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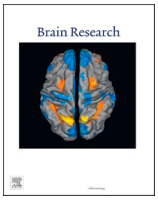

Research paper

The changes of neurogenesis in the hippocampal dentate gyrus of SAMP8 mice and the effects of acupuncture and moxibustion

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ABSTRACT

Background: Influenced by the global aging population, the incidence of Alzheimer's disease (AD) has increased sharply. In addition to increasing β-amyloid plaque deposition and tau tangle formation, neurogenesis dysfunction has recently been observed in AD. Therefore, promoting regeneration to improve neurogenesis and cognitive dysfunction can play an effective role in AD treatment. Acupuncture and moxibustion have been widely used in the clinical treatment of neurodegenerative diseases because of their outstanding advantages such as early, functional, and benign two-way adjustment. It is urgent to clarify the effectiveness, greenness, and safety of acupuncture and moxibustion in promoting neurogenesis in AD treatment.

Methods: Senescence-accelerated mouse prone 8 (SAMP8) mice at various ages were used as experimental models to simulate the pathology and behaviors of AD mice. Behavioral experiments, immunohistochemistry, Western blot, and immunofluorescence experiments were used for comparison between different groups.

Results: Acupuncture and moxibustion could increase the number of $PCNA^+$ DCX^+ cells, Nissl bodies, and mature neurons in the hippocampal Dentate gyrus (DG) of SAMP8 mice, restore the hippocampal neurogenesis, delay the AD-related pathological presentation, and improve the learning and memory abilities of SAMP8 mice.

Conclusion: The pathological process underlying AD and cognitive impairment were changed positively by improving the dysfunction of neurogenesis. This indicates the promising role of acupuncture and moxibustion in the prevention and treatment of AD.

1. Introduction

Geriatric disorders account for an increasing proportion of various diseases as the pace of population aging accelerates globally, and have been the focus of medical attention (Li et al., 2020). Alzheimer's disease (AD), as an insidious-onset neurodegenerative condition with progressive diminutions in learning and memory abilities, is the most typical one among geriatric disorders (Arai et al., 2019). AD seriously damages the health of the elderly and its related deaths have been markedly increasing year by year (Kavitha et al., 2022; Cui et al., 2020). In China, its incidence among the population aged over 60 years old is as high as 3.2 % (Fernandes et al., 2022). Even with the unremitting exploration of molecular and genetic mechanisms of AD, there are no medications that can cure this disorder, and only some symptoms can be alleviated by proper drugs.

The pathological hallmarks of AD include abnormal amyloid-β (Aβ) deposition, neurofibrillary tangles (NFTs), and excessive neuronal loss (Cataldo et al., 2004). In its pathological process, excessive Aβ deposition and phosphorylation of tau protein can both cause varying degrees of neuronal loss (Pino et al., 2017). While mammalian hippocampal neurons have a very limited ability to generate new neurons (Abbott et al., 2020), the human hippocampus retains the ability to produce neurons throughout life. Adult human neurogenesis in the hippocampus is an intrinsic process through which neural stem cells (neural precursor

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cells) drive the production of new neurons in the substratum of the DG. Besides, the hippocampus is the most closely aligned part of the brain to memory formation and is also associated with learning function. Therefore, hippocampal neuronal regeneration has become an increasingly popular focus in the research of learning, memory, and related diseases (Furcila et al., 2019). Mounting studies have confirmed neuronal regeneration in the peripheral nervous system of adult mammals (Moreno-Jiménez et al., 2019) and the reduction in the number of neurons in the hippocampus and the presence of altered neurogenesis throughout the whole process of AD (Bihelek, 2019; Li et al., 2016). It is of great significance to study the multiple stages of neurogenesis and seek proper or effective interventions for AD. Some scholars hold the view that the damage of new hippocampal neurons appeared earlier than Aβ deposition, neuronal loss, and inflammation (Shi et al., 2020). Stimulating endogenous neuronal regeneration can modulate or hamper Aβ aggregation and help restore learning and memory functions. These findings suggest the pathogenesis of AD could be partly attributed to the damage to hippocampal neurogenesis. Relevant domestic and international studies have confirmed neuronal regeneration is regulated by various transcription and growth factors and manifested or affected by several biomarkers, such as DCX (doublecortin), NeuN (a neuronal nuclear antigen), PCNA (proliferating cell nuclear antigen) and APP (Delprato et al., 2022; Moreno-Jiménez et al., 2021; Flor-García et al., 2020; Tobin et al., 2019; Zheng et al., 2013). Induction of extensive transcriptional and epigenetic changes in damaged neurons (Zhou et al., 2015) and promotion of the proliferation and differentiation of adult hippocampal neural progenitor cells through the Shh signaling pathway (Jia et al., 2017) have shown favorable results among AD patients. It is encouraging that compensation for the damage or loss of neurons via promoting neuronal regeneration and maintaining brain homeostasis would be helpful in the prevention and treatment of AD.

