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Research Article

HAEE tetrapeptide as a promising neuroprotector: comparison with Piracetam in an experimental model of Alzheimer's disease

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Abstract

Introduction: During the experiment, the effectiveness of tetrapeptide HAEE and the comparison drug Piracetam in correcting neurodegenerative disorders in Alzheimer's disease was investigated in a model of transgenic mice.

Materials and Methods: transgenic mice of the APPswe/PS1dE9 (APP/PS1) line were used in the study. The animals were injected with HAEE and Piracetam, after which behavioral tests were performed ("Open Field", "Light-Dark Chamber", "Novel Object Recognition", "Barnes Maze"). Additionally, gene expression was analyzed using real-time PCR. The histological study included staining of brain sections with Congo red, followed by counting amyloid plaques in the cortex and hippocampus.

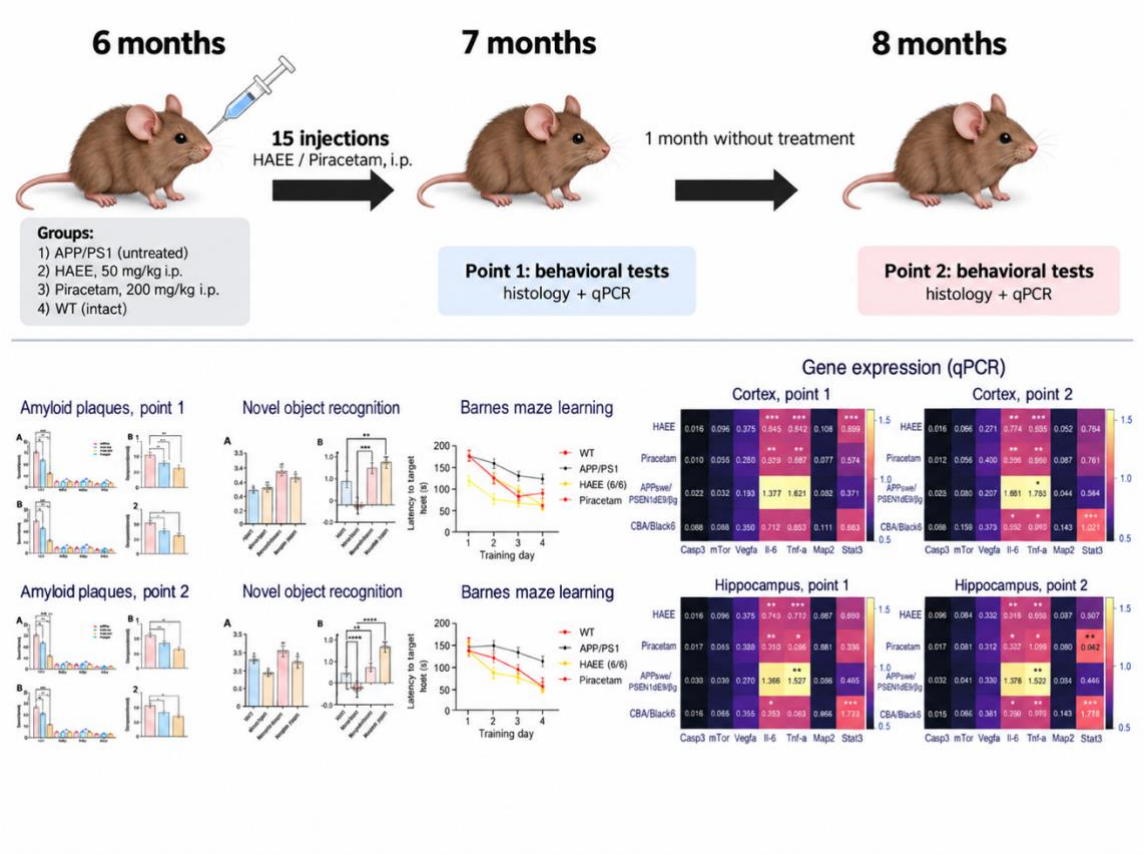
Results and Discussion: The administration of HAEE and Piracetam led to an improvement in cognitive performance, with both groups demonstrating memory recovery to the level of intact controls. Histological analysis showed an increase in the number of amyloid plaques in the positive control animals and a reduction in the therapeutic groups by an average of 40%. Gene expression analysis showed that HAEE and Piracetam modulate key molecular pathways associated with neuroplasticity, inflammation, and cellular survival. Changes in the expression of the Stat3, mTor, Vegfa, and Casp3 genes have been observed, reflecting the activation of compensatory neuroprotective mechanisms, as well as the relative stabilization of the pro-inflammatory markers Il-6 and Tnf-a.

Conclusion: HAEE tetrapeptide at a dosage of 50 mcg per 1 kg and Piracetam at a dosage of 200 mcg/kg have a comprehensive positive effect on cognitive functions, molecular and morphological parameters in Alzheimer's disease. The results obtained in the animal model confirm its high potential as a promising neuroprotective agent, comparable to or superior to classical nootropic drugs.

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Graphical Abstract



Keywords

alzheimer's disease; beta-amyloid; behavioral testing; gene expression

Introduction

Alzheimer's disease (AD) is the most common form of dementia and is a progressive neurodegenerative disease accompanied by impaired memory, cognitive functions, and behavioral responses (De Strooper et al. 2016; Long and Holtzman 2019; Welikovitch et al. 2025) The pathogenesis of AD is based on a complex of molecular and cellular disorders, among which the accumulation of β-amyloid (Aß) in the form of extracellular amyloid plaques, as well as the formation of neurofibrillary tangles consisting of hyperphosphorylated tau protein, is central (De Strooper et al. 2016; Welikovitch et al. 2025).

According to the amyloid hypothesis, an imbalance between Aß production and clearance leads to its aggregation and toxic effects on neurons, which initiates a cascade of pathological processes, including synaptic dysfunction, neuroinflammation, and apoptosis (De Strooper et al. 2016; Long and Holtzman 2019; Welikovitch et al. 2025). Experimental studies in transgenic models confirm that the accumulation of Aß is closely associated with cognitive impairment and the development of neurodegeneration (Almohmadi et al. 2025; Han et al. 2025).

Transgenic mice, in particular, of the APPswe/PS1dE9 line, characterized by early accumulation of Aß and severe cognitive impairment, are widely used to study the mechanisms of AD and evaluate the effectiveness of therapeutic approaches (Almohmadi et al. 2025; Han et al. 2025). This model allows reproducing key aspects of the disease, including the formation of amyloid plaques in the cortex and hippocampus, which makes it a convenient tool for preclinical research.

Despite the accumulated information about the mechanisms of development of Alzheimer's disease, modern pharmacotherapy does not yet have a sufficient number of drugs that can significantly

affect the course of the disease. Most of the drugs used are mainly focused on reducing the severity of symptoms (Lysikova et al. 2023). Traditional nootropics, including Piracetam, affect neurometabolic processes and synaptic transmission, but their effect on the main pathogenetic links of the disease remains limited (Malykh and Sadaie 2010; Korokin et al. 2023).

One of the promising areas of the search for new therapeutic agents has become the study of peptide compounds capable of simultaneously acting on several molecular targets. Their multi-target action may encompass key processes involved in the development of Alzheimer's disease, including amyloidogenesis, neuroinflammation, and neuroplasticity (Bach et al. 2020; Zhang et al. 2021). It is precisely the possibility of a complex effect on various links of the pathological process that makes it possible to consider peptides as a promising basis for the creation of neuroprotective drugs.

Special attention is drawn to the HAEE tetrapeptide, which has demonstrated in experimental studies the ability to reduce amyloid load and improve cognitive functions. The data obtained indicate that HAEE promotes beta-amyloid clearance and has a pronounced neuroprotective effect in models of Alzheimer's disease (Patrakhanov 2024). In addition, the results of additional studies indicate the involvement of HAEE in the regulation of molecular processes associated with neuroinflammation (Mukhina 2025).

The aim of this study was to study the effect of HAEE tetrapeptide and Piracetam on cognitive function, amyloid load, and expression of genes associated with neuroinflammation and neuroplasticity in transgenic APP/PS1 mice used as a model of Alzheimer's disease.

Materials and Methods

The compound under investigation

The composition represents an efficient tetrapeptide delivery [Acetyl]-His-Ala-Glu-Glu-[Amide] (HAEE) (VladMiVa Experimental Plant JSC, Russia,), used as a medicinal substance through the blood-brain barrier (BBB) for the treatment of neurodegenerative diseases, including Alzheimer's type dementia (Alzheimer's disease). A pharmaceutical composition for the treatment of neurodegenerative diseases containing an effective amount of HAEE peptide in the equimolar complex HAEE-Zn-HSA with zinc and human serum albumin. The reference drug was Piracetam (Borisov Plant of Medical Preparations JSC, Belarus).

Animals

The animals were kept in the SPF vivarium of Belgorod State National Research University (NRU BelSU, Russia) under artificially regulated daylight conditions (12 hours of dark and 12 hours of daylight) at temperatures from +22 to +26 °C and had free access to food and water. The work was guided by the ethical principles of the treatment of laboratory animals in accordance with the European Convention for the Protection of Vertebrates Used for Experimental and Other Scientific Purposes (ETS No. 170). All painful manipulations with animals were carried out in accordance with regulatory standards.: Directive 2010/63/EU of the European Parliament and of the Council of the European Union of 22 September 2010 on the protection of animals used for scientific purposes. The study was approved by the Commission for control over the maintenance and use of laboratory animals of the National Research University ”BelSU”, expert opinion No. 01-06i/24 dated 06/03/2024.

