



Original Article



Acupuncture Protects Brain Regions in an Alzheimer's Disease Mouse Model by Inhibiting Apoptosis and Reducing Tau Protein

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Abstract

Background and objectives: Acupuncture treatment on the DU channel has shown therapeutic effects for Alzheimer's disease (AD), but the underlying mechanisms are not yet clear. The purpose of this study was to comprehensively observe the protective effects of acupuncture on different brain regions in AD model mice, providing laboratory evidence for clinical acupuncture intervention in AD.

Methods: Eleven senescence-resistant strain 1 male mice were used as the normal control group. The senescence-accelerated prone strain 8 (SAMP8) male mice were used as AD model mice. Thirty-three SAMP8 mice were randomly divided into three groups: AD model group (group M), drug treatment group, and acupuncture treatment group (group A). The effect of acupuncture on learning and memory capabilities of SAMP8 mice was assessed by the Morris water maze test. Nissl staining was employed to provide a general view of the brain structure in AD model mice. Additionally, Western blot analysis was used to quantify Caspase-3 and tau protein levels.

Results: In the spatial navigation test, the ratio of time mice spent in the goal quadrant in group M remained low, even lower than 25%. The ratio of time spent in the goal quadrant by mice in the acupuncture group on day 4 was higher than that on day 1 ($P < 0.01$). There was a trend indicating that the time ratio of mice in the acupuncture group during the probe trial was higher than in group M, though there was no statistically significant difference. Most traces of mice in group A were in the goal platform quadrant and across the platform in different, yet effective, ways. Compared to group M, most of the cells in the frontal cortex, hippocampus, and temporal cortex of mice in group A were round with clear stratification, regular arrangement, and increased Nissl bodies. The content of Caspase-3 in the frontal cortex and hippocampus of mice in the acupuncture group was lower than in group M ($P < 0.01$, $P < 0.05$). The content of tau in the hippocampus and temporal cortex of mice in group A was lower than in group M ($P < 0.05$; $P < 0.01$).

Keywords: Acupuncture; DU channel; Alzheimer's disease; Neurons; Apoptosis; Tau protein.

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Conclusions: Acupuncture at the DU channel can improve learning and memory abilities to a certain degree by reducing apoptosis in the frontal cortex and hippocampus and decreasing tau deposition in the hippocampus and temporal cortex of AD model mice.

Introduction

Alzheimer's disease (AD) ranks among the most common neuro-

degenerative conditions, impacting a vast number of individuals globally. The incidence of new cases tends to rise as individuals grow older.^{1,2} Hence, there is a pressing demand for the development of novel and more potent treatment to treat AD. As a natural therapeutic approach with multiple benefits, cost-effectiveness, and minimal side effects, acupuncture has demonstrated its efficacy in enhancing the cognitive abilities of individuals with AD.^{3,4} The DU channel holds a significant position and role in the treatment of brain diseases in traditional Chinese medicine. By needling the acupoints on the DU channel, effective holistic regulation and treatment of brain diseases can be achieved. The fundamental etiology of AD is rooted in the depletion of vital essence and the weakening of cerebral marrow, with the superficial manifestation of excessive symptoms attributed to the congestion of phlegm in the brain's collateral pathways. There is an inseparable connection between the DU channel and the brain, both in terms of anatomical distribution and functional interaction. Acupuncture applied to the DU channel can stimulate the circulation of qi and blood to expel pathogenic factors, as well as invigorate the body's healthy qi and blood to nourish the brain.^{5,6} Our previous studies have demonstrated that acupuncture at the DU channel on AD mice has a positive effect on improving cognitive function and delaying the AD process.⁶⁻⁸

As memory impairment is among the first symptom of AD, brain regions associated with memory impairments should be prioritized in AD research. The key neuropathological features of AD include amyloid-beta plaque deposition outside cells, the accumulation of neurofibrillary tangles within cells, and neuronal degeneration. Given the robust association between tau protein pathology and cognitive decline, tau is increasingly recognized as a principal factor in neurodegeneration that leads to the death of nerve cells.^{9,10} Removing tau protein has been shown to safeguard brain functionality against apoptotic processes.¹¹ This study explored the mechanism of acupuncture at the DU channel for treating AD by observing its comprehensive effect on tau deposition and apoptosis in the frontal cortex, hippocampus, and temporal cortex of AD mice, aiming to provide laboratory evidence for the acupuncture treatment of AD.

