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Research Article

Study of the neuroprotective properties of the heteroreceptor EPOR/CD131 agonist of peptide structure in tau-proteinopathy modeling

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Abstract

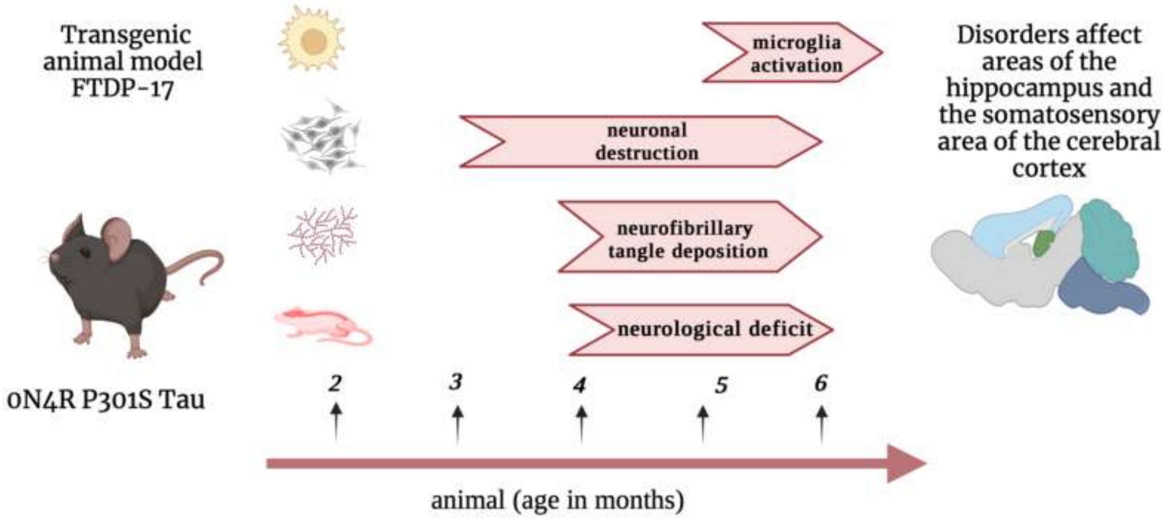
Introduction: Tau proteinopathy is a pathology associated with the activation of post-translational modifications and interactions of pathophysiological cascades of neuroinflammation with hyperphosphorylation of Tau aggregates. Therefore, preference is given to agents that have properties in reducing or slowing down the processes of neuroinflammation and post-translational modifications in the brain.

Material and Methods: The study was conducted on male and female homozygous individuals of a transgenic murine line with overexpression of mutant human Tau gene (P301S) and a background wild mouse line C57Bl/6J. To assess the progression of Tau proteinopathy, behavioral tests were used at two control time points, and the last one measured the level of neuroinflammation markers and tau-proteinopathy.

Results: In the group of P301S mice treated with ARA-290, an improvement in the phenotypic picture of Tau proteinopathy was demonstrated compared with intact animals. In the Barnes circular maze test, mice showed a decrease in the total distance traveled and the latent time spent on the platform, which indicates a rapid entry into the shelter. In the O-shaped maze test, the group maintained a fairly high level of spontaneous exploratory behavior. In the vertical rod test, the animals recorded the best time indicators that they needed to turn and maintain balance compared to the intact group. A statistically significant decrease in the level of GSK-3β and an increase in CDK5 and PP2A were revealed, which indicates a dephosphorylating effect on Tau protein, as well as markers of neuroinflammation. NF-KB and TNF-α were significantly reduced by 57% and 32%, respectively, compared to the intact group.

Conclusion: In the model of transgenic P301S murine line with overexpression of the mutant human Tau gene, the peptide agonist of the EPOR/CD 131 heteroreceptor demonstrated neuroprotective properties, which were confirmed by indicators of behavioral tests and markers of neuroinflammation and tau-proteinopathy.

Graphical abstract



Keywords

tau-proteinopathy, neuroinflammation, post-translational modifications, frontotemporal dementia, parkinsonism, ARA-290

Introduction

To date, tau-proteinopathy is a pathology that occurs mainly in the elderly, but recently the incidence among the young population has increased. This affects the quality of life and leads to early disability, which subsequently negatively affects the socio-economic sphere of countries (Dinda et al. 2019).