The animals were divided into experimental groups:

– APPswe/PS1dE9/Blg (APP/PS1) – transgenic control (without therapy) (n=10);
– HAEE(IV) – transgenic animals treated with HAEE tetrapeptide intraperitoneally (n=10);
– Piracetam – transgenic animals treated with the reference drug Piracetam (n=10);
– C3H/C57BL6 (WT) – wild type animals (n=10).

During the study, the drug was administered to the studied groups of animals aged 6 months. In the first group, the drug tetrapeptide HAEE was administered intraperitoneally at a dosy of 50 mcg per 1 kg of mouse weight. The second group was given Piracetam at a dosy of 200 mcg/kg. A control group of transgenic animals (positive control) and a group of healthy mice (negative control) were injected with saline intraperitoneally. The drugs were administered in a circadian manner, every 48 hours, seven days a week, for one month, then at the 1st point of the experiment, half of the group (n=5) was selected for further histological analysis of the mouse brain. The remaining half of the animals did not receive drugs for a month, then a histological examination of the brain was performed.

Histological analysis of the brain

The animals were subjected to terminal anesthesia, the brain was dissected and fixed in a Carnois solution (6 parts 96% ethyl alcohol, 3 parts chloroform, 1 part glacial acetic acid) overnight. The

tissue was dehydrated by sequentially passing through ethyl alcohols with increasing concentration: 75% 1 hour, 96% (I) 5 minutes, 96% (II) 45 minutes, 100% (I) 5 minutes, 100% (II). Next, it was incubated for 30 minutes in a mixture of 100% ethyl alcohol-chloroform (1:1), 1 hour chloroform (I), left overnight in chloroform (II), after which the tissues were impregnated with paraffin (3 shifts of 1 hour) at 600C. The paraffin blocks were prepared at the Leica EG1160 filling station (Leica Biosystems, United Kingdom). Paraffin sections with a thickness of 8 microns were mounted on slides with a polylysine coating.

Five glasses consisting of 10 brain slices were formed from a 400-micron-thick area of the brain, and every fifth brain slice was placed on each slide. Next, the sections were de-waxed in xylene for 20 minutes and rehydrated by sequential incubation in ethyl alcohol:10 minutes in 100%, 5 minutes in 95%, and 5 minutes in 50%, then washed three times in deionized water for 5 minutes. The sections were stained with a solution of Congo red dye (0.5% Congo red in 50% ethyl alcohol) for 5 minutes and differentiated in a solution of 0.2% KOH in 80% ethyl alcohol for 1 minute, washed three times in deionized water for 5 minutes and enclosed in a water-based medium Immu-Mountm (Thermo Scientific).

Behavioral testing

The "Open Field" test

The animal under study was placed in the "Open Field" installation (NPC Open Science, Russia) and the movements of the animal were recorded. The installation is a square chamber with a 50 × 50 cm base made of opaque plexiglass. The animal's behavior was assessed using one parameter that characterizes the behavior of mice – motor activity. EthoVision software (Noldus Information Technology, Netherlands) allows you to automatically obtain indicators of the parameters selected for analysis: distance traveled, activity, average speed of all movements in cm/sec. Each animal was tested for 5 minutes at 40 lux.

Novel Object Recognition Test

This is a simple behavioral test based on the innate exploratory behavior of rodents. The test is divided into three phases: habituation, training/adaptation, and the testing phase. On the first day of the test, the animal is placed in an empty 50 × 50 cm arena (OpenScience, Krasnogorsk, Russia) to explore for 5 min under standard room lighting. The second day is the adaptation phase, when the animal is placed in the same arena with two identical objects. On the third day, the testing phase, the animal is placed in the arena with one familiar object from the previous phase and one novel object. The following parameters were recorded: the number of approaches to the novel and familiar objects and the time spent near each.

The following indicators were recorded: the number of trips to the novel and familiar object and the time spent near each, the preference index (PI) calculated according to formula 1, and the discrimination index (DI) calculated according to formula 2.

$$\mathrm{PI} = \left(\frac{\mathrm{Tn}}{\mathrm{Tn} + \mathrm{To}}\right) * 100 \text{ , (1)}$$

$$\mathrm{DI} = \frac{\mathrm{Tn} - \mathrm{To}}{\mathrm{Tn} + \mathrm{To}}, \text{ (2)}$$

PI – preference index; DI – discrimination index; Tn – time to explore a novel object; To – time to explore an familiar object.

Barnes Maze Test

This test was used to study spatial learning and memory in animals. The goal of the Barnes Maze (OpenScience, Krasnogorsk, Russia) is for the mouse to explore the space and remember the location of the escape hole using the configuration of distal visual cues placed around the testing area. The setup is a circular platform 122 cm in diameter, containing 40 holes, each 5 cm in diameter, one of which serves as the exit (escape box). The distal visual cues consisted of four black-and-white images with different shapes and patterns, positioned in the north, south, west, and east directions. Video recording was performed for 5 min. Measurements included the total distance traveled by the animal, movement speed, and finding the exit within the allotted time (Polikarpova et al. 2022).

Gene expression analysis

Brain structures were isolated on ice immediately after decapitation of an animal under injection of anesthesia. All tissues were directly placed in tubes with a lysing buffer from the RNeasy Mini Kit (250) RNA isolation kit (Quagen, Germany), located on ice, where they were subjected to ultrasonic homogenization. The further RNA isolation procedure was carried out following the instructions for the kit. Purified solutions with RNA were analyzed spectrophotometrically by a

NanoDrop OneC device (Thermo Scientific, USA) and diluted to a single concentration of 100 ng/μL.

Reverse transcription was performed using the MMLV RT reagent kit (Eurogen, Russia) according to the manufacturer's instructions. 1 microgram of each RNA sample was used for reverse transcription. The synthesized cDNA was used as a template in a real-time PCR reaction using a set of reagents 5X qPCRmix-HS SYBR (Eurogen, Russia). Specific primers were used to determine the expression level of the genes of interest (GOI): Stat3, Map2, Il-6, mTOR, Tnf-a, Vegfa, and Casp3. Gapdh was selected as the reference gene (REF).

The relative quantitative assessment of the expression of the target genes was carried out according to formula 3:

$$2\text{-}\Delta CT*100, \text{ где } \Delta Ct = Ct(GOI) - \Delta Ct(REF) \quad (3)$$

Table 1. Oligonucleotides for PCR analysis of gene expression

Genes	Forward primer	Reverse primer
Casp3	5'-AGCTTGGAACGGTACGCTAA-3'	5'-GAGTCCACTGACTTGCTCCC-3'
mTor	5'-CAGTTCGCCAGTGGACTGAAG-3'	5'-GCTGGTCATAGAAGCGAGTAGAC-3'
Vegfa	5'-CCTCCACCATGCCAAGTGGTCC-3'	5'-ACCAGGGTCTCAATCGGACGGC-3'
Il6	5'-TAGTCCTTCCTACCCCAATTTCC-3'	5'-TTGGTCCTTAGCCACTCCTTC-3'
Tnf-a	5'-TCCAGGCGGTGCCTATGT-3'	5'-GCCCCTGCCACAAGCA-3'
Map2	5'-CTGGACATCAGCCTCACTCA-3'	5'-AATAGGTGCCCTGTGACCTG-3'
Stat3	5'-GACATTCCCAAGGAGGAGGC-3'	5'-CTTGGTCTTCAGGTACGGGG-3'
Gapdh	5'-CCGGGGTCCCAGCTTAGGTTCA-3'	5'-CGGCCAAATCCGTTCACACCGA-3'

Statistical analysis

Statistical analysis was performed using GraphPad Prism 8.0 software (GraphPad Software, San Diego, CA, USA). Data are presented as mean ± SEM unless otherwise indicated. Normality of distribution was assessed using the Shapiro–Wilk test. For normally distributed data, comparisons between groups were performed using two-way ANOVA with genotype and TBI as the main factors, followed by Tukey's multiple-comparisons post hoc test when appropriate. For non-normally distributed data, the Kruskal–Wallis test followed by Dunn's multiple-comparisons test was used.

For behavioral tests involving repeated measurements across training days, such as the Barnes maze acquisition phase, repeated-measures two-way ANOVA or a mixed-effects model was used, with day as the within-subject factor and experimental group as the between-subject factor. If missing values were present, a mixed-effects model was applied. Post hoc comparisons were corrected for multiple testing.

Correlation analyses between behavioral parameters and amyloid pathology were performed using Pearson's correlation for normally distributed data or Spearman's rank correlation for non-normally distributed data. The threshold for statistical significance was set at $p < 0.05$. Results with $0.05 \leq p < 0.10$ were interpreted as trends and not as statistically significant differences. No data points were excluded from the analysis unless they met the predefined technical exclusion criteria.

Results

In the cerebral cortex, drug HAEE (Fig. 1) exhibits a statistically significant anti-amyloid effect already at the first time point of the study. It reduces the total number of plaques by 17.3% in the HAEE group and by 46% in the Piracetam group compared to the control group ($p = 0.0150$, $p = 0.0018$) and significantly reduces the number of small deposits by 30% and 63%, respectively (<100 μm², $p = 0.0015$; $p < 0.0001$). The effect of HAEE is inferior to that of Piracetam.

In the hippocampus, HAEE significantly reduces the number of small plaques by 45% ($p < 0.0001$), but the total number of deposits decreases only at the level of the trend (33%; $p = 0.0857$), which may be due to insufficient statistical power or to the fact that in the hippocampus at an early stage, the contribution of small plaques to the overall load is less than in the cortex.

Piracetam, by contrast, already at an early stage produces a significant reduction of both small plaques by 72.7% ($p < 0.0001$) and their total number by 55% ($p = 0.0076$).