Materials and methods

Experimental animals

The experimental mice, including the senescence-accelerated prone strain 8 (SAMP8) and the senescence-resistant strain 1, with an average weight of approximately 30.0 ± 2.0 grams, were all purchased from the Experimental Animal Center (Animal Lot: SCXK(Jing)2014-0003). These mice were housed at the Experimental Animal Center of BUCM, where the temperature was strictly controlled at $24 \pm 2^\circ\text{C}$ and a 12-h light/dark cycle, which was in accordance with the natural habits of mice, was maintained. Each cage housed one mouse to prevent adult male mice from biting each other. They had free access to sterile drinking water and a standard pellet diet. All mice were acclimated to the environment for five days before the experiments began. Uniform dietary and housing conditions were maintained to avoid discrepancies in results that could arise from variations in breeding conditions. All procedures were conducted in compliance with the "Principles of Laboratory Animal Care" established by the National Institutes of Health and the legislation of the People's Republic of China regarding the use and care of laboratory animals.

Animal cohort and manipulation

Eleven senescence-resistant strain 1 mice were used as the control group (group C). Thirty-three SAMP8 mice were evenly segregated into three experimental groups, with 11 mice per group: the AD model group (group M), the drug treatment group (group D), and the acupuncture treatment group (group A). For group D, the mice received Donepezil (Eisai China Inc., Batch No. H20050978) through oral gavage at a dosage rate of $0.65 \mu\text{g}$ per gram of body weight. Mice in group A were immobilized in a homemade mouse bag during acupuncture treatment. At the Shui Gou (GV26) acupoint, a swift and accurate puncture was performed on the mouse. At the Baihui (GV20) and Yintang (GV29) acupoints, the acupuncture needle was inserted into the subcutaneous tissue of the mouse using an oblique insertion technique. The locations of these acupoints were determined according to the "Laboratory Animal Acupuncture Atlas". The needle insertion depth was 4–5 mm. The needles were secured with tape and linked to the Hans electroacupuncture apparatus. They were stimulated using a sparse waveform set at a frequency of 2 Hz, a voltage of 2 V, and a current of 0.1 mA. Acupuncture treatment was administered for 15 min daily for 15 consecutive days, while group C or group M received no treatment. The mice in groups C, M, and D were subjected to the same 15-m restriction as group A. The experimental methods used in this study are consistent with the methods outlined in Jiang's paper.⁴ During the intervention period, one mouse in group M and one in group A died, and a total of 42 mice completed the study.

Learning and memory behavioral assessment

The water maze apparatus consisted of a circular pool, a video camera, and a platform. The pool had a height of 50 cm and a diameter of 120 cm. The water depth in the pool was 30 cm, and the platform height was the same as the water depth. The video camera was mounted on the roof of the pool, linked to a recording device that enabled the automatic collection of data. The pool's plane was divided into four parts by two perpendicular and horizontal lines passing through the center of the circle, analogous to four quadrants. In the third quadrant of the pool, the movable circular platform was placed. Four different-shaped markers were placed on the inner walls of the pool above the water level, one in each of the four quadrants, to help the mice remember the position of the platform by using these markers as cues. After each experiment, the mice were helped to dry off and were kept warm.

Spatial navigation test

After 15 days of intervention, mice from each group underwent the spatial navigation test. The mice were placed into the pool with their heads facing the pool wall, starting from the midpoint of the pool groove in the third and fourth quadrants, respectively. Each mouse was given 60 s to find the platform. Each mouse underwent two tests per day for a total of five consecutive days.

Spatial exploration test

The day after the spatial navigation test ended, a spatial exploration experiment was conducted on each group of mice. The platform in the pool was removed, making it impossible for the mice to locate the actual physical platform. The mice were placed into the pool from quadrant I (opposite to the original platform's position). The number of times the mice attempted to swim towards the position of the platform in the third quadrant from the spatial navigation test, as well as the swimming distance, were used as indicators to evaluate the learning and memory capabilities of the mice.

Nissl staining

After the Morris water maze, three mice per group in Groups M and A, and four mice per group in Groups C and D were randomly chosen and anesthetized. The brains of the mice were extracted for Nissl staining. After fixation, the brains underwent dehydration treatment and were soaked in xylene until completely transparent. The transparent brains were then embedded in paraffin wax. The brains were sectioned into 10-micrometer-thick slices. The brain slices underwent 1% toluidine blue solution staining for the background for 2 m, followed by 1% Tar Purple solution staining for the Nissl bodies for 2 m. The slices were then dehydrated, made transparent, and sealed. Stained brain sections were observed under a microscope (Olympus Corporation, magnification, $\times 40$).