Tau-proteinopathy is a pathology characterized by abnormal hyperphosphorylation of the Tau protein due to the activation of post-translational modifications, but it is also worth noting the important role of the interaction of pathophysiological cascades of neuroinflammation with hyperphosphorylation of Tau aggregates (Lecordier et al. 2021; Limorenko et al. 2021). Glial cells are able to exert beneficial and anti-inflammatory effects under normal and pathological conditions (phagocytosis, steroid release, free radical depletion, and cell repair) (Larson et.al 2022; Pinto et al. 2023), while cytokine release and free radical generation leads to the death of neuronal tissue and causes disruption of synaptic transmission in neurons (Vogels et al. 2019; Wu and Zhang 2023). The active form of microglia and astrocytes increases the content of cytokines, interleukins and chemokines, which enhance the progression of tau-proteinopathology and contribute to the development of neuroinflammation (Mahady et al. 2023).

In preclinical studies, in order to model tau-proteinopathy (frontotemporal dementia with parkinsonism linked to chromosome 17, FTDP-17), researchers chose transgenic P301S mice with overexpression of the mutant human Tau gene. In this P301S model, homozygous individuals develop synaptic pathology and microgliosis in hippocampal structures at 12 weeks of age, followed by loss of neurons and formation of neurofibrillary tangles. As a result, at the age of 24 weeks, they develop synaptic dysfunction and cognitive impairment. It is worth noting that there is a chronic denervation of motor neurons, as a result of which motor symptoms gradually increase: weakness in the muscles of the limbs, tremor, hunched posture, paralysis of the hind limbs, which indicates a neurological deficit (Ivanov et al. 2020).

In pharmacotherapy of tau-proteinopathy (FTDP-17), preference is given to agents that have properties to reduce or slow down the processes of neuroinflammation and post-translational modifications in the brain. Non-hematopoietic derivatives of erythropoietin (ARA-290 or cibenitide), a peptide that mimics alpha helix of erythropoietin B, is involved in the activation of the innate repair receptor (O'Leary et al. 2019; Belyaeva et al. 2020; Meyer et al. 2020; Antsiferov et al. 2021; Xu et al. 2022). ARA-290 has cytoprotective and immunomodulatory effects, without direct hematopoietic effects. Binding of EPO to the heterodimeric receptor promotes the activation of phosphorylation of janus kinase 2 and three main signaling pathways: STAT5 (Signal transducer and activator of transcription 5), PI3K/Akt (intracellular signaling pathway, the central components of which are phosphoinositide-3 kinase and AKT kinase), mitogen-activated protein kinases that participate in simultaneous regeneration and at the same time – in the inhibition of apoptosis and inflammation cascades (Dinda et al. 2019; Al-Onaizi et al. 2022; El-Ganainy et al. 2022).

The aim of the research is to study the neuroprotective properties of the heteroreceptor EPOR/CD131 agonist of peptide structure in a model of a transgenic P301S murine line with overexpression of the mutant human Tau gene.

Materials and Methods

Animals

In the experiment, the manipulations on animals were performed in accordance with the international standards (European Convention for the Protection of Vertebrate Animals Used for Experimental and Other Scientific Purposes) and requirements of good laboratory practice (GLP). Experimental studies were approved by the BSU Bioethical Commission (Minutes №15/10 of 29.10.2021). The experiment was performed on 60 males and females of homozygous transgenic mice with overexpression of the mutant human Tau gene (P301S) and on 20 males and females of the background wild mouse line C57Bl/6J at the age of 8 weeks. The time for the start of experimental work is due to the absence of the debut of the phenotypic pattern and characteristic pathomorphological changes in the structures of the brain and spinal cord for tau-proteinopathology. To identify the dependence of the course of tau-proteinopathology on gender, individuals of both sexes were included in the study. The animals were kept on the basis of an experimental biological clinic (vivarium with SPF conditions) of Belgorod State National Research University.