HAEE has a statistically significant anti-amyloid effect in the cerebral cortex (Fig. 2): it reduces the total number of β-amyloid plaques by 13.6 % ($p = 0.0272$) and, primarily, decreases the number of small deposits by 18.5 % (<100 μm², $p < 0.0001$). A similar trend is observed in the hippocampus: the number of small plaques significantly decreases by 36% ($p < 0.0001$), and the overall amyloid load decreases at the borderline significance level of 32.5% ($p = 0.0518$).

The effect of HAEE is size-specific; it acts primarily on the early stages of amyloidogenesis (small, presumably recently formed plaques) without affecting already formed deposits of medium, large, and giant size. This may indicate the ability of HAEE to inhibit the de novo formation of plaques rather than to induce regression of mature conglomerates.

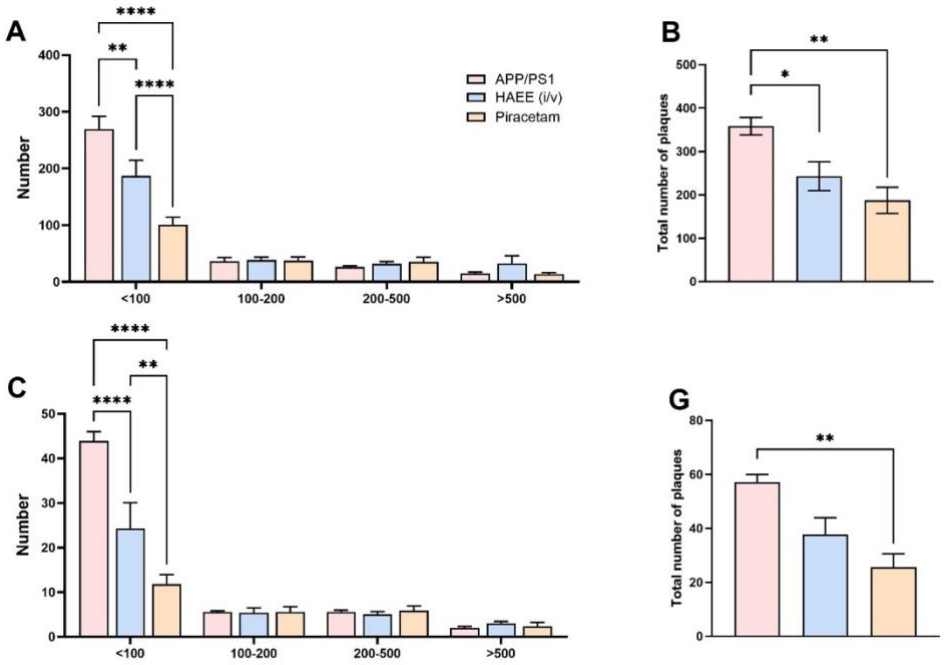


Figure 1. The distribution of plaques by size 1 month after the start of therapy in (**A**) the cortex and (**C**) the hippocampus. The plaques are arranged in the following gradation: <100 μm^2, 100–200 μm^2, 200–500 μm^2, and >500 μm^2. The total number of plaques 1 month after the start of therapy in the (**B**) cortex and (**G**) hippocampus. ***Note:*** The values are presented as the mean ± the standard error of the mean. * – $p < 0.05$, ** – $p < 0.01$, *** – $p < 0.001$, **** – $p < 0.0001$ (one-way ANOVA post hoc comparisons).

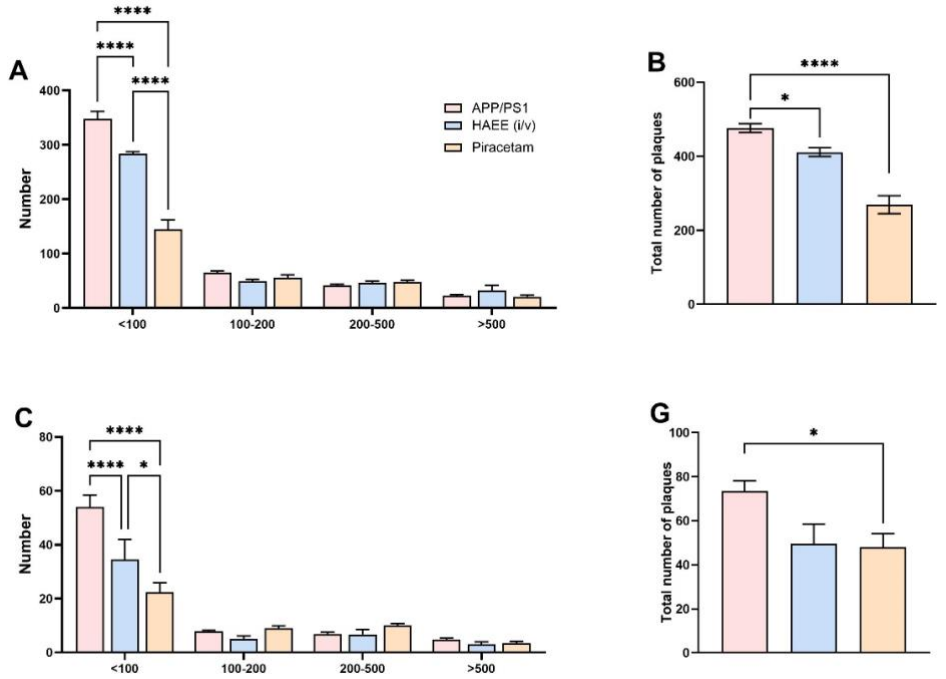


Figure 2. Plaque size distribution 2 months after initiation of therapy in (**A**) cortex and (**B**) hippocampus. The plaques are arranged in the following gradation: <100 μm^2, 100–200 μm^2, 200–500 μm^2, and >500 μm^2. The total number of plaques 1 month after the start of therapy in (**B**) the cortex and (**G**) the hippocampus. ***Note:*** The values are presented as the average ± standard error of the average. * – $p<0.05$, *** – $p<0.001$, **** – $p<0.0001$. (one-way ANOVA post hoc comparisons).

Comparison with the reference drug Piracetam shows that HAEE is inferior to it in terms of the severity of the anti-amyloid effect in both the cortex (34.4%) and the hippocampus (3%). Differences between the treated groups are highly significant for small plaques ($p < 0.0001$ in the cortex and in the hippocampus ($p < 0.05$). Nevertheless, it retains a significant positive effect compared to positive control.

At the first stage of the experiment, there were no statistically significant changes in locomotor activity in the mice of the positive control and the groups receiving the studied compounds, compared with the negative control. The absence of differences in anxiety indicators showed that neither the transgenic genotype, nor the intravenous administration of Piracetam and HAEE had a modulating effect on the emotional status of animals. There was aarked tendency to increase the distance traveled in the HAEE (i/v) group ($p=0.0871$). It can be considered as a potential effect of the compound, requiring evaluation in dynamics.

At the second stage of the experiment, there were no statistically significant differences in locomotor activity and anxiety behavior between the groups. At the same time, the positive control animals and the Piracetam group showed a tendency to increase their movement speed and distance traveled, but statistical reliability was not achieved. Throughout the entire follow-up period, the HAEE group's (i/v) values remained comparable with the negative control in all the parameters studied.

The results of the "Open Field" test indicate the temporal dynamics of behavioral effects. At an early stage, the indicators of all groups corresponded to such in the control level, whereas by the second stage, multidirectional trends appeared: in animals of positive control and in the Piracetam group, a moderate increase in locomotor activity was observed. The HAEE group (i/v) maintained a profile comparable to the negative control throughout the experiment. This confirms the need for additional assessment of locomotor activity in other behavioral tests.

At the first stage of the experiment, there were no statistically significant differences between the groups. The positive control, as well as the animals treated with Piracetam and HAEE (i/v), did not differ from the negative control in terms of the time spent in the light compartment, the number of crossings, the latency of the first approach, and the total distance traveled. The data obtained ($p>0.05$ in all comparisons) allow us to conclude that under the conditions of this experiment neither the transgenic genotype nor the studied compounds had a modulating effect on the level of anxiety and locomotor activity of animals.

At the second stage of the experiment, as at the first point, there were no statistically significant differences between the groups in any of the studied parameters. The positive control, as well as the animals treated with Piracetam and HAEE (i/v), did not differ from the negative control in terms of the time spent in the light compartment, the latency of the first approach, the number of crossings and the total distance traveled. The data obtained ($p >0.05$ in all comparisons) allow us to conclude that after a while neither the transgenic genotype nor the studied compounds had a modulating effect on the level of anxiety and locomotor activity of animals, which is completely consistent with the results of the first point of the experiment.

The analysis of the speed in the "Novel object recognition" movement test did not reveal statistically significant differences between the groups on any of the days ($p>0.05$ for all comparisons). All animals moved at a comparable speed, which makes it possible to exclude the influence of motor disorders on the interpretation of cognitive indicators.

An in-group analysis of the distance traveled did not reveal any differences between the first and second days of testing in any of the groups. Consequently, the motor activity of the animals remained stable throughout the test.

In the "Novel object recognition" test (Fig. 3), the most pronounced impairments were detected in animals of positive control. Their discrimination index was 5 times lower than the negative control index ($p=0.0401$), and compared with the Piracetam and HAEE groups (i/v), it was 12 and 12.5 times lower, respectively ($p=0.0010$ and $p=0.0015$). At the same time, the locomotor activity of the positive control did not differ from the indicators of the negative control; therefore, the revealed cognitive deficit cannot be explained by motor disorders.

In the Piracetam and HAEE (i/v) groups, motor activity, on the contrary, was increased, but this did not affect the results of object recognition. The discrimination indices in both groups corresponded to the level of healthy control and statistically significantly exceeded the indicator of positive control.