Western blot analysis

The remaining seven mice from each group were anesthetized, and fresh tissues from the frontal lobe, hippocampus, and temporal lobe were collected for subsequent detection. Sodium dodecyl sulfate-polyacrylamide gel electrophoresis was performed with separating and stacking gels. After the fresh tissues of experimental animals were ground and centrifuged, a cell lysis solution was added. The protein concentration was measured by the Bradford method. The samples were adjusted to equal concentrations before loading, the voltage was adjusted, and electrophoresis was performed. Once the electrophoresis was completed, the gel was removed, and the electrophoretic transfer cell was assembled. The electrophoretic transfer cell was placed in cold water, and the transfer was conducted at a constant current of 100 mA overnight. After the polyvinylidene fluoride membrane was removed and cleaned, it was soaked in blocking solution for 1 h. The polyvinylidene fluoride membrane was sealed with blocking solution containing the primary antibody (USA, Proteintech, Caspase-3, mouse, 1:1,000; USA, ABCAM, tau, chicken, 1:500; GAPDH Rabbit Polyclonal antibody Cat. No. 10494-1-AP, 1:2,000) and allowed to stand at 4°C overnight. After the incubation with the primary antibody was completed, the secondary antibody (1:2,000; 1:2,000; 1:2,000) was added and incubated for 1 h. The HRP-ECL luminescent solution was added, and the X-ray film was exposed in a darkroom, developed, and fixed. The marker was standardized, and analysis and scanning were performed to compare the relative expression levels (Caspase-3/GAPDH and tau/GAPDH grayscale values) of each group.

Statistical analysis

Data analysis was conducted using the SPSS software package, version 20.0, with results presented as the mean \pm standard deviation. For the results of the time ratio of mice spent in the spatial exploration test, tau, and Caspase protein content, if the experimental data from groups C, M, D, and A conformed to the normal distribution, a test for homogeneity of variances was performed. If the variances were homogeneous, the one-way analysis of variance (ANOVA) was used for comparison. If the variances were not homogeneous, robust analysis with equal means (Welch's test) was used. For comparisons between the two groups, the least significant difference test was employed. If the data did not conform to the normal distribution, non-parametric tests were used, and for pairwise comparisons between groups, the intergroup paired comparison method was applied. For the results of the ratio of time mice spent in the goal quadrant and swimming speed in the spatial navigation test over five consecutive days, a general linear model with repeated measures and a multivariate process was applied for repeated measures ANOVA and multivariate ANOVA. First, Mauchly's test of sphericity was used to determine whether there

was a correlation among repeated data. If there was a correlation ($P \leq 0.05$), multivariate ANOVA was performed. Between-subject variation was calculated to analyze whether the treatment factor had an effect. Within-subject variation was calculated to analyze whether the time factor had an effect and whether there was an interaction effect between time and treatment factors. Pairwise comparisons were made for each group at each time point using the paired t-test method for multiple comparisons of repeated measures data (Bonferroni method). Pairwise comparisons were made between each group at each time point using the method of multivariate ANOVA. Results were considered statistically significant if $P < 0.05$ for differences between groups.

Results

Influence of acupuncture on learning and memory capabilities

Figure 1a shows that the ratio of time the mice spent in the goal quadrant changed over time ($P = 0.026$), and the ratio of time spent in the goal quadrant by group C increased gradually. In group C, the ratio of time spent in the platform quadrant on day 3, day 4, and day 5 was higher than on day 1 ($P < 0.01$; $P < 0.01$; $P < 0.01$). However, the ratio of time spent in the goal quadrant by mice in group M remained consistently low, even below 25%. In group D, the ratio of time spent in the platform quadrant increased from day 1 to day 5 ($P < 0.01$). For group A, the ratio of time spent in the goal quadrant on day 4 was greater than on day 1 ($P < 0.01$).

Figure 1b shows that on days 1 and 2, there were no notable differences in the ratio of time spent between groups C, M, D, and A. On days 3, 4, and 5, group M spent a smaller proportion of time in the goal quadrant compared to group C ($P < 0.01$; $P < 0.05$; $P < 0.01$). The time ratio of group D was higher than that of group M ($P < 0.01$). Although there were no significant differences, the time ratio in group A showed a trend of being higher than in group M on days 4 and 5 ($P > 0.05$, $P > 0.05$). The time ratio in group A was higher than that of group D on days 2 and 4 ($P > 0.05$, $P > 0.05$), but lower on days 3 and 5 ($P > 0.05$).