Acupuncture and moxibustion are typical techniques in oriental medicine and have been widely used to treat neurodegenerative diseases in clinical practice. A recent systematic review has discussed the potential effects of acupuncture therapy on adult neurogenesis and neurotrophic factors in AD (Shin et al., 2017). Acupuncture could significantly improve the clinical symptoms of AD patients and exert synergistic effects when combined with donepezil hydrochloride (Zhao et al., 2017). It could promote the proliferation of endogenous neural stem cells, regulate the effects of exogenous ones on neuropeptides, and improve the dysfunction of neurogenesis (Sun et al., 2021). Moxibustion was reported to improve the spatial learning and memory abilities in dementia rats and promote the reconstruction of neurogenesis (A R et al., 2021). A recent *meta*-analysis has reviewed the evidence-based effects of moxibustion in the treatment of AD (Koo et al., 2010). Accumulating evidence suggested that acupuncture and moxibustion could serve as an effective alternation for early prevention and treatment of AD and improvement of relevant physical functions. Both therapies have bidirectional benign regulatory effects in the treatment of AD. However, the underlying mechanisms remain largely unclear.

Since SAMP8 (senescence-accelerated mouse prone 8) mice are an ideal model for age-related cognitive decline and AD, we in this study used SAMP8 mice of different months as the AD model to explore the effects of acupuncture and moxibustion on neurogenesis. We investigated the expression of neurogenesis-related biomarkers including DCX, NeuN, PCNA, and pathological characteristics-related biomarkers tau protein, APP, and Nissl bodies in the hippocampus, as well as the behavioral performance of SAMP8 mice, in order to provide a more experimental basis for the prevention and treatment of AD via acupuncture-moxibustion.

2. Materials and methods

2.1. Animals, grouping, and interventions

Three-month-old male SAMP8 mice and SAMR1 (senescence-accelerated mouse resistant 1) mice weighting 24–28 g, six-month-old male SAMP8 mice and SAMR1 mice weighting 33–37 g and ten-month-old male SAMP8 and SAMR1 mice weighing 30–35 g were purchased from Peking University Health Science Centre (Beijing, China) for this study. These mice were individually caged in a specific pathogen-free animal room (maintained at 24 ± 1 °C) on a 12/12 h light/dark cycle, with free access to food and water *ad libitum*. All animals in this study were treated strictly according to the Guidelines for the Care and Use of Laboratory Animals by the National Institutes of Health, and every possible effort was made to minimize animal suffering. The animal experiments were approved by the Animal Ethics Committee of the Hubei University of Chinese Medicine (approval No. HUCMS-110332194164424311).