When assessing the distance traveled, normalization of motor activity in the experimental groups was revealed. In contrast to the first point, where the experimental groups Piracetam and HAEE (i/v) demonstrated pronounced hyperlocomotion, there are no statistically significant differences at the second point.

The analysis of movement speed, as in the first point, did not reveal statistically significant differences between the groups on any of the days ($p>0.05$ for all comparisons). All animals moved at a comparable speed, which makes it possible to exclude the influence of motor disorders on the interpretation of cognitive indicators.

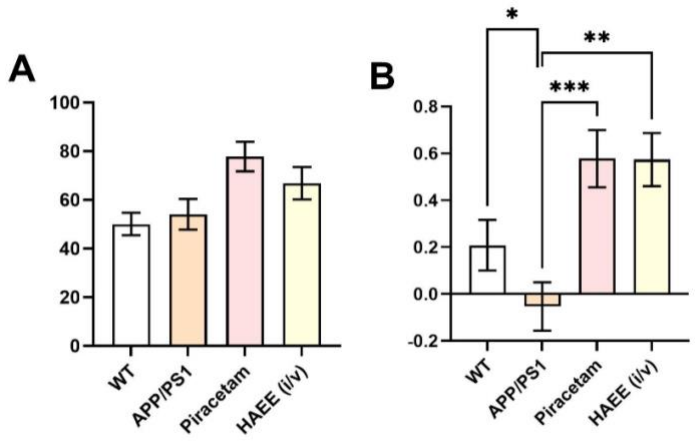


Figure 3. Analysis of cognitive indicators in the "Novel object recognition" test 1 month after the start of therapy. A is the preference index, B is the discrimination index. ***Note:*** The values are presented as the average ± standard error of the average. * – p<0.05,** – p<0.01, *** – p<0.001. (one-way ANOVA post hoc comparisons).

The discrimination index, considered as the most sensitive indicator of "recognition" memory, revealed a pronounced separation of the studied groups (Fig. 4). In animals of positive control, this indicator was lower than in WT, Piracetam and HAEE (i/v) by 0.543, 0.593 and 1.093, respectively. Differences with WT

were of high statistical significance (p=0.0004), as were differences with the Piracetam (p=0.0025) and HAEE (i/v) (p<0.0001) group. This pattern indicates a persistent cognitive deficit in positive control animals.

The dynamics of the indicators also indicates a gradual deterioration of memory in animals of the pathological group. The administration of Piracetam and HAEE (i/v), on the contrary, ensured the preservation of memory parameters at the level of healthy control; in addition, the values in both therapeutic groups were statistically significantly higher than those of positive control.

According to the preference index, there was no statistically significant difference between the groups. Nevertheless, in animals of the positive control, there was a tendency to decrease this indicator compared with the negative control (p=0.1224) and the Piracetam group (p=0.0960). The values obtained in the HAEE group (i/v) were closer to the level of positive control. The lower sensitivity of the preference index compared to the discrimination index is a characteristic feature of this test.

The dynamics between the points demonstrates progressive memory impairment in the positive control. If at the first point the differences from the negative control on the discrimination index were at the level of p=0.0401, by the second point the significance of the differences increased to p=0.0004, which confirms the development and consolidation of the pathological phenotype characteristic of the Alzheimer's disease model.

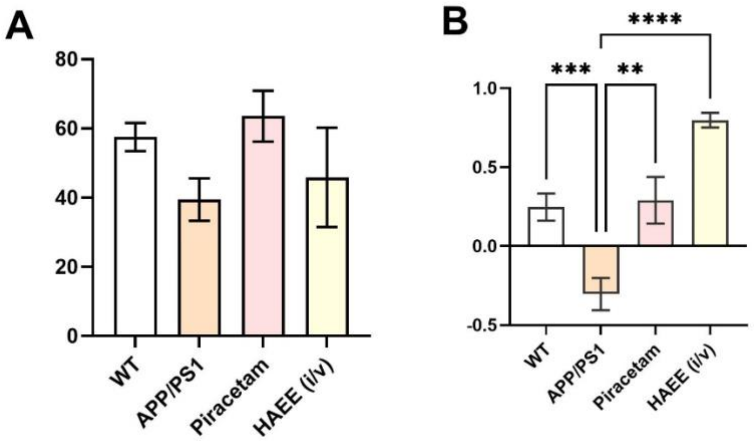


Figure 4. Analysis of cognitive indicators in the "Novel object recognition" test 2 months after the start of therapy. A is the preference index, B is the discrimination index. ***Note:*** The values are presented as the average ± standard error of the average. ** – p<0.01, *** – p<0.001, ****– p<0.0001 (one-way ANOVA post hoc comparisons).

The key result is memory retention in the treated animals. Throughout the experiment, cognitive performance in the Piracetam and HAEE groups (i/v) did not differ from that of the healthy controls.

At the second experimental point, motor activity also returned to normal. The increased locomotion observed in the treated groups at the first stage disappeared, which made it possible to evaluate the cognitive effects of the late stage of the experiment with less influence of the motor factor.

In the "Barnes Maze" test (Fig. 5), typical learning dynamics was revealed in animals of negative control. The latency time decreased consistently and statistically significantly by 46 seconds during the transition from the first to the second day ($p<0.0001$), from the second to the third – by 45 seconds ($p=0.0005$), and when comparing the first and fourth days – by 106 seconds and amounted to 68 seconds before entering the hole ($p<0.0001$). The absence of significant differences between the third and fourth days ($p=0.8585$) indicates the achievement of a plateau and consolidation of the formed skill.

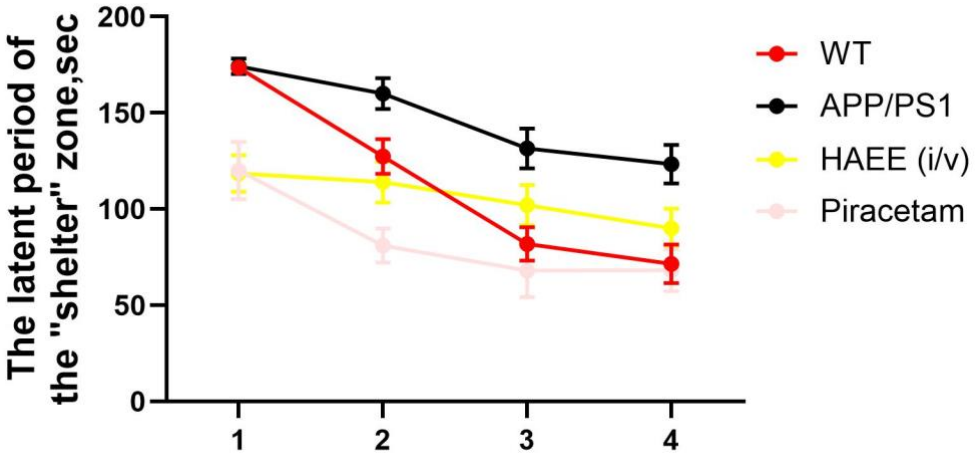


Figure 5. The results of training in the "Barnes Maze" 1 month after the start of therapy. The latent period of the "shelter" zone, with days of training in the "Barnes Maze" test shown on the X-axis.

The positive control animals (APP/PS1) were trained with impairments. A statistically significant reduction in latency was noted only when comparing the first and third days – by 43 seconds ($p=0.0009$), as well as the first and fourth days – by 51 seconds ($p=0.0003$). This dynamic indicates a lack of progress in memorizing a spatial landmark and corresponds to the phenotype of Alzheimer's disease.

Piracetam provided a gradual improvement in spatial learning, although pronounced dynamics appeared only towards the end of testing. When comparing the first and fourth days, the latency decreased by 50 seconds ($p=0.0099$). There were no statistically significant differences between the neighboring days – the first and second, the second and third, the third and fourth. At the same time, the general tendency to reduce search time indicates a slow but consistent skill formation.

In the HAEE (i/v) group, improvement was already evident at an early stage. Latency was significantly lower when comparing the first and second days – by 40 seconds ($p=0.0071$), and the first and fourth days – by 57 seconds ($p=0.0192$). The absence of significant differences between the second, third, and fourth days indicates that the plateau was quickly reached by the second day and the formed level of learning was maintained.

An intergroup comparison of the indicators for individual days also revealed a consistent pattern. On the second day, APP/PS1 animals spent 33 seconds longer on completing the task than WT ($p=0.0404$), which indicates an early learning disability. In the Piracetam group, latency was 79 seconds lower than in the positive control ($p<0.0001$) and 46 seconds lower than in the WT ($p=0.0050$). In the HAEE (i/v) group, the indicator was also 46 seconds lower than in APP/PS1 ($p=0.0054$), with no differences from WT ($p=0.7829$), indicating normalization of results already at this stage.

By the third day, the difference between positive and negative control reached 50 seconds ($p=0.0025$). Piracetam maintained its advantage over APP/PS1: latency was 29 seconds lower ($p=0.0073$), and the indicators corresponded to the WT level ($p=0.8297$). In the HAEE group (i/v), the average value was better than that of APP/PS1, but there was no statistically significant difference ($p=0.2005$); at the same time, the indicators remained comparable with WT ($p=0.4678$).

On the fourth day, APP/PS1 animals remained deficient compared to WT (p=0.0024). In the Piracetam group, latency was 55 seconds lower than in the positive control (p=0.0046), and there were no differences from such in WT (p=0.9956). For HAEE (i/v), there was an improvement trend relative to APP/PS1 (p=0.0968), while maintaining comparable indicators with WT (p=0.5674). A direct comparison of the two compounds revealed no differences on any of the training days (p>0.05 in all cases), suggesting similar efficacy of Piracetam and HAEE (i/v).

When estimating the latency time in the shelter zone test (Fig. 6A), animals of the positive control spent 3.3 times more time on the first visit to the target zone than animals of the negative control (p=0.0375). This confirms the presence of a pronounced spatial memory deficit. The indices of the Piracetam and HAEE groups (i/v) did not significantly differ from either the positive or negative control and occupied an intermediate position.

A similar pattern was found when calculating visits to the former shelter area (Fig. 6B): animals of positive control made 1.85 times fewer visits than animals of negative control (p=0.006). Such dynamics indicates an impaired consolidation of the memorial trail and a decrease in interest in the spatially associated zone. No significant differences with positive control were found in either therapeutic groups (p>0.9999 in both comparisons); however, their indicators did not differ from the level of intact control either, which indicates a partial normalization of search behavior.

In terms of the total time spent in the shelter area (Fig. 6C), in the positive control group it was significantly 2.5 times lower than in the negative control group (p=0.0293), confirming the stability of cognitive deficits in transgenic animals.

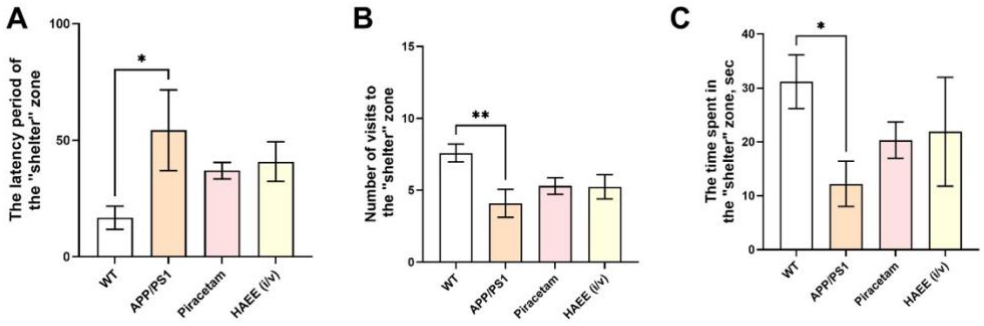


Figure 6. Test day of mice in the "Barnes Maze" test. The latency period of the "shelter" zone (**A**), the number of visits to the "shelter" zone (**B**), the time spent in the "shelter" zone (**C**). ***Note:*** The values are presented as the average ± standard error of the average. * – p<0.05, ** p – < 0.01 (one-way ANOVA post hoc comparisons).

The assessment of the time spent in the target area did not reveal a statistically confirmed improvement in the experimental groups. In the Piracetam group, there was only an increase in this indicator compared to the positive control (p=0.2191), which did not reach the significance level. The HAEE group's (i/v) values did not differ from the positive control (p>0.9999) either. At the same time, no significant differences from negative controls were found in any of the experimental groups. A significant variation in the data may reflect incomplete recovery of the function under study.

The results of the three parameters combined indicate a persistent pronounced deficit of spatial memory in animals of positive control. In the Piracetam and HAEE groups (i/v), the indicators occupied an intermediate position between such in the pathological and intact controls: they did not reach the level of healthy animals, but the severity of the disorders was less than that of the positive control. The combination of the absence of statistically significant differences with positive control and simultaneous comparability with WT suggests a partial therapeutic effect, manifested in a tendency to improve spatial memory without its full restoration to physiological norm. The movement speed and distance traveled during all four days of training did not differ between the groups (p>0.05) either. The comparable values of these indicators in all animals make it possible to exclude the influence of motor disorders on the results of spatial memory assessment.

In the negative control group, a latency reduction curve characteristic of successful learning was formed (Fig. 7). Compared with the first day, latency decreased by 47 seconds by the third (p=0.0016), and by the fourth – by 80 seconds (p<0.0001). A significant reduction was also noted when comparing the second and fourth days – by 61 seconds (p=0.0002), and the third and fourth days – by 33 seconds (p=0.0339). There were no statistically significant differences between the first and second days (p=0.3190), whereas when comparing the second and third days, there was a tendency to decreased latency (p=0.0739). This dynamics indicates a gradual and steady formation of spatial skill with the achievement of a plateau by the fourth day.

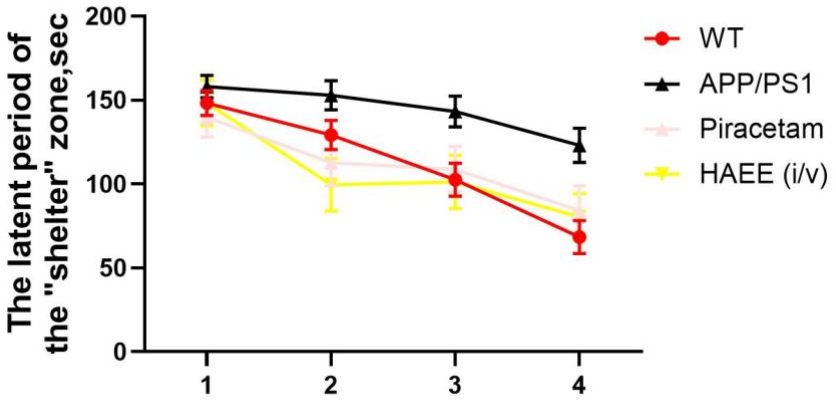


Figure 7. The results of the "Barnes Maze" training 2 months after the start of therapy. The latent period of the "shelter" zone, with days of training in the "Barnes Maze" test shown on the X-axis.

In animals of positive control, there was a marked impairment of spatial learning. A statistically significant reduction in latency was found only when comparing the first and fourth days ($p=0.0349$). No significant differences were found between the adjacent days of training – the first and second, the second and third, the third and fourth ($p>0.05$), indicating the inability of animals to form a stable spatial footprint.

There was a more favorable learning dynamics in the Piracetam group. Latency decreased significantly when comparing the first and third days – by 31 seconds ($p=0.0473$), as well as the first and fourth days – by 59 seconds ($p=0.0317$). When comparing the first and second days ($p=0.3849$), as well as the second and third and third and fourth days ($p>0.05$), no significant differences were found. Taken together, these data confirm the overall positive trend towards training.

In the HAEE group (i/v), signs of improvement appeared earlier than in the other experimental groups. Latency decreased statistically significantly when comparing the first and second days – by 40 seconds ($p=0.0071$), and the first and fourth days – by 57 seconds ($p=0.0192$). The absence of significant differences between the second, third and fourth days indicates that the plateau was quickly reached by the second day and the achieved result was maintained afterwards.

A comparison of the indicators between the groups on individual days of study revealed consistent dynamics of intergroup differences. On the second day, latency in the HAEE (i/v) and Piracetam groups was significantly lower than in APP/PS1 ($p=0.0158$ and $p=0.0236$, respectively).

By the third day, the differences between the groups became more noticeable: APP/PS1 differed from WT ($p=0.0374$), and the Piracetam group differed from APP/PS1 ($p=0.0061$). For HAEE (i/v), when compared with APP/PS1, there was only a trend ($p=0.0771$). On the fourth day, the same dynamics persisted: the difference between APP/PS1 and WT was $p=0.0006$, between Piracetam and APP/PS1 – $p=0.0509$, and between HAEE (i/v) and APP/PS1 – $p=0.0479$.

A direct comparison of the Piracetam and HAEE groups (i/v) at no experimental point revealed statistically significant differences, indicating comparable therapeutic activity of the compounds. At the first stage, both experimental groups were characterized by higher latency, but by the second point their indicators were leveled. This increases the credibility of the identified therapeutic effect.

In general, a comparison of the results between experimental points shows the progression of cognitive impairment in animals of positive control. At the same time, the use of Piracetam and HAEE (i/v) was accompanied by a steady and gradually increasing effect on spatial learning and memory.

The movement speed and distance traveled in animals of all groups remained comparable ($p>0.05$). Therefore, the identified intergroup differences should be attributed to the peculiarities of the cognitive state, and not to changes in motor activity.

In animals of the positive control, the latency period was 2.5 times longer than in the intact control (Fig. 8A; $p=0.0027$), and the number of visits to the target zone declined 7 times (Fig. 8B; $p=0.0012$). These results confirm the presence of persistent and progressive cognitive impairments.

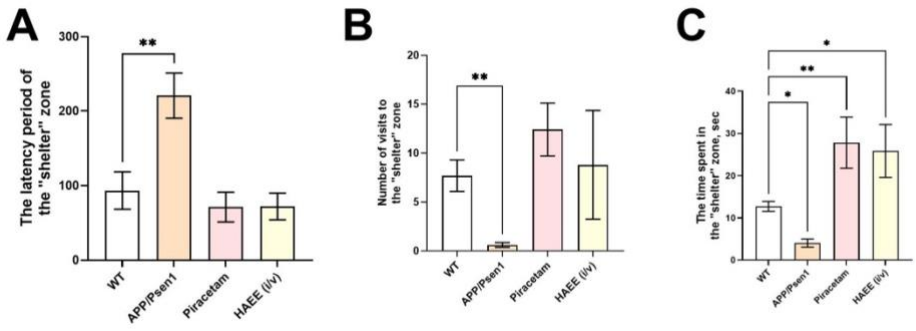


Figure 8. Test day of mice in the "Barnes Maze" test. The latency period of the "shelter" zone (**A**), the number of visits to the "shelter" zone (B), the time spent in the "shelter" zone (**C**). ***Note:*** The values are presented as the average ± standard error of the average. * – p<0.05, ** – p<0.01. (one-way ANOVA post hoc comparisons).

At the second experimental point, animals treated with Piracetam and HAEE (i/v) showed better results in all three main memory indicators compared with such in the positive control. The latency period in both groups was 3 times lower than in the positive control (p=0.0075 and p=0.0079, respectively). The number of visits to the target area increased 12-fold in the Piracetam group and 8-fold in the HAEE group (i/v) (p<0.0001 and p=0.0042, respectively). The time spent in the target area was also 6 times longer in both groups (p<0.0001). At the same time, the indicators did not differ from the level of intact animals, which indicates a complete restoration of spatial memory under the influence of the studied compounds.

The absence of differences in locomotor activity at the second experimental point confirms that the identified improvements were related specifically to memory recovery, and not to changes in motor behavior.

At the first point, there was no statistically significant improvement in the indicators in the groups of studied compounds compared with such in the positive control. By the second point, Piracetam and HAEE (i/v) provided complete restoration of spatial memory: both groups significantly outperformed the positive control in all assessed parameters and reached the level of healthy animals. The similar effectiveness of the compounds at a late stage of the experiment indicates their potential in correcting cognitive impairments in Alzheimer's disease.

Thus, the dynamics between the points indicates the progressive nature of disorders in positive controls and the ability of prolonged intravenous administration of Piracetam and HAEE not only to prevent, but also to fully restore spatial memory.

Gene expression is represented by comparing the cerebral cortex and the hippocampus at different time points in the experiment.

A comparative analysis of gene activity (Fig. 9) between two time control points showed that in the group of mice with intraperitoneal HAEE therapy, there was a decrease in the expression of Vegfa genes (by 47.6%, p=0.9987), Map2 (by 48.1%, p>0.05 ns), mTOR (by 46.3%, ns), and Tnf-a (by 13.7%, ns). However, the expression of Stat3, Il-6, and Casp3 remained at almost the same levels.

In the group of animals with intraperitoneal administration of Piracetam, an almost imperceptible increase in the expression of Vegfa (by 7.8%, ns), mTOR (by 8.2%, ns), and Map2 (by 13.7%, ns) genes can be noted. There was also a tendency to increase the activity of Casp3 (by 19.5%, ns) and Stat3 (by 9.9%, ns). The expression of Il-6 and Tnf-a genes did not change.

In the untreated control group (K-, APPswe/PSEN1dE9/Blg), the expression of pro-inflammatory factors Il-6 and Tnf-a remained virtually unchanged over time, while the expression of genes involved in regulating neuroplasticity (Stat3) and maintaining neuron structure (Map2) decreased by 7.0% (mean diff = -0.027136, ns) and 12.9% (mean diff = -0.006294, ns), at the second point of the experiment. The level of Vegfa expression also decreased slightly by 7.6% along with a decrease in the activity of the mTOR gene (by 24.6%, ns). The activity level of Casp3 increased by 13.2%.

In the control group without pathology (K+, CBA/Black6), the changes were minimal and stable: only a slight increase in the level of mTOR expression (by 9.9, ns) could be noted, while the remaining genes stayed at approximately the same level of activity.

In the comparative analysis between the groups (post-hoc Tukey multiple comparison test) at the first control point, the most pronounced differences were observed in the activity levels of pro-inflammatory genes. In the HAEE study group, the expression levels of Il-6 and Tnf-a were significantly lower than in the negative K-control group (Il-6: mean diff = 0.6337, p<0.001; Tnf-a: mean diff = 0.8173, p<0.01). The same observation is true for the group of mice, who received

the comparison drug Piracetam (Il-6: mean diff = 0.6379, p<0.01; Tnf-a: mean diff = 0.7078, p<0.01) In addition, in the K-group, the expression of these genes significantly exceeded the indicators of the positive K+ control (Il-6: mean diff = 0.6917, p<0.001; Tnf-a: mean diff = 0.8839, p<0.001). The Vegfa level at the first point was highest in the HAEE group and significantly exceeded the values in K- (mean diff = 0.2326, p<0.01). Stat3 expression in the K-group was significantly lower than in mice treated with HAEE tetrapeptide (mean diff = 0.5043, p<0.001), and less pronounced decreased compared to the Piracetam group (mean diff = 0.3274, p<0.05).

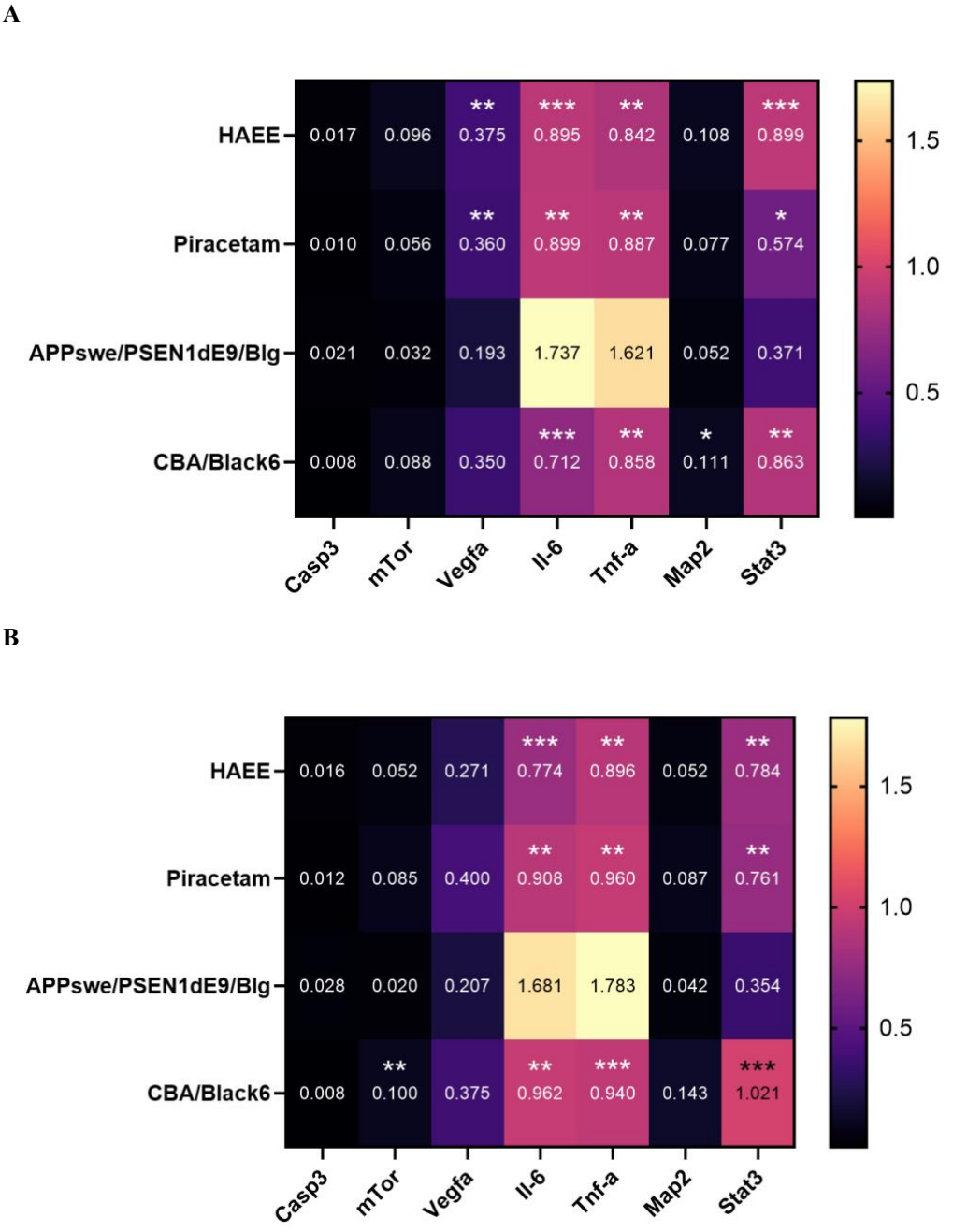


Figure 9. Heat map of the relative levels of gene expression in the cerebral cortex of experimental mice: A) at the first control point of tissue sampling; B) at the second control point. The table shows the average values of relative gene expression normalized by the Gapdh reference gene using the 2-ΔCt formula for seven genes of interest (Casp3, mTOR, Vegfa, Il-6, Tnf-a, Map2, Stat3) in four experimental groups: 1) intact animals of the control positive group (K+) of wild-type CBA/Black6 mice; 2) intact animals with the APPswe/PSEN1dE9/Blg mutation – the control negative group (K-); 3) mutant mice with Piracetam comparison drug therapy; 4) mutant mice with HAEE tetrapeptide therapy. The color scale indicates the level of relative expression, where dark blue (0.5 and below) is the expression of a very low level relative to the Gapdh household gene, shades from pink to bright yellow (1.0 and above) are the expression of a moderately higher or much higher level relative to the Gapdh household gene. ***Note:*** The asterisks indicate the values of the statistical significance of the difference compared to the experimental group: * – p<0.05, ** – p<0.01, *** – p<0.001.

At the second control point (Fig. 10), significant differences in proinflammatory cytokines remained. Group K- continued to show the highest levels of Il-6 and Tnf-a compared to all other groups: significantly higher HAEE (Il-6: mean diff = 0.639, p<0.001; Tnf-a: mean diff = 0.596, p<0.01) and Piracetam (Il-6: mean diff = 0.692, p<0.01; Tnf-a: mean diff = 0.851, p<0.01), as well as above K+ (Il-6: mean diff = 0.742, p <0.01; Tnf-a: mean diff = 0.903, p<0.001). The expression of mTOR at the second point was significantly lower in the treatment groups, as well as in the K- group compared with K+ (p<0.01). There was also a marked increase in Stat3 activity in the HAEE and Piracetam groups relative to the K- group (p<0.01). Vegfa values were aligned between the groups, and no significant intergroup differences in the Casp3 and Map2 genes were detected at the second point (p>0.05).

Thus, the negative K-control consistently demonstrated the most pronounced pro-inflammatory profile at both time points, whereas HAEE and Piracetam therapy significantly weakened the expression of Il-6 and Tnf-a, bringing their levels closer to those of the intact K+ group. HAEE had a more pronounced effect on reducing mTOR and Vegfa, while Piracetam was characterized by a relatively higher level of mTOR.

Correlation analysis revealed positive correlations between the genes Casp3/Il-6 (r=0.89), Casp3/Tnf-a (r=0.92), mTOR/Stat3 (r=0.81), Vegfa/Map2 (r=0.95), Vegfa/Stat3 (r=0.96), Il-6/Tnf-a (r=0.98), and Map2/Stat3 (r=0.96). A negative correlation was found between Casp3/Vegfa (r=-0.75), Vegfa/Il-6 (r=-0.91), Vegfa/Tnf-a (r=-0.87), Il-6/Map2 (r=-0.86), Il-6/Stat3 (r=-0.79), Tnf-a/Map2 (r=-0.82), and Tnf-a/Stat3 (r=-0.75) (all at p<0.05).

In all groups, there was a general trend towards an increase in the expression of Il-6 and Tnf-a against the background of a decrease in Vegfa and Map2. The most pronounced changes (increased proinflammatory cytokines Il-6 and Tnf-a with a decrease in Vegfa and Map2) were observed in the K-negative control group (F=172.6 for column factor in ANOVA, p<0.001). Intraperitoneal administration of HAEE was associated with a moderate decrease in plasticity (decrease in Vegfa and Map2 by 47-48%, ns), but with a more significant interaction of factors (p<0.001) compared with such in Piracetam group. The HAEE and Piracetam therapy groups demonstrated Il-6 and Vegfa levels closer to normal (K+) compared with K-; at the same time, Piracetam was characterized by relatively lower Tnf-a levels and higher mTOR levels (mean diff vs HAEE mTOR =0.01687, ns).

Analysis of the dynamics of changes in gene expression showed that in the group of mice with intraperitoneal HAEE therapy, there was a decrease in the expression of Casp3 genes (by 38.8%, p=0.947), Stat3 (by 45.9%, p<0.05), Map2 (by 27.6%, ns), Vegfa (by 11.2%, ns). At the same time, there are trends towards an increase in the expression level of the mTOR gene (by 10.9%, ns), as well as increased expression of the Il-6 genes (by 14.9, P=0.8556) and Tnf-a (by 15.9%, ns).

In the group of animals with intraperitoneal administration of Piracetam, the changes were less pronounced. It is possible to note an increase in the expression of the Casp3 (by 10.7%, ns), mTOR (by 22.2%, ns), Stat3 (by 10.4%, ns), Map2 (by 12.2%, ns), Vegfa (by 6.9%, ns) genes, while the activity of Il-6 genes and Tnf-a decreased slightly: by 2.3% (mean diff = -0.019615, ns) and 15.4% (mean diff = -0.17678, ns), respectively.

In the control negative group without treatment (K-, APPswe/PSEN1dE9/Blg), the expression of pro-inflammatory factors Tnf-a and Il-6 increased over time by 6.8% (mean diff = 0.098116, ns) and 7.0% (mean diff = 0.09475, ns), respectively, while the expression of genes Those involved in the regulation of neuroplasticity (Stat3) and the maintenance of the structure of neurons (Map2) decreased by 6.9% to the second point of the experiment (mean diff = -0.03151, ns) and increased by 5.9% (mean diff = 0.003307, ns), respectively. The activity level of Casp3 increased by 15.7% (mean diff = 0.004417, ns), while the activity of the Vegfa and mTOR genes remained practically low.

In the control positive group without pathology (K+, CBA/Black6), the changes were minimal and stable: a slight decrease in Casp3 (by 2.5%), Il-6 (by 2.5%), Tnf-a (by 2.8%), along with a relatively significant increase in mTOR (by 21.5%, ns) and almost unchanged levels of activity of the Vegfa, Map2 and Stat3 genes.

In the comparative analysis between the groups (post-hoc Tukey multiple comparison test) at the first control point, the most pronounced differences were observed in pro-inflammatory genes. In the negative control group, K-levels of Il-6 and Tnf-a were significantly higher than in the HAEE group (Il-6: mean diff = -0.5131, p<0.01; Tnf-a: mean diff = -0.6387, P < 0.001) and in the Piracetam group (Il-6: mean diff = -0.3025, p<0.01; Tnf-a: mean diff = -0.3023, p=0.033). In addition, in the K-group, the expression of these genes significantly exceeded the K+ positive control (CBA/Black6) (Il-6: mean diff = 0.4576, p<0.01; Tnf-a: mean diff = 0.5417, p<0.001). The Stat3 level in the K-group was significantly lower than in the K+ group (mean diff = -0.7590, p<0.001), and therapy with HAEE tetrapeptide and Piracetam resulted in an increase in Stat3 activity by 72.0% (p<0.01) and 101.2% (p<0.01), respectively. There were no significant

intergroup differences in the expression levels of the Casp3, mTOR, Vegfa, and Map2 genes for the treatment groups (all comparisons p>0.05).

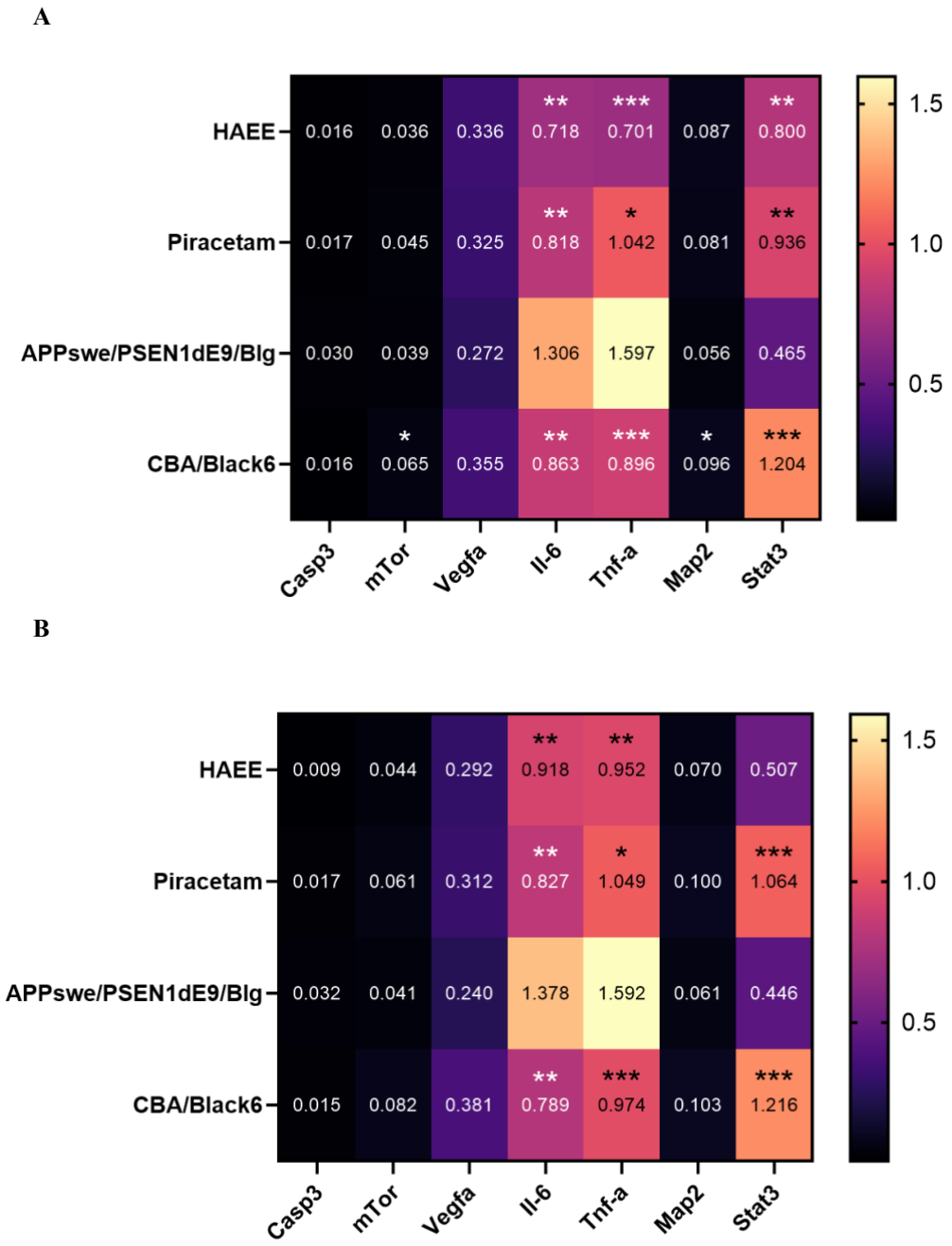


Figure 10. Heat map of the relative levels of gene expression in the hippocampus of the brain of experimental mice: A) at the first control point of tissue sampling; B) at the second control point. The table shows the average values of relative gene expression normalized by the Gapdh reference gene using the 2-ΔCt formula for seven genes of interest (Casp3, mTOR, Vegfa, Il-6, Tnf-a, Map2, Stat3) in four experimental groups: 1) intact animals of the control positive group (K+) of wild-type CBA/Black6 mice; 2) intact animals with the APPswe/PSEN1dE9/Blg mutation – the control negative group (K-); 3) mutant mice with Piracetam comparison drug therapy; 4) mutant mice with HAEE tetrapeptide therapy. The color scale indicates the level of relative expression, where dark blue (0.5 and below) is the expression of a very low level relative to the Gapdh household gene, shades from pink to bright yellow (1.0 and above) are the expression of a moderately higher or much higher level relative to the Gapdh household gene. ***Note:*** The asterisks indicate the values of the statistical significance of the difference compared to the experimental group: * – p<0.05, ** – p<0.01, *** – p<0.001.