Figure 1c and d illustrate variations in swimming velocity during the spatial navigation trial. Figure 1c shows that the swimming speed of each group did not change over time ($P > 0.05$). Figure 1d indicates that groups M, D, and A exhibited lower swimming speeds compared to group C ($P < 0.01$; $P < 0.01$; $P < 0.01$) on each day. However, there were no significant differences among groups M, D, and A over the five days.

Figure 1e shows that the time ratio in group M was lower than 0.25, while the ratios in groups C, D, and A were higher than 0.25. There was a trend that all were higher than group M, but no statistical difference was found. Figure 1f shows the strategy used by mice in different groups to search for the platform in the spatial exploration test. The more times the mice swam, the redder the color. Mice in group C swam more frequently in the goal platform quadrant compared to the other three quadrants, with swimming traces showing a circular pattern around the goal platform. Mice in group M mostly swam near the point where they were released into the water, not in the goal platform area. Mice in groups D and A swam across the platform in different but effective ways, with most traces in the goal platform quadrant.

The overall impact of acupuncture on neurons across various brain regions

Figure 2 shows an overview of the frontal cortex, hippocampus, and temporal cortex of mice stained with Nissl staining. Yellow

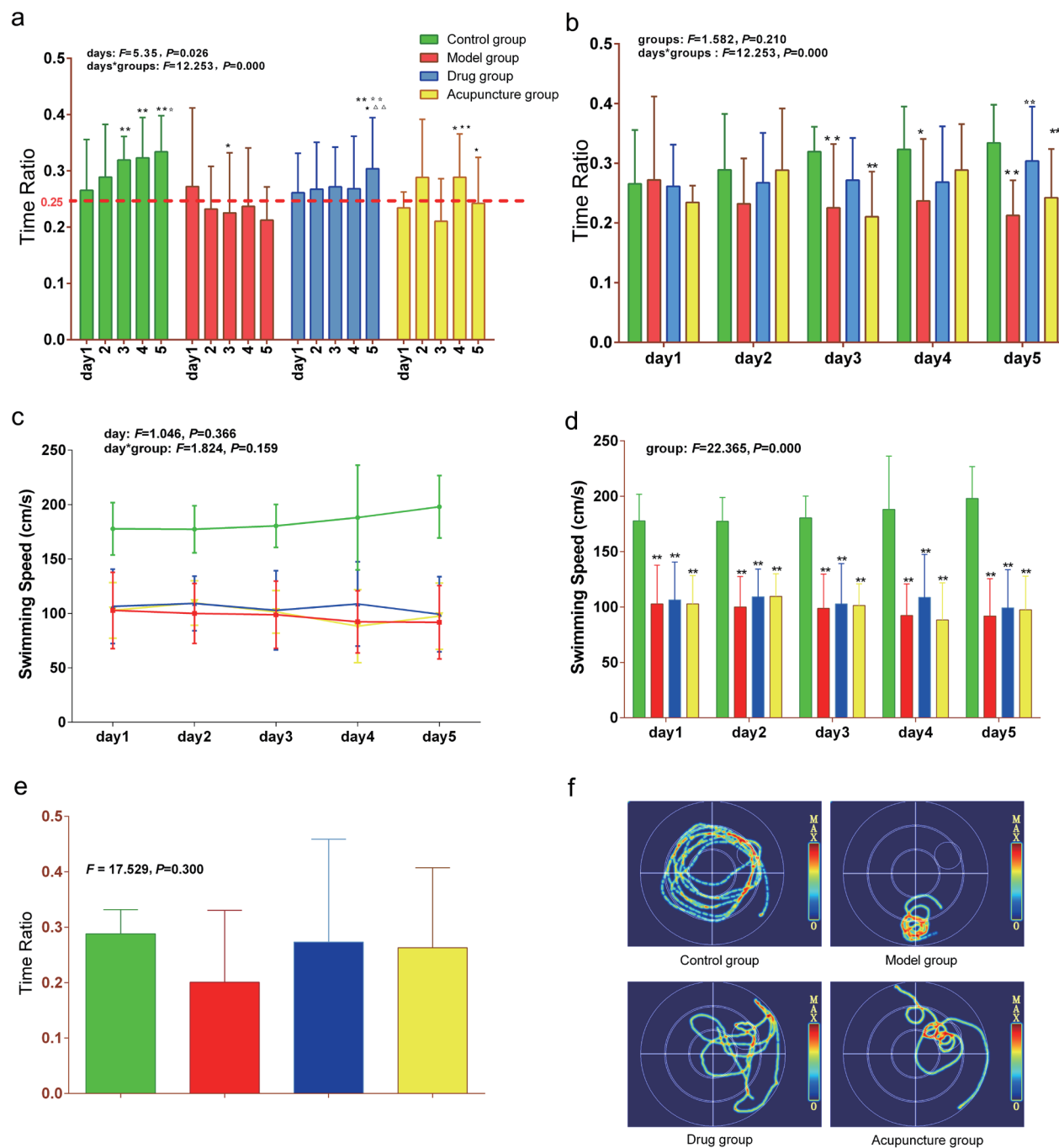