Experiment plan

For the experiment, the animals were divided into four groups: 1) intact group of P301S mice (aqua pro injection in the amount of 100 μL subcutaneously once every two days for 4 weeks); 2) group with treatment with piracetam (at a dose of 200 mg/mL in the amount of 175 μL solution subcutaneously once every two days for 4 weeks); 3) group with ARA-290 treatment (at a dose of 20 μg/kg in a volume of 100 μL of solution subcutaneously once every 3 days for 4 weeks) and 4) group of negative control C57Bl/6J mice (aqua pro injection in a volume of 100 μL subcutaneously once every two days for 4 weeks)

Behavioral tests were performed at two time points: first – at 12 weeks of age and second – at 20 weeks of age:

1) The Barnes Circular Test is designed to study spatial memory in mice. It is a rounded surface with a diameter of 1.2 meters with 40 holes around the perimeter and raised one meter above the floor. Then, it was expected that the mice would learn to determine the location of a shelter located under a dark evacuation box. Bright light generated by four 100-watt lamps was used for negativing stimulation during the task. Four different colored paper figures were placed around the room as visual cues. During the memorization phase, mice were allowed to explore the maze for 3 minutes per challenge (a total of 4 trials per day) for 4 consecutive days. On day 5, a spatial memory test was performed using a test in which mice were given 1 minute to find shelter. To assess spatial memory, we measured: speed, time spent searching for shelter, and distance traveled. The behavior of mice was recorded and analyzed using Noldus Ethovision XT software (Noldus Information Technology, USA).

2) The O-shaped maze test, which is used to assess the level of anxiety and determine the activity index. It is a white ring, 6 cm wide and 45 cm in outer diameter, with alternating sections of open and closed walls. At the beginning of the study, mice were placed in a closed section for 5 minutes, during the test, the time spent in the open section, the number of exits and peeks in the open section, the time of the first exit to the open section, and the total distance traveled in the open section were recorded. The data was analyzed by the Noldus Ethovision XT video tracking program (Noldus Information Technology, USA).

3) The vertical rod test is used to analyze coordination movements in mice. The mouse is placed head-up on top of a vertical rod that is 50 cm high and 1 cm in diameter. During the test, the time of turning the head down and the time of descent from the rod are recorded; each animal has 3 attempts.

Enzyme-linked immunosorbent assay (ELISA) of neuroinflammation and tau-proteinopathy markers

Physiological phosphorylation of Tau depends mainly on the balance of protein kinase and phosphatase activity. Accordingly, an imbalance between these enzymes is present in tau-proteinopathy, represented by lower phosphatase activity and increased kinase activity. To assess protein levels in the cerebral cortex and hippocampus in accordance with the instructions of the ELISA kits, glycogen synthase kinase 3β (Cloud-Clone Corp., USA), protein phosphatase A2 (R&D Systems, USA), cyclin-dependent kinase 5 (Cloud-Clone Corp., USA), nuclear factor Kappa B (Cloud-Clone Corp., USA), tumor necrosis factor alpha (R&D systems, USA) were measured using a tablet enzyme immunoassay reader (ELISA) (Stat Fax 2200, Awareness Technologies, USA). Three-fold samples of the supernatant of homogenized tissues were taken from 3 mice from each group. The concentration was calculated according to the mean value and standard deviation according to ANOVA with Bonferroni correction, where $p<0.05$. All experimental procedures were performed in accordance with the manufacturer's instructions.

Statistical data processing

All the results obtained were statistically processed using GraphPad Prism 8.0 software (California, USA); a two-way ANOVA was used, followed by a post hoc Tukey test. The normality of the distribution was checked using the Kolmogorov-Smirnov test (n=10 mice) $p<0.01$; $p<0.001$; $p<0.0001$; ns.

Results and Discussion

Behavioral tests

Based on the data obtained in the round-robin Barnes test for the first and second control time points, mice of the third group treated with ARA-290 statistically reliably confirmed a high level of spatial cognitive functioning, based on the following indicators: a decrease in the total distance traveled and latent time spent on the platform, as well as an increase in speed at this distance due to the rapid entry into the zone where there is a shelter, compared to the intact group. In addition, the second group treated with piracetam showed moderate indicators, what can be evidence of spatial and cognitive dysfunction due to the accumulation of Tau protein aggregates in the brain, and the obtained indicators were statistically higher than in the group of intact animals (Fig. 1).