At the second control point, significant differences in proinflammatory cytokines remained and partially intensified. Group K- continued to show the highest levels of Il-6 and Tnf-a compared to all other groups: statistically significantly higher than in HAEE group (Il-6: mean diff = -0.5969, p<0.01; Tnf-a: mean diff = -0.6078, p<0.01) and in Piracetam group (Il-6: mean diff = -0.6168, p<0.01; Tnf-a: mean diff = -0.5772, p<0.05), and also higher than in K+ group (Il-6: mean diff = 0.6727, p< 0.001; Tnf-a: mean diff = 0.6652, p<0.01). Stat3 expression at the

second point was significantly lower in the K- group compared with such in Piracetam group (mean diff = -0.6299, p<0.001) and K+ group (mean diff = -0.8325, p<0.001), without statistically significant differences with the HAEE tetrapeptide group. No significant intergroup differences were found for the Casp3, mTOR, Vegfa, and Map2 genes (all p>0.05).

Thus, the negative K-control consistently demonstrated the most pronounced pro-inflammatory profile at both time points, whereas HAEE and Piracetam therapy significantly weakened the expression of Il-6 and Tnf-a, bringing their levels closer to those of the intact K+ group. HAEE had a more pronounced effect on Stat3 reduction, while Piracetam was characterized by relatively lower levels of Tnf-a and higher levels of Vegfa. Correlationa analysis revealev positive correlations between genes Casp3/Il-6 (r=0.93), Casp3/Tnf-a (r=0.91), mTor/Stat3 (r=0.78), Vegfa/Map2 (r=0.97), Vegfa/Stat3 (r=0.97), Il-6/Tnf-a (r=0.97), and Map2/Stat3 (r=0.97). A negative correlation was found between Casp3/Vegfa (r=-0.72), Vegfa/Il-6 (r=-0.89), Vegfa/Tnf-a (r=-0.85), Il-6/Map2 (r=-0.84), Il-6/Stat3 (r=-0.77), Tnf-a/Map2 (r=-0.80), and Tnf-a/Stat3 (r=-0.72) (all at p<0.05 ns).

In all groups, there was a general tendency to increase the expression of Il-6 and Tnf-a against the background of a decrease in Stat3 and Map2 levels. The most pronounced profile of changes – increased expression of proinflammatory cytokines Il-6 and Tnf-a with simultaneous decrease in Stat3 and Map2 – was observed in the negative control group K− (F=12.94 for Tnf-a, according to ANOVA, p<0.001).

Intraperitoneal administration of HAEE was accompanied by a moderate pro-inflammatory shift: Il-6 and Tnf-a levels increased by 14-16%, but the differences did not reach statistical significance (ns). At the same time, the decrease in Stat3 expression was more pronounced compared with the Piracetam group (p<0.001). In both therapeutic groups, Il-6 and Stat3 levels were closer to the normal K+ control compared to K−. Piracetam was also characterized by relatively lower levels of Tnf-a and higher levels of Vegfa. The difference in Vegfa compared to HAEE was 0.05792 and was not statistically significant.

Discussion

The totality of the data obtained confirms the reproducibility of the pathological phenotype in transgenic APPswe/PSEN1dE9/Blg mice. The animals showed cognitive impairments and increased amyloid load in the cortex and hippocampus. This corresponds to the previously published characteristics of this model, in which the accumulation of beta-amyloid is accompanied by a gradual deterioration in cognitive functions. The revealed relationship between an increase in the number of amyloid plaques and a decrease in the results of behavioral tests further confirms the key importance of amyloid pathology in the development of neurodegenerative changes (De Strooper et al. 2016; Long and Holtzman 2019; Welikovitch et al. 2025).

Both compounds studied improved cognitive performance, but the dynamics of their effects differed. Against the background of the use of Piracetam, the improvement developed gradually, which is consistent with its known mechanism, mainly associated with the modulation of neurometabolic processes and synaptic transmission (Malykh and Sadaie 2010; Korokin et al. 2023). HAEE, on the contrary, provided faster recovery of cognitive functions, especially in spatial learning tests. This may indicate its more pronounced effect on the processes of neuroplasticity.

Chronic administration of HAEE to APPswe/PS1dE9/Blg mice was accompanied by a moderate but statistically significant antiamyloid effect. The drug's effect was most stable against small beta-amyloid plaques with an area of less than 100 microns. This result was reproduced in the cortex and hippocampus, both at early and late observation. The decrease in total amyloid load reached statistical significance in the cortex, while it manifested itself primarily as a trend in the hippocampus.

In terms of effect magnitude, HAEE was inferior to Piracetam, but superior to the untreated control. The results suggest that HAEE is able to slow down the formation of new amyloid deposits, but does not lead to regression of already formed plaques. To more accurately assess the role of HAEE in changing the course of amyloidosis, additional studies are needed with an increase in sample size and/or an extension of the follow-up period (Almohmadi et al. 2025; Han et al. 2025).

The preservation of the HAEE effect after discontinuation of therapy deserves special attention. At the delayed experimental point, animals treated with Piracetam tended to partially lose the previously achieved result. At the same time, the HAEE group maintained a reduced amyloid load and stable cognitive performance. Such stability of action may indicate a combination of symptomatic and pathogenetic effects in HAEE. Similar properties have previously been described for peptide compounds capable of modifying the course of

neurodegenerative processes (Bach et al. 2020; Zhang et al. 2021). The molecular results indicate a significant involvement of neuroinflammation in the development of the identified pathological changes. In the group of animals with pathology, the expression of pro-inflammatory markers Il-6 and Tnf-a increased, which corresponds to modern ideas about the role of the inflammatory response in the progression of Alzheimer's disease (De Strooper et al. 2016; Welikovitch et al. 2025). The introduction of HAEE was accompanied by a more pronounced reduction in the levels of these genes to normal compared with Piracetam, which indicates its ability to limit the neuroinflammatory response.

Simultaneously, changes in the expression of Stat3, Map2, mTOR, and Vegfa indicate the involvement of processes related to neuroplasticity, cell survival, and restoration of the functional state of neurons. The stronger effect of HAEE on these molecular parameters is consistent with its multi-target mechanism, suggesting an effect on several pathogenetic links of the disease at once (Bach et al. 2020; Zhang et al. 2021).

The data obtained correspond to the results previously published in the journal Research Results in Pharmacology. These studies have shown that HAEE tetrapeptide reduces amyloid load and improves cognitive function in experimental models of Alzheimer's disease (Patrakhanov 2024). Publications in the same journal also confirm the promise of peptide compounds for the creation of new neuroprotective agents (Polikarpova et al. 2022).

In general, the results of this work indicate a more pronounced and prolonged neuroprotective effect of HAEE compared with Piracetam. The effects of HAEE were manifested simultaneously at the behavioral, morphological and molecular levels, which justifies the expediency of its further study in preclinical and clinical studies. The results obtained support the prospects of peptide compounds as the basis for pathogenetically oriented therapy of Alzheimer's disease.

Conclusion

The present study demonstrated that tetrapeptide HAEE at a dose of 50 mcg per 1 kg with a frequency of 1 administration within 48 hours and a course of 30 days has a pronounced neuroprotective effect in a model of Alzheimer's disease in transgenic mice of the APPswe/PSEN1dE9/Blg line. Its use led to an improvement in cognitive functions, a decrease in amyloid load in the cortex and hippocampus, as well as normalization of gene expression associated with neuroinflammation and neuroplasticity. HAEE has been shown to be superior to Piracetam in terms of the rate of development and stability of the therapeutic effect. Unlike Piracetam, which has a predominantly symptomatic effect, HAEE shows signs of pathogenetically oriented effects, including effects on the processes of amyloidogenesis and neuroinflammation. Of particular importance is the preservation of the HAEE effect after discontinuation of therapy, which indicates its ability to modify the course of the neurodegenerative process. The results obtained are consistent with current ideas about the prospects of peptide compounds as a basis for the development of new approaches to the treatment of Alzheimer's disease.

HAEE tetrapeptide is a promising candidate for further preclinical and clinical studies aimed at developing effective pathogenetic therapies for Alzheimer's disease.

Additional Information

Conflict of interest

The authors declare that they have no conflicts of interest.

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Ethics statement

The animals were kept in the SPF vivarium of Belgorod State National Research University (NRU BelSU, Russia) under artificially regulated daylight conditions (12 hours dark and 12 hours daylight) at temperatures from +22 to +26 °C and had free access to food and water. The work was guided by the ethical principles of the treatment of laboratory animals in accordance with the European Convention for the Protection of Vertebrates Used for Experimental and Other Scientific Purposes (ETS No. 170). All painful manipulations with animals were carried out in accordance with regulatory standards.: Directive 2010/63/EU of the European Parliament and of the Council of the European Union of 22 September 2010 on the protection of animals used for scientific purposes. The study was approved by the Commission for control over the maintenance

and use of laboratory animals of the National Research University "BelSU", expert opinion No. 01-06i/24 dated March 6, 2024.

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The figures were created with BioRender.com.

Data availability

All relevant data generated and analyzed during this study are included in this article.

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