Fig. 1. The effect of acupuncture on the learning and memory abilities of senescence-accelerated prone strain 8 (SAMP8) mice tested by the Morris Water Maze. (a, b) The time ratio that mice spent in the goal quadrant of the total 60 s. (c, d) The swimming speed of mice during the entire test (cm/s). (a, c) In the same group, compared to day 1, * $P < 0.05$, ** $P < 0.01$; compared to day 2, ☆ $P < 0.05$; ☆☆ $P < 0.01$; compared to day 3, ★ $P < 0.05$; ★★ $P < 0.01$; compared to day 4, △ $P < 0.05$; △△ $P < 0.01$. (b, d) At the same time point, compared to the control group, * $P < 0.05$, ** $P < 0.01$; compared to the model group, ☆ $P < 0.05$; ☆☆ $P < 0.01$; compared to the drug group, ★ $P < 0.05$; ★★ $P < 0.01$. (e) The time ratio of mice spent in the spatial exploration test. (f) The strategy that mice in different groups used to find the platform in the spatial exploration test. Control group, $n = 11$; Model group, $n = 10$; Drug group, $n = 11$; Acupuncture group, $n = 10$.

arrows point to examples of healthy cells rich in Nissl bodies, while red arrows point to examples of damaged cells with unclear stratification. Observations revealed that Nissl bodies were present in neurons across all groups, characterized by deep blue

staining of the cytoplasm with granular Nissl bodies. In group C, cell morphology in the frontal and temporal cortex was plump with clear nucleoli, round or oval in shape. In group M, many cells were stained deep blue with unclear stratification and

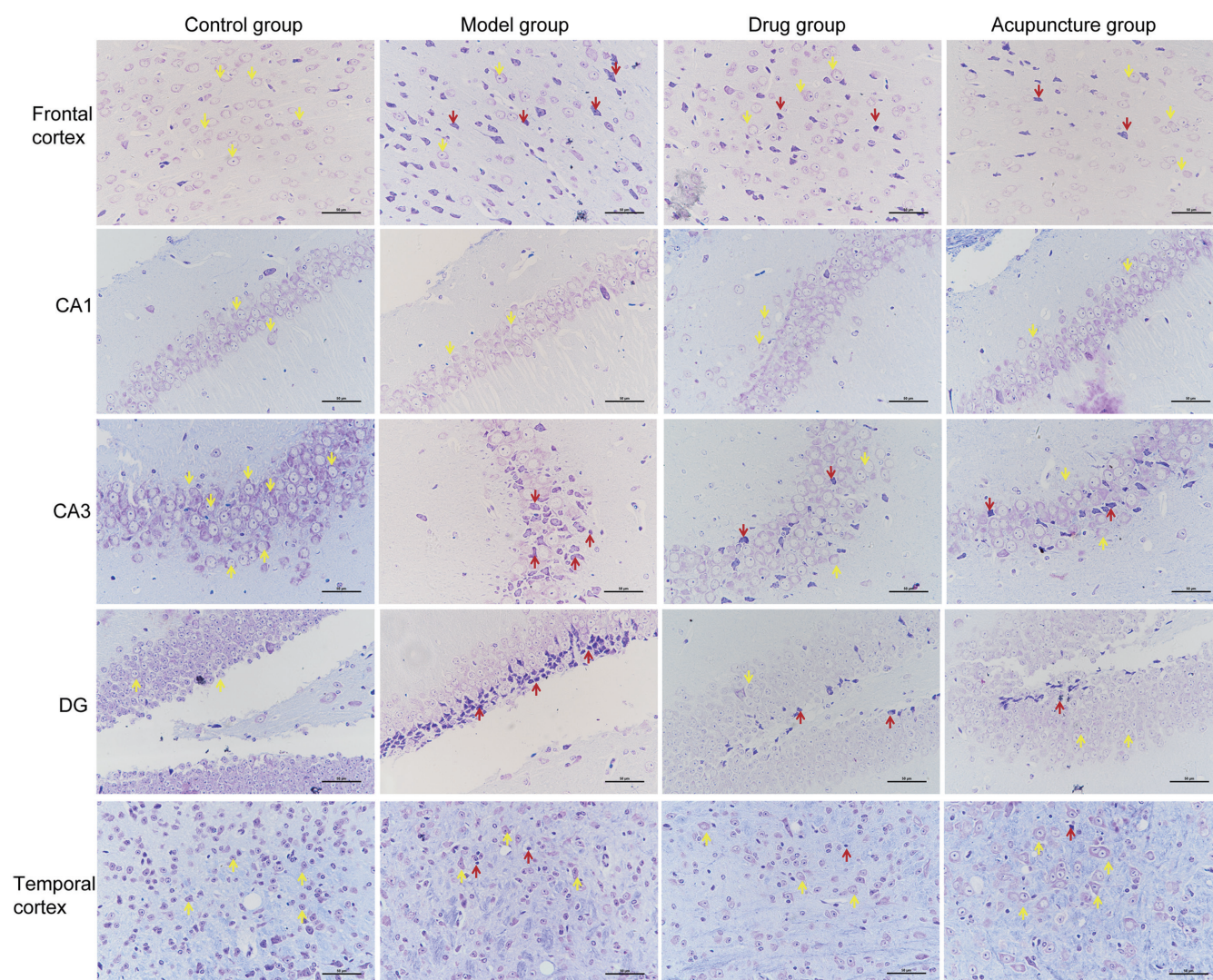


Fig. 2. The effect of acupuncture on neurons in different brain regions. The yellow arrows mark healthy cells that are rich in Nissl bodies. The red arrows mark damaged cells with unclear stratification. Control group, $n = 4$; Model group, $n = 3$; Drug group, $n = 4$; Acupuncture group, $n = 3$. CA1, cornu ammonis 1; DG, dentate gyrus. The scale bar represents a length of 50 μm .

had conical or polygonal shapes, with reduced Nissl bodies. In groups D and A, most cells were round with clear stratification, and a small number of cells were stained deep blue with unclear stratification. To clearly show the hippocampus, the cornu ammonis 1 (CA1), CA3, and dentate gyrus (DG) regions were presented individually. In group C, cells in CA1, CA3, and DG were regularly distributed with clear stratification. In group M, the CA3 and DG areas exhibited cellular damage, with an irregular pattern of cell distribution, indistinct layering, diminished Nissl body presence, and thinning of the cellular layers. In groups D and A, there were some damaged cells, but much less than in group M.

The effect of acupuncture on the content of Caspase-3 and tau protein across various brain regions

Figure 3 shows the content of Caspase-3 and tau proteins across various brain regions of mice tested by Western blot. It was found that the content of Caspase-3 in the frontal cortex and hippocampus

of group M was higher than that in group C ($P < 0.01$, $P < 0.01$). The content of Caspase-3 in the frontal cortex and hippocampus of group D was lower than that in group M ($P < 0.05$, $P < 0.05$). After acupuncture treatment, the content of Caspase-3 in the frontal cortex and hippocampus was also lower than that in group M ($P < 0.01$, $P < 0.05$). For Caspase-3 protein levels in the temporal cortex, there were no statistical differences between the groups ($P > 0.05$). The contents of Caspase-3 in the frontal cortex, hippocampus, and temporal cortex of group A were lower than those of group D ($P > 0.05$, $P > 0.05$, $P > 0.05$).

As for tau protein levels in the frontal cortex, there were no statistical differences among the four groups ($P > 0.05$). The hippocampal Tau levels in group M were elevated compared to those in group C ($P < 0.01$). The content of Tau in group A was lower than that in group M ($P < 0.05$). Group M exhibited increased tau protein levels in the temporal cortex relative to group C ($P < 0.01$). Both groups D and A had reduced tau protein levels in the temporal cortex compared to group M ($P < 0.01$, $P < 0.01$). No statistically

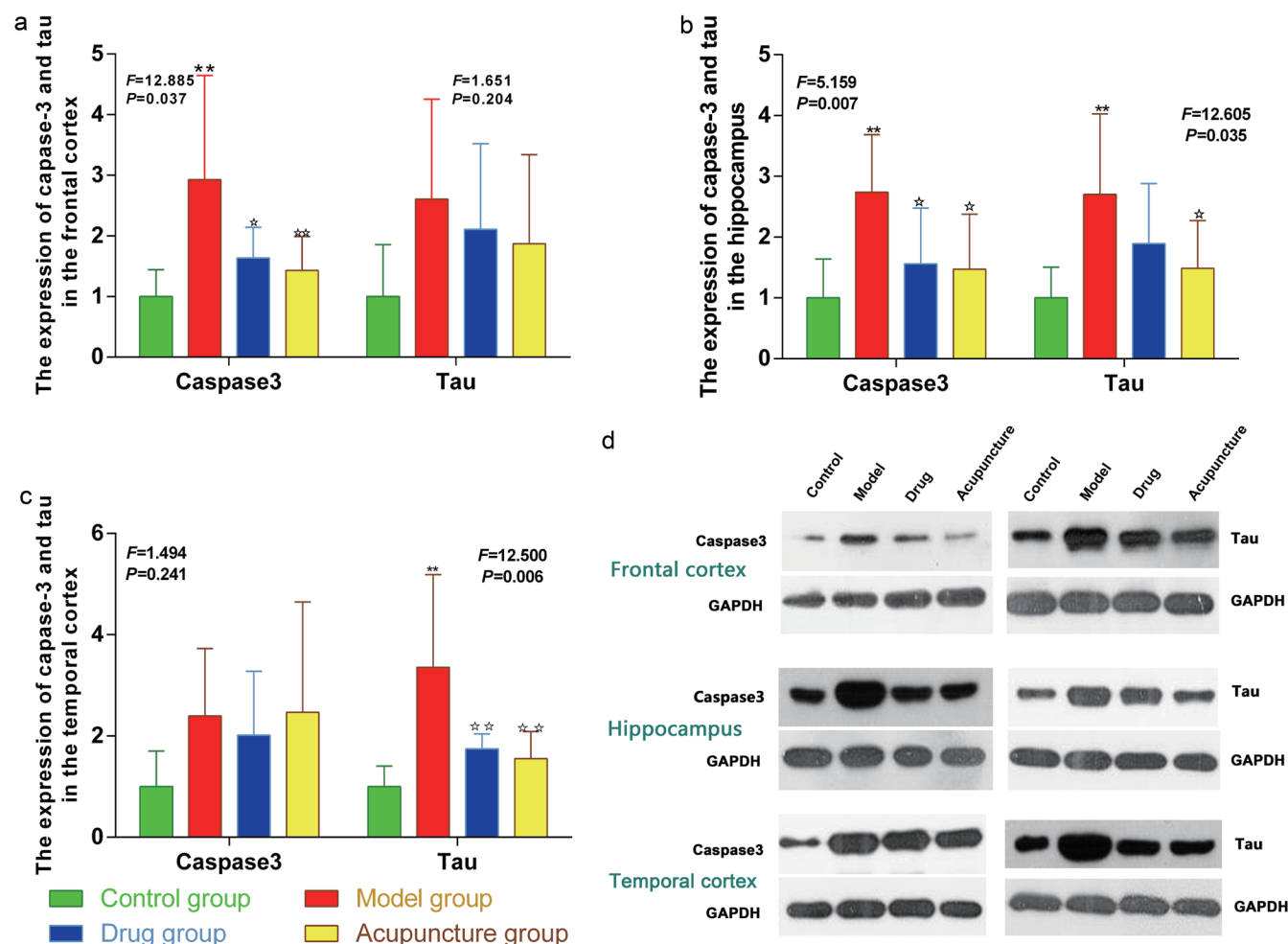


Fig. 3. The effect of acupuncture on the content of Caspase-3 and tau protein in the frontal cortex, hippocampus, and temporal cortex. Compared to the control group, * $P < 0.05$, ** $P < 0.01$; compared to the model group, ☆ $P < 0.05$; ☆☆ $P < 0.01$; compared to the drug group, ☆ $P < 0.05$; ☆☆ $P < 0.01$. $n = 7$.

significant differences were observed between groups D and A ($P > 0.05$).

Discussion

The Morris water maze serves as a de facto standard for effectively and objectively assessing cognitive capabilities.¹² This study described that the time ratio of mice in group M spent in the goal quadrant remained low, even below 25%, during both the hidden platform trial and the probe trial, which is consistent with a previous study.¹³ In contrast, the ratio of time mice in group A spent in the goal quadrant was above 25% on most of the five days, with day 4 showing higher values compared to day 1. Hence, this study suggests that acupuncture at the DU channel can enhance the cognitive capabilities of AD mice.

Learning and memory capabilities are manifestations of brain function that depend on the collective contributions of multiple brain regions.¹⁴ The frontal cortex, a crucial neural hub for cognition and behavioral control, is among the areas most affected in AD.¹⁵ For several decades, the hippocampus has been recognized as a pivotal region for the creation of cognitive maps and is necessary for tasks involving the organization and flexible expression of

memories.¹⁶ Substantial evidence indicates that hippocampal damage typically leads to deficits in spatial learning and memory performance.¹⁷ The temporal cortex plays a particularly crucial role in episodic memory, with its reduced thickness being indicative of a heightened risk for AD.¹⁸ The frontal cortex, hippocampus, and temporal cortex are all cognition-related brain regions. Neurons in the brain are the fundamental functional units that integrate and transmit signals in response to various stimuli. Liu *et al.*¹⁹ showed that Qingxin Kaiqiao Fang could protect the hippocampal neuronal morphology of AD mice. Yang *et al.*²⁰ showed that salidroside attenuated neuronal damage in the cortex and hippocampus. Li *et al.*²¹ showed that acupuncture protected the neurons in the dentate gyrus of the hippocampus, medial septum, and vertical limb of the diagonal band. This study indicates that acupuncture at the DU channel protected neurons in the three main brain regions, including the frontal cortex, hippocampus, and temporal cortex.

Numerous investigations have shown that tau protein is toxic to neurons and is considered a primary factor in neurodegeneration that leads to neuronal cell death.^{22,23} Apoptosis is a major physiological process of cell death. Characteristics of apoptotic neuronal demise include nuclear pyknosis, condensation of the cytoplasm, and fragmentation and condensation of chromatin.²⁴ Reducing

apoptosis helps ameliorate cognitive dysfunction. Zang *et al.*²⁵ suggested that nicergoline inhibited apoptosis in the hippocampus of AD mice. Yang *et al.*²⁶ found that moxibustion improved cognitive function in vascular dementia rats by inhibiting the apoptosis of hippocampal neurons. Zhang *et al.*²⁷ proved that acupuncture attenuated the cognitive defects of SAMP8 mice by inhibiting the apoptosis of hippocampal neurons. The results of this study suggest that acupuncture at the DU channel possesses a multi-target effect, including curbing apoptotic processes in the frontal cortex and hippocampus and reducing tau accumulation in the hippocampus and temporal cortex.

It is also worth noting the depression-like behavior of AD model mice in this study, and that acupuncture at DU channel could alleviate this depression-like behavior. The majority of the traces of AD model mice were not in the goal platform area but near the point where they were released into the water. This suggests that the AD model mice had a reduced desire to survive. After treatment with drugs and acupuncture, most of the traces of the mice were in the goal platform quadrant and across the platform quadrant, following different but effective routes. This phenomenon indicates that the impaired learning ability of SAMP8 mice may be associated with depression,²⁸ which is consistent with findings in a number of clinical AD patients who also suffer from depression.²⁹ Acupuncture has the potential to enhance AD mice's capacity for learning and memory, as well as alleviate depression-like behavior, similar to other treatments.^{3,30}

The study suggests that acupuncture could improve cognitive function by inhibiting apoptosis and reducing tau protein levels, but the specific biological pathways and mechanisms were not explored in depth. This is a limitation of this study. Future studies will delve into the biological pathways involved in acupuncture's intervention in AD.

Conclusions

Acupuncture at the DU channel can improve learning and memory abilities, as well as depression-like behavior, to a certain degree by reducing apoptosis in the frontal cortex and hippocampus and by decreasing the deposition of tau in the hippocampus and temporal cortex of AD model rats. Further research should focus on the multiple effects and mechanisms of acupuncture for AD, as well as its impact on accompanying symptoms such as depression-like behavior.

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Conflict of interest

The authors declare no potential conflicts of interest.

Author contributions

Interpretation of results, writing of the manuscript (HLT, XW),

data analysis (YJL, YSG) study design, review and approval of the manuscript (ZGL, JJ), and experiment performance (ZDW, SW, HL, JYY, JHZ, RSD, JYR). All authors have approved the final version and publication of the manuscript.

Ethical statement

The experimental protocol applied in this study was approved by the Ethics Committee for Animal Experimentation of Beijing University of Chinese Medicine (ID: BUCM-4-2018111701-4045). All procedures complied with the Animal Research: Reporting of *In Vivo* Experiments guidelines and were performed according to the guidelines of the National Institutes for Animal Research. All researchers in this study were certified by the Animal Experimentation Center of Beijing University of Chinese Medicine.

Data sharing statement

The data used to support the findings of this study are available from the corresponding author upon request.

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