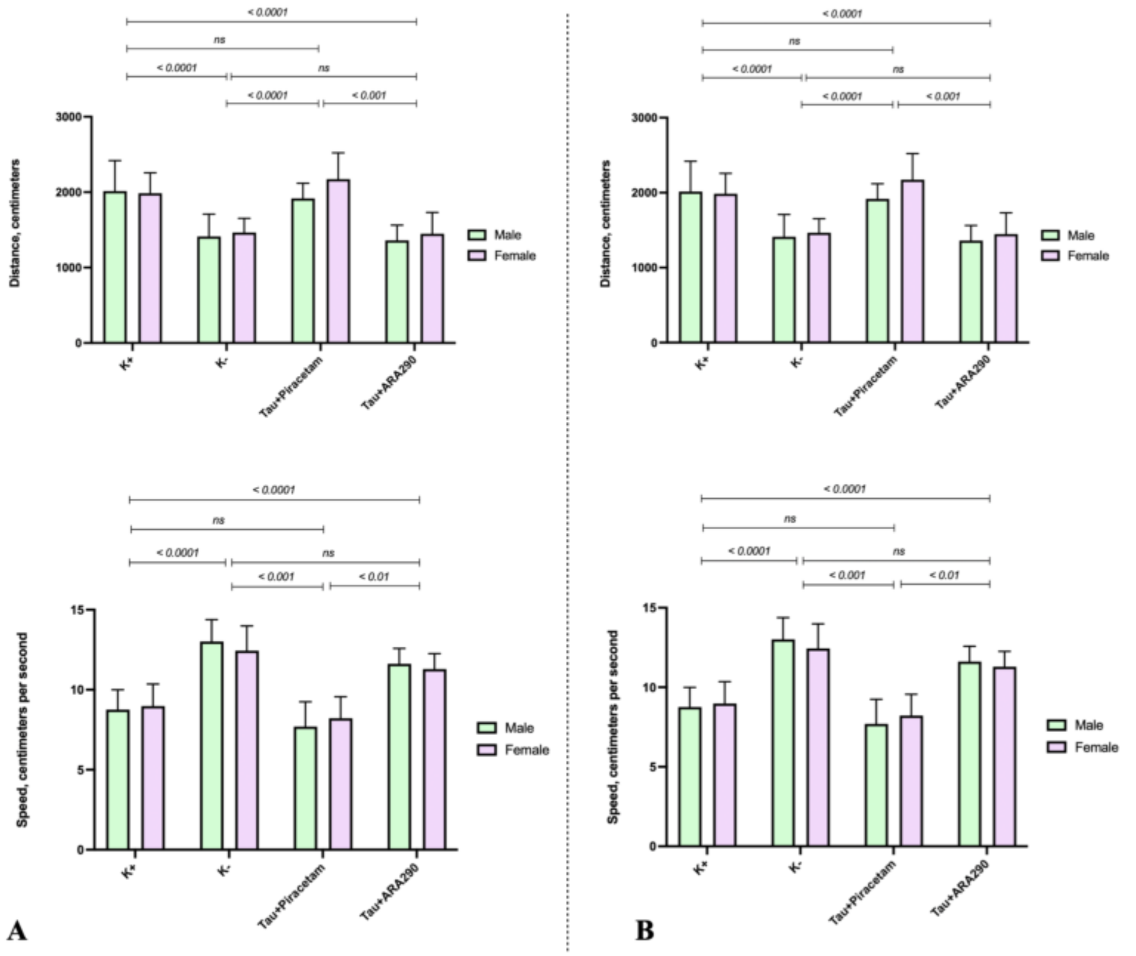


Figure 1. The distance and speed indicators in the Barnes circular test on the test day for the first time control point (**A**) and the second time control point (**B**).

Due to the use of the O-shaped maze test at two time control points, it can be concluded that the third group of P301S mice treated with ARA-290 retained a fairly high level of spontaneous research behavior – this was confirmed by the time spent in the open section and the time out of this section. The results are comparable to the fourth group of negative controls, which indicates low anxiety and preserved cognitive abilities. It is worth noting that the second group of P301S mice treated with piracetam also demonstrated a high level of spontaneous exploratory behavior, but the statistics are not as close to the negative control group as in the fourth group using ARA-290 (Fig. 2).

Based on the data obtained in the Vertical Rod test for the first and second control time points, the following conclusions can be drawn: in the first intact group of P301S mice, positive dynamics of the time for turning along the rod was observed throughout the test, but short time intervals were recorded for maintaining balance, and therefore a drop occurred after a short time, compared to the negative control and groups that received treatment this was more pronounced at the second control point. In the third group of P301S mice treated with ARA-290, normal indicators of the time it took them to turn and maintain balance were recorded, and the delay time on the rod was statistically longer compared to the intact group and the group, receiving piracetam treatment (Fig. 3).