As shown in Fig. 1, SAMP8 mice aged 3 months were randomized into the 3 M−ACU (acupuncture) group and 3 M−Model group, and SAMR1 mice aged 3 months were used as the 3 M−Control group (12 mice per group). Similarly, 6 M−ACU group, 6 M−Model group, 6 M−Control group, 10 M−ACU group, 10 M−Model group, and 10 M−Control group (12 mice per group) were made for comparison. The mice in the 3 M−ACU group, 6 M−ACU group, and 10 M−ACU group were administrated with acupuncture at bilateral GV20 and HT7 acupoints and moxibustion at bilateral BL23 acupoints according to a widely recognized mouse acupoint atlas (Park et al., 2017). The location of the three acupoints was also clearly described in a systematic review of animal-based studies on the effects of acupuncture on AD (Babcock et al., 2021). As to GV20 acupoint, the acupuncture needles (0.16 mm * 7 mm; Huanqiu Acupuncture Medical Appliance Co., Ltd., Suzhou, China) were inserted horizontally at a 15-degree angle into the skin/acupoint to a depth of 1–2 mm; and to HT7 acupoint, the needles were inserted vertically (90 degrees) into the skin/acupoint to a depth of 1–2 mm. The needles were slightly twisted clockwise and counter-clockwise evenly within 180–270 degrees. The twisting was repeated 40–45 times within one treatment time (30 s). The acupuncture treatment was performed once a treatment day. The operator for the manual acupuncture stimulation was well-trained before this study. As to moxibustion stimulation for BL23 acupoint, the burning end of a moxa stick (4 mm in diameter; Linxiang Huaxiangai Biotechnology Co., Ltd., Hunan, China) was suspended at 2–3 cm above the skin/acupoint (local temperature was maintained at 41 ± 0.5 °C). Moxibustion was performed 15 min a time and once a treatment day. One treatment course lasted 6 days and 4 courses were given to the treated mice, with an interval of one day between the courses. The mice in the control groups and model groups received no acupuncture or moxibustion stimulation but were grabbed at the same treatment time and fixed with the same method as the acupuncture groups.

2.2. Morris water maze test

Morris water maze test was performed in all SAMP8 mice and SAMR1 mice on the next day after the above interventions to evaluate their spatial learning and memory. All mice were subjected to place navigation test and spatial probe test. Briefly, the formal positioning navigation training was conducted once daily for 5 consecutive days. For each hidden platform trial, one mouse was immersed in the water from one of the four entry points of four different quadrants, facing the wall of the circular black pool (120 cm in diameter and 50 cm in height). The time duration from entering the water to climbing onto the hidden platform in the fixed quadrant within 1 min was recorded as escape latency (s), namely, the mouse's performance. The default value was 60 s if one mouse did not reach the target platform within 1 min. Each mouse received four platform trials per day to reach a mean value for escape latency. The space exploration test was carried out on the next day after the positioning navigation training. The mice were placed into the water at any entry point of the non-original platform quadrants in the absence of the target platform. The number of times of crossing the original platform within 1 min was recorded. The WMT-100 Morris

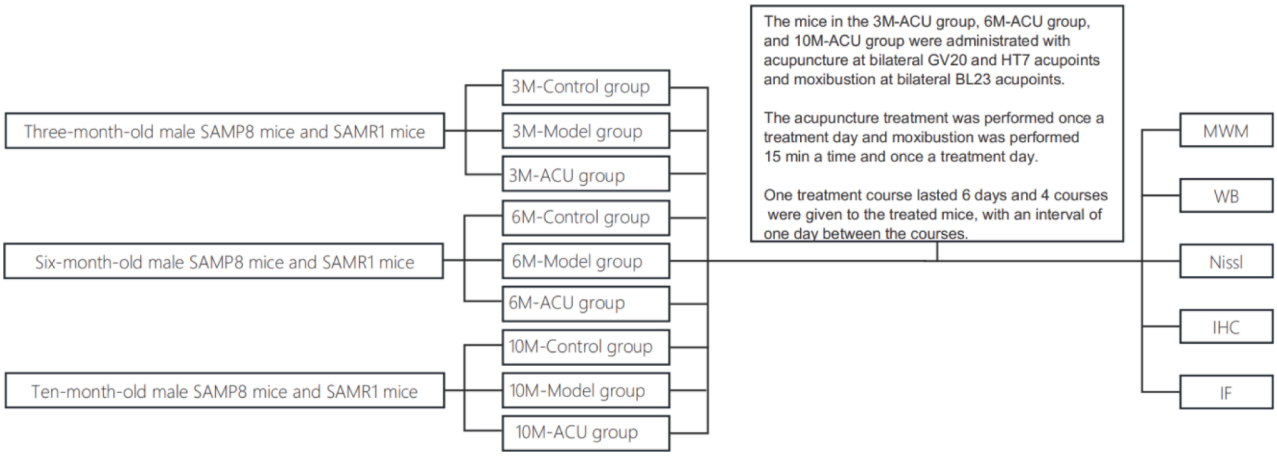

Fig. 1. Experimental design of the study.

video tracking system (Taimeng Technology Co., Ltd., Chengdu, China) was used for data recording and analysis for the Morris water maze test.

2.3. Western blotting

Fresh hippocampal tissues were rapidly isolated from the SAMP8 mice and SAMR1 mice and placed in cryopreservation tubes. The tubed samples were placed in liquid nitrogen and then stored in a refrigerator at − 80 °C until western blot analysis. Hippocampal tissues were rinsed 3 times with pre-cooled phosphate-buffered saline (P1010; Solarbio, Beijing, China), and then quickly cut into small pieces and placed in a homogenizer. The cells in the tissue homogenate were lysed by radioimmunoprecipitation buffer (R0010; R0010; Beyotime, Shanghai, China) complemented with 1 % phenylmethylsulfonyl fluoride (ST506; Beyotime, Shanghai, China). After centrifugation at 12,000 r/min at 4 °C for 15 min, the supernatant was collected as the total protein solution. The total protein concentration was determined using the BCA protein assay kit (P0012; Beyotime, Shanghai, China). For each sample, 50 μl supernatant was processed by the sodium dodecyl sulphate–polyacrylamide gel electrophoresis and transferred to the methanol-soaked polyvinylidene fluoride membrane. The membrane was then blocked with skim milk (P0216; Beyotime, Shanghai, China) in Tris-buffered saline with Tween® 20 (TBST). After the blocking, the membrane was incubated with the primary antibodies against Tau-ps262 (ab131354; Abcam, USA) with a dilution ratio of 1:1000, APP (25524–1-AP; Proteintech, Wuhan, China) with the dilution ratio of 1:1000, and β-actin (20536–1-AP; Proteintech, Wuhan, China) with the dilution ratio of 1:10000 overnight at 4℃. The membrane was subsequently incubated with goat anti-rabbit IgG antibody with the dilution ratio of 1:3000 conjugated with horseradish peroxidase for 2 h. The membrane was washed by TBST before and after each incubation with the above antibodies. The membrane was then treated with an ECL detection kit (BeyoECL Moon, P0018FS; Beyotime, Shanghai, China). The protein bands were obtained through the Bio-Rad® Image Analysis System, and their gray values were analyzed using Image J software. The relative protein content was shown by the ratio of the target band gray value to the β-actin band gray value.

2.4. Nissl staining

After successful anesthesia, cardiac perfusion fixation of the treated mice was achieved with 0.9 % sodium chloride solution (ST341-500 ml; Beyotime, Shanghai, China) and subsequent buffered 4 % paraformaldehyde (P0099-500 ml; Beyotime, Shanghai, China). The hippocampus was isolated from the collected brains of the mice and postfixed in 4 % paraformaldehyde overnight. The hippocampal tissues were dehydrated and embedded in paraffin blocks for sectioning. Paraffin slices were dewaxed to water. Staining the slices with toluidine blue (G1032-500 ml; Servicebio, Wuhan, China), soaking the slices in dyeing solution for 5 min, washing with water, slightly differentiating with 1 % glacial acetic acid (G10000218-500 ml; Servicebio, Wuhan, China), washing with tap water to stop the reaction, controlling the degree of differentiation under microscope, and drying the slices in oven after washing with tap water. Then put the slices in clean xylene and transparent for 5 min, and seal the slices with neutral gum. Observe and take pictures under a microscope. Nissl bodies of the hippocampus were dark blue with a light blue background. The counts of Nissl bodies per area (cell counts / mm^2) were recorded.

2.5. Immunohistochemistry test

The paraffin-embedded sections were dewaxed and rehydrated after drying at 37 °C overnight. For antigen retrieval, the hydrated sections were placed in sodium citrate buffer (P0081; Beyotime, Shanghai, China) and heated with a microwave oven until boiling for 15 min, followed by natural cooling at room temperature. The sections were then incubated with 3 % hydrogen peroxide solution at 37 °C for 25 min to quench endogenous peroxidase activity, followed by washing with phosphate-buffered saline buffer solution 3 times. Afterward, the sections were incubated with primary antibodies against NeuN (ab177487; Abcam, USA) with the dilution ratio of 1:3000 overnight at 4 °C, followed by incubation with the horseradish peroxidase-conjugated goat anti-rabbit IgG antibody for 2 h. The tissue sections were stained with the 3,3′-diaminobenzidine substrates (P0202; Beyotime, Shanghai, China) and counterstained with hematoxylin, and dehydrated with ethanol and xylene to prepare for slide mounting. The images were viewed by a bright-field microscope, and the staining intensity of each image was quantified using Image-Pro Plus 6.0 software. The integrated optical density (IOD) and its values of the positive area (IOD/area = mean optical density (MOD)) of each image were determined and obtained as representative NeuN staining density. Higher mean optical density indicates stronger positive expression. The counts of $NeuN^+$ cells per area (cell counts / mm^2) were recorded. A higher count value indicates a stronger positive expression.

2.6. Immunofluorescence double-labeling method

The paraformaldehyde-fixed paraffin-embedded sections of hippocampal tissues of mice were dewaxed and rehydrated, followed by antigen retrieval as per the above-described procedures. After natural cooling, the sections were placed in phosphate-buffered saline and washed on a decolorizing shaking table. Incubation with the primary

antibodies against PCNA (ab18197; Abcam, USA) with the dilution ratio of 1:3000 mixed with DCX (bs-20797R; Bioss, Beijing, China) with the dilution ratio of 1:500 was conducted separately overnight at 4 °C. Subsequent incubation with their two corresponding Cy3-conjugated goat anti-mouse IgG antibodies was conducted separately for 50 min at room temperature in the dark. The nucleus was counterstained with DAPI (4′6-diamidino-2-phenylindole; C1002; Beyotime, Shanghai, China). After washing with phosphate-buffered saline three times, the cells on the slides were sealed with an anti-fluorescence quenching sealing solution (P0126; Beyotime, Shanghai, China). The image of each slide (section) was viewed by a fluorescence microscope, and the count of $PCNA^+DCX^+$ cells in each image was obtained by the Image-Pro Plus 6.0 software. The labeling density was defined as the counts of $PCNA^+DCX^+$ cells per area (cell counts / mm^2). A higher labeling density value indicates a stronger positive expression.

2.7. Statistical analysis

All the data analyses were conducted by GraphPad Prism (Version 7.03; GraphPad Software Inc., La Jolla, CA, USA). For the normally distributed data with homogeneous variance, a one-way analysis of variance was used for comparisons among multiple groups, and Fisher's least significant difference test was used for pair comparisons. Tamhane's T2 test was performed for pair comparisons of the normally distributed data with unequal variances. A non-parametric test was used for data that did not conform to normal distribution. Data are presented as mean ± SEM unless otherwise stated. A probability value of less than 0.05 was considered statistically significant.

3. Results

3.1. Behavioral performance

Acupuncture and moxibustion could obviously improve the learning and memory ability of SAMP8 mice aged 3 months and 6 months, but they could not effectively improve the learning and memory ability of 10-month-old SAMP8 mice. As shown in Fig. 2, the average escape latency of the 3 M−ACU group was significantly shorter than that of the 3 M−Model group ($P < 0.01$), and its number of times of crossing the original platform within 1 min was significantly bigger than that of the 3 M−Model group ($P < 0.05$). The same goes for the comparisons of the 6 M−ACU group with the 6 M−Model group ($P < 0.01$, $P < 0.05$, respectively). Similarly, the average escape latency of the 10 M−ACU group was shorter than that of the 10 M−Model group, and its number of times of crossing the original platform within 1 min was bigger than that of the 10 M−Model group, but the differences were not statistically significant. Additionally, these results indicate longer average escape latency but fewer times of crossing the original platform among the SAMP8 mice with the increase of their month age.

3.2. Number of neurons in hippocampal DG

To examine the number of neurons in hippocampal DG, we quantified the number of mature neuron marker immunoreactive cells. As shown in Fig. 3, the number of $NeuN^+$ cells in the 3 M−ACU group was significantly higher than that of the 3 M−Model group ($P < 0.01$). The same goes for the comparisons of the 6 M−ACU group with the 6 M−Model group ($P < 0.05$). Similarly, the number of $NeuN^+$ cells in the 10 M−ACU group was higher than that of the 10 M−Model group ($P > 0.05$), but the differences were not statistically significant. Overall, in the hippocampal DG of the SAMP8 mice, the number of $NeuN^+$ cells decreased with mouse month age (10 M−Control group < 6 M−Control group < 3 M−Control group; 10 M−Model group < 6 M−Model group < 3 M−Model group; 10 M−ACU group < 6 M−ACU group < 3 M−ACU group).

3.3. Number of proliferating immature neurons in hippocampal DG

To determine the number of proliferating immature neurons in hippocampal DG, we quantified the number of $PCNA^+$ DCX^+ cells. As shown in Fig. 4, the number of $PCNA^+$ DCX^+ cells of the 3 M−ACU group was significantly higher than that of the 3 M−Model group (both $P < 0.01$). The same goes for the comparisons of the 6 M−ACU group with the 6 M−Model group (both $P < 0.05$). Similarly, the number of $PCNA^+$ DCX^+ cells of the 10 M−ACU group was higher than that of the 10 M−Model group ($P > 0.05$), but the differences were not statistically significant. Overall, in the hippocampal DG of the SAMP8 mice, the number of $PCNA^+$ DCX^+ cells decreased with mouse month age (10 M−Control group < 6 M−Control group < 3 M−Control group; 10 M−Model group < 6 M−Model group < 3 M−Model group; 10 M−ACU group < 6 M−ACU group < 3 M−ACU group).

3.4. Number of Nissl bodies in hippocampal DG

To detect the damage of neurons in hippocampal DG, we quantified the number of Nissl bodies. As shown in Fig. 5, the number of Nissl bodies in hippocampal DG of the 3 M−ACU group was significantly higher than that of the 3 M−Model group ($P < 0.01$). The same goes for the comparisons of the 6 M−ACU group with the 6 M−Model group ($P < 0.05$). Similarly, the number of Nissl bodies in hippocampal DG of the 10 M−ACU group was higher than that of the 10 M−Model group ($P > 0.05$), but the differences were not statistically significant. Overall, in the hippocampal DG of the SAMP8 mice, the number of Nissl bodies decreased with mouse month age (10 M−Control group < 6 M−Control group < 3 M−Control group; 10 M−Model group < 6 M−Model group < 3 M−Model group; 10 M−ACU group < 6 M−ACU group < 3 M−ACU group).

3.5. Levels of APP and p-tau protein in hippocampal DG

To evaluate the pathological features of AD in hippocampal DG of SAMP8 mice and the effect of acupuncture, the levels of APP and p-tau were detected. As shown in Fig. 6, the relative expression levels of APP and p-tau in hippocampal DG of the 3 M−ACU group were significantly lower than that of the 3 M−Model group (both $P < 0.01$). The same goes for the comparisons of the 6 M−ACU group with the 6 M−Model group (both $P < 0.05$). Similarly, the levels of APP and p-tau in hippocampal DG of the 10 M−ACU group were lower than that of the 10 M−Model group ($P > 0.05$), but the differences were not statistically significant. Overall, in the hippocampal DG of the SAMP8 mice, the relative expression levels increased with mouse month age (3 M−Control group < 6 M−Control group < 10 M−Control group; 3 M−Model group < 6 M−Model group < 10 M−Model group; 3 M−ACU group < 6 M−ACU group < 10 M−ACU group).

4. Discussion

Substantial evidence suggests impaired neurogenesis in Alzheimer's disease in the adult brain, contributing to learning and memory deficits characterizing the disease (Disouky and Lazarov, 2021). New neurons play an essential role in learning as well as memory encoding within the hippocampal DG, and halting neuronal loss and enhancing neurogenesis have been indicated as a promising therapeutic strategy for AD treatment (Moreno-Jiménez et al., 2019; Kim et al., 2022). Improvement of neurodegenerative diseases including AD has been indeed seen in the application of acupuncture-moxibustion in recent decades. Our study used SAMP8 mice, an ideal model for spontaneous age-related cognitive decline in AD (Ma et al., 2011; Morley et al., 2012), to investigate the expