLDF-6 on leaning parameters in passive avoidance test in the rats with βA 25–35 and ibotenic acid co-injection into the hippocampus. Medians, upper, and lower percentiles are presented. Each group consists of 10 rats. *p<0.05 compared to sham operated group, ^{*tr}–p<0.1 compared to sham operated group, $^{\#\#}p$<0.01 compared to control group, $^{\#tr}p$<0.1 compared to control group, $^{\&tr}p$<0.1 compared to group receiving 250 μg/kg of native peptide.

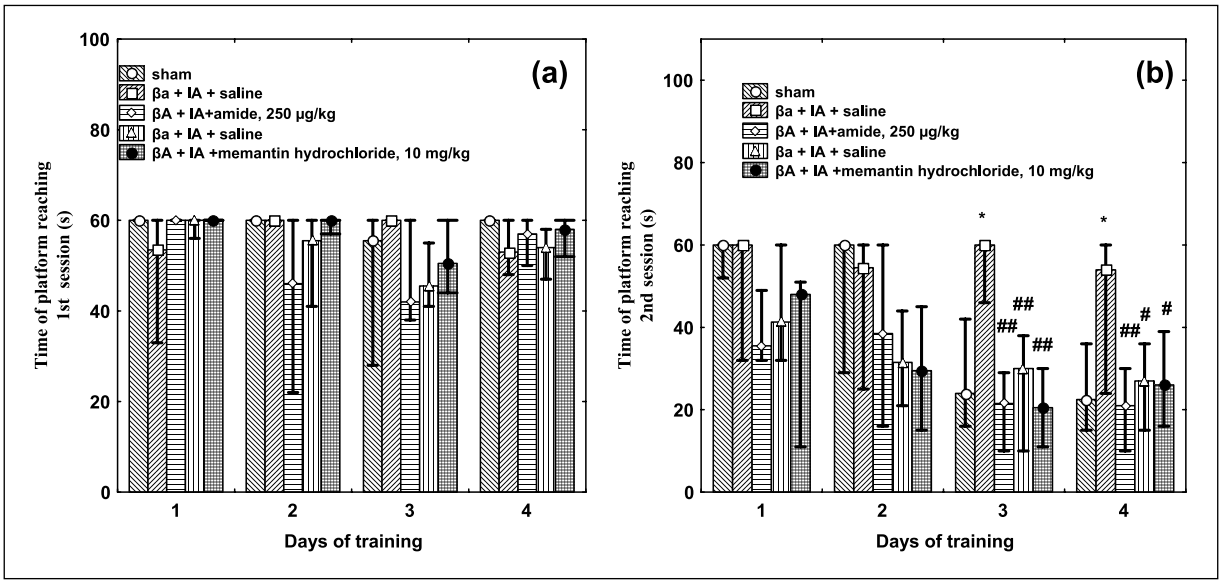

Figure 11. Morris maze test study of the effects of two forms of HLDF-6 on leaning parameters in the rats with βA 25–35 and ibotenic acid co-injection into the hippocampus; (a) first training session, (b) second training session. Medians, upper, and lower percentiles are presented. Each group consists of 10 rats. *p<0.05 compared to sham operated group, $^{\#}p$<0.05 compared to control group, $^{\#\#}p$<0,01 compared to control group.

drug. The concentration of the amide form of the peptide used was much lower than that of the reference drug.

Morris maze. In this series of experiments, we tested the working spatial conditioned memory, the maintenance of which depends on the integrity of hippocampus functions. The main characteristic of the working memory in this model is the time to reach the platform at the second attempt on each of the training days. Analysis of this parameter did not reveal any statistically important differences between the groups, either on the first (Kruskal–Wallis test: H(5, N=50)=4.639090, p=0.2002) or second (Kruskal–Wallis test: H(5, N=50)=4.600772, p=0.2035) training day. These days are during the stage of search strategy formation, which does not depend on hippocampus functions. At the same time, considerable intergroup differences were revealed on training days 3 (Kruskal–Wallis test: H(5, N=50)=10.6842, p=0.305) and 4 (Kruskal–Wallis test: H(5, N=50)=9.49829, p=0.0494) (Figure 11).

Analysis using the Mann–Whitney test revealed a statistically significant increase in the time to reach the platform at the second

attempt on training days 3 and 4 in the control rats. Injection of both amide and carboxylate forms of the HLDF-6 peptide at a dose of 250 μg/kg considerably decreased the time to reach the platform in the second attempt on training days 3 and 4 in the control rats. In the case of the HLDF-6-NH_2 peptide, the value was lower than that of the HLDF-6-OH peptide. The time to reach the platform in animals treated with the HLDF-6-NH_2 peptide was similar to that in animals treated with the reference agent (memantine hydrochloride), although the concentration of the former was 40 times lower.

Discussion

The practical application of peptides is known to be limited due to their low biological stability. One of the rationales to overcome these difficulties is modification of the peptide structure.

Previous study of biologically active HLDF-6 peptide has revealed that it has a short period of life (half-life time is two minutes). Pharmacokinetic studies with radioactive peptide have demonstrated that the major pathway of biodegradation of the peptide is its cleavage with dipeptidyl carboxypeptidase. The reaction results in the formation of N-terminal TGEN tetrapeptide and C-terminal HR dipeptide. We have suggested that modification of the carboxyl group of C-terminal arginine would protect the peptide from the cleavage. We modified the C-terminal carboxylate group of the HLDF-6 peptide to an amide. Modification resulted in a four-fold increase in the life time of the modified peptide in plasma. The increased biological stability of the HLDF-6-NH_2 peptide allowed hope for its higher biological activity. Further studies confirmed our suggestion.

A comparative study of the neuroprotective and nootropic activity of pharmaceutical substance samples based on the HLDF-6-OH and HLDF-6-NH_2 peptides was conducted. The study was performed in healthy male rats using models of a neurodegenerative disorder, AD, and a cerebrovascular pathology, ischemic stroke (Storozheva et al., unpublished).

Accumulation of the amyloid protein in the form of soluble monomers is the key stage in AD pathogenesis. The beta-amyloid 25–35 fragment is known to be a functional peptide of the amyloid protein, exhibiting the same neurotrophic and neurotoxic properties as the whole protein. Therefore, central injections of βA 25–35 in animals are considered able to model AD. Along with the morphological and neurochemical changes, the injections cause cognitive dysfunction manifested in the animals' behavior. Injection of a single dose of beta-amyloid 25–35 fragment was shown to be valid as a model of not only amnesia but also the whole complex of neuropsychiatric symptoms of the early stages of AD (Abdurasulova et al., 2012). Considering that memory impairment in Alzheimer-type dementia can be mediated through a decrease in cholinergic effects on the cerebral cortex as a result of de-afferentation in the background of cell death in the nucleus basalis of Meynert, injection of the beta-amyloid fragment in this very structure of the brain is an informative and adequate AD model.

A comparative analysis of the data obtained in the model of AD induced by injection of beta-amyloid 25–35 fragment into the nucleus basalis of Meynert demonstrates that the neuroprotective efficiency of the HLDF-6-NH_2 peptide, evaluated by the rate of reactivation of cognitive functions, reliably surpasses the efficiency of the carboxylate form of the peptide. The difference between the pharmaceutical substances' effects is most pronounced in the cued version of the spatial memory test in the Morris maze. This learning depends on the integrity of basal ganglia and neocortex interaction (Packard, 2009). After βA 25–35 treatment the acquisition stage was impaired, although consolidation did not suffer. At the same time, this acquisition impairment was very robust to the drug protective effects. Both the neuromidin-treated group and the group treated with 250 μg/kg of HLDF6-NH2 displayed the trend to increase platform searching rate relative to the control animals, while HLDF6-OH was almost fully ineffective. Also, expressed difference between the two forms of peptide was observed in passive avoidance, which depends on the integration of the amygdala, hippocampus, and neocortex (Ambrogi Lorenzini et al., 1999). In this test the difference was more obvious when the lowest doses were compared. Less difference between amide and carboxylate was seen in object recognition, which is thought to be the most dependent on cortex activity (Ahn and Lee, 2015). These differences in the effects of HLDF6 forms should be studied using other behavioral tests, as well as histochemical and immunocytochemical techniques. It should be noted that a U-shaped form of the dose–response curve was observed for the HLDF-6-NH_2 peptide in AD modelled by injection of βA 25–35 into the nucleus basalis of Meynert. Our data was in agreement with the hypothesis of Nieto-Escámez et al. (2004), that NBM plays an important role during the acquisition phases but not in the execution of spatial navigation (Nieto-Escámez et al., 2004). Some data also indicated that, after brain injury, the performance in the cued version of the Morris water maze test was disturbed, mainly at the stage of acquisition (Wagner et al., 2013). These authors also showed that naïve rats reached a plateau of performance at a significantly higher level than in the non-cued version. Our results were quite similar.

Another proof of the greater efficiency of HLDF6-NH_2 compared to HLDF6-OH was obtained from the study of ChAT activity, which is a measure of the integrity of cholinergic projections into the cortex.

Another in vivo rat model of AD utilizes the intra-brain injection of beta-amyloid 25–35 and ibotenic acid, which causes neuron death, receptor decline, and impairment of cognitive functions (Morimoto et al., 1998). This model was also used in our study.

A higher activity of the HLDF-6-NH_2 peptide was confirmed in the second model. For example, in animals receiving injections of the HLDF-6-NH_2 peptide, the increment in the latency to enter the dark sector in the passive avoidance task tended to be considerably higher than in the groups of animals receiving the HLDP-6-OH peptide or the reference drug. The effects of the HLDF-6-NH_2 peptide in the AD models were comparable to those of the reference drug, or even exceeded the latter. For example, compared with memantine hydrochloride (10 mg/kg), the peptide tended to demonstrate a higher activity in the AD model in the conditioned passive avoidance task. Importantly, the peptide exerts its effects at much lower doses than the reference drugs.

At the same time, in the working memory task in the non-cued water maze, which is more dependent on hippocampal function, the difference in effects of the peptide forms was insignificant The significant progress of performance in this model which was observed only from the third day of training has been seen in other studies (Shoham et al., 2003; Weinstock M, 2003). This

somewhat surprising situation needs further study of the mechanisms of HLDF-6 action. It can be proposed that, besides the neuroprotective effects, the peptide acts as a nootropic drug and acts not only on impaired cells, but also improves also the functional activity of unimpaired structures. This suggestion, however, needs to be proved in a future study.

In our previous work we have shown that native HLDF-6-OH peptide influences the synthesis of steroid hormones. In particular, the peptide decreased the level of dihydrotestosterone content in the blood plasma of rats and also in the culture medium of hippocampal neurons, which increased significantly upon treatment with βA 25–35 fragment and ibotenic acid (Kostanyan et al., 2006). Dihydrotestosterone is the product of testosterone reduction by 5α-reductase. 5α-reductase is a membrane-bound enzyme found in the testicles, adrenal glands, and neurons of the cerebellum and hippocampus. Its activity strongly depends on the ordering of the lipid environment in the membrane (Weisser et al., 2001). Beta-amyloid peptides are known to decrease the fluidity of synaptosomal membranes, and ibotenic acid considerably increases this effect (Li et al., 2005). We have shown that HLDF-6-OH inhibits the expression of 5α-reductase of type I (Rzhevsky et al., 2005). In addition, HLDF-6-OH increases the fluidity of lipid membranes and, owing to this property, could prevent the hyper activation of 5α-reductase by beta amyloid (Kostanyan et al., 2000).

Dihydrotestosterone stimulates the binding of NMDA to the receptors of pyramidal neurons in the CA1 area of the hippocampus in males. This effect is strictly specific for this area of the hippocampus (Miguel-Hidalgo et al., 2002). The first pathological changes characteristic of AD are observed precisely in the pyramidal NMDA-sensitive neurons of the hippocampus. NMDA receptors are one of the most important targets for the action of nootropic and neuroprotective medical preparations, because they play a crucial role in the regulation of survivability and death rates of nerve cells (Bartlett and Wang, 2013; Gilmour et al., 2012; Myhrer 2003). Thus, the protective effect of HLDF-6-OH on the neurons of the rat hippocampus is probably associated with its ability to block the testosterone to dihydrotestosterone metabolism and, consequently, to influence the functioning of NMDA receptors.

Study of the receptor component of the HLDF-6-NH_2 peptide mechanism of action was performed by comparative analysis of density changes in the serotonin, $GABA_A$, and NMDA-glutamate receptors on the membranes of inbred C57B1/6 and Balb/c mouse prefrontal cortices and hippocampuses under the action of subchronic peptide administration. Mice of the Balb/c line are characterized by initial cognitive deficit and a lesser density of the receptors of interest.

Upon subchronic introduction of the peptide, the Bmax value showing the density of the corresponding receptors increased only in the case of NMDA-glutamate receptors. Thus, the five-fold systemic administration of the 100 μg/kg dose of the HLDF-6-NH_2 peptide increased the binding site density of [G-^{3}H] MK-801 with NMDA receptors in the Balb/c mice hippocampuses by 21% compared to the intact control. The density of NMDA receptors in Balb/c mouse hippocampuses approached the corresponding value of the reference group, represented by C57B1/6 mice, in which the preparation was ineffective. Subchronic introduction of the Thr-Gly-Glu-Asn-His-Arg-NH_2 peptide resulted in a non-selective density decrease in 5-HT_{2A} serotonin receptors and produced no effect on the nicotine nACh-receptors and $GABA_A$ receptors in the prefrontal cortices of both lines (Zolotarev et al., 2014b).

Thus, the results obtained from radio ligand analysis studies of the HLDF-6-NH_2 peptide effects on neuroreceptor processes showed that the hippocampal glutamatergic system, particularly the NMDA-type receptors, was involved with high probability in the mechanism of its pharmacological effects.

Recently, the neuroprotective effects of several proteins (erythropoietin, the main fibroblast growth factor) were demonstrated in animals using the beta-amyloid injection model of AD. However, under conditions of intranasal administration, improvement of cognitive functions was observed in a considerably longer treatment (12–17 days) when compared with the protocol for the HLDF-6 peptide injections that we used (Tazangi et al., 2014; Zhang et al., 2014). A decrease in the duration of the therapeutic treatment is a considerable advantage, because it decreases the risk of adverse effects. Moreover, proteins are much more immunogenic than short peptides; therefore, the risk of allergic reactions and other unwanted consequences upon administration of proteins is much higher. Also important is the fact that the cost of peptide formulations, as a rule, is much lower than that of proteins.

Altogether, the data obtained allow for the conclusion that both forms of the HLDF-6 peptide reactivated impaired cognitive functions in the AD models under study, but the amide form was significantly more efficient. Similar data were obtained in the model of a cerebrovascular pathology, that is, ischemic stroke (Storozheva et al., unpublished). Hence, our suggestion of higher biological activity of the peptide with the protected C-terminal carboxyl group was confirmed. A dose of 250 μg/kg resulted in practically complete restoration of the functions (statistically significant differences compared with the control and no differences with sham-operated animals or exceeding the latter values). Therefore, a dose of 250 μg/kg HLDF-6-NH_2 peptide was established as an efficient therapeutic dose.

Based on these data the amide form of the HLDF-6 peptide (HLDF-6-NH_2) was chosen as the subject for further preclinical and clinical studies.

Preclinical studies of the HLDF-6-NH_2 peptide demonstrated that a therapeutic agent based on the substance exhibited no immunogenicity, was characterized by high efficiency, was not toxic, and was safe at a dose equivalent to a 10-fold therapeutic dose for humans (Rzhevsky et al., unpublished).

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