Enzyme-linked immunosorbent assay of neuroinflammation and tau-proteinopathy markers

In P301S mice treated with ARA-290 at a dose of 20 mg/kg, the following results were obtained: a statistically significante decrease in the level of GSK-3b ise4.42±0.56 ng/mg compared to the intact group of P301S which received an equivalent amount of water solution for injection. It is also worth noting that at that time, the group of mice treated with piracetam was 70% lower than GSK-3b. Surprisingly, in the group of mice treated with ARA-290, a significant increase in CDK5 levels was recorded, which was 9.80±0.70 ng/mg compared to the groups of intact animals and negative controls. The piracetam-treated group of mice showed that CDK5 levels were 50% lower in the negative control group. In the intact group, the level of PP2A protein was significantly reduced by 35% compared to the negative control group and was 0.54±0.19 nmol/mg. In the group of mice treated with ARA-290 resulted in a significant increase in PP2A by 181% compared to the intact group, and the indicator was 0.98±0.16 nmol/mg, which indicates a dephosphorylating effect on the Tau protein; in the second group of mice treated with piracetam, it was 0.54±0.11 nmol/mg, where $p<0.05$ in comparison with the negative control group.

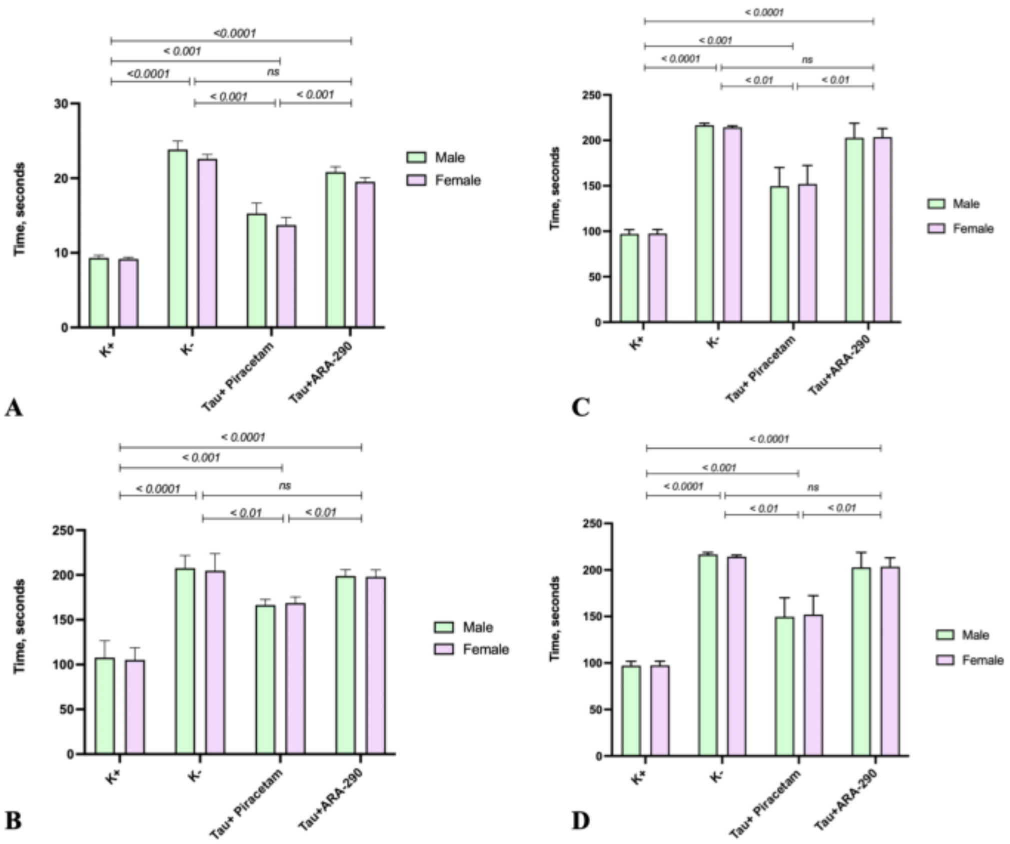


Figure 2. O-shaped maze test results: latent exit time to the open section for the first time control point (**A**) and time spent in the open section for the first time control point (**B**) latent exit time to the open section for the second time control point (**C**) and time spent in the open section for the second time control point (**D**).

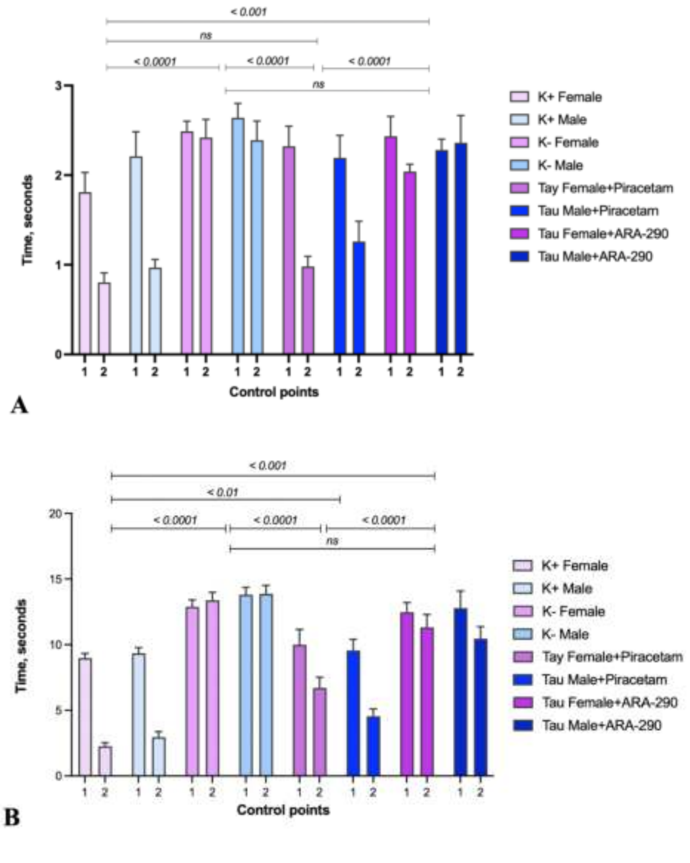


Figure 3. Results of testing the "Vertical rod" at two control time points, where the time of rotation on the rod (**A**) and the total time spent on the rod (**B**).

To assess the effect of ARA-290 on neuroinflammation caused by tau-proteinopathy, NF-kB and TNF-α levels were evaluated. Hyperphosphorylation of Tau leads to activation of the transcription factor NF-kB, which subsequently leads to increased expression of proinflammatory cytokines in the form of TNF-α. The group of intact animals showed an increased level of neuroinflammatory markers: NF-kB kb and TNF-α, which were 7.84±1.34 ng/mg and 386.00±26.32 pg/mg, respectively, which is 286% and 501% more than in the negative control group with $p<0.05$. The group of P301S mice with treatment ARA-290 (20 mcg /kg) showed a significant reduction in NF-KB and TNF-α values by 57% and 32%, respectively, compared to the intact group and amounted to 4.46±0.44 ng/mg and 124.00±21.62 pg/mg, respectively, while in the piracetam-treated group, the indicators were 6.86±0.77 ng/mg and 231.00±36.81 pg/mg, with $p<0.05$, compared to the negative control group. From the above data, it can be concluded that piracetam exerted an anti-inflammatory effect and changed the balance of tau-proteinopathy indicators, such as GSK-3b, CDK-5, and PP2A, but was statistically significantly inferior to the indicators from the group of mice treated with ARA-290.

Conclusion

The above data of the study results show that the peptide agonist of the EPOR/CD131 heteroreceptor in the model of the transgenic P301S mouse line with overexpression of the mutant human Tau gene demonstrated neuroprotective properties. This was statistically confirmed by the indicators of behavioral responses in the tests: Barnes circular maze, O-shaped maze and vertical rod, which record spatial-cognitive, motor and adaptive activities in animals.

The most pronounced anti-inflammatory effect was observed when measuring the levels of NF-kB and TNF-α, but also the marker of tau-proteinopathy was reduced – GSK-3β, which is responsible for physiological phosphorylation of Tau protein, while CDK5 and PP2A were increased, which indicates the dephosphorylation of pathological Tau aggregates.

Conflict of interests

The authors declare no conflict of interests.

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Data availability

All of the data that support the findings of this study are available in the main text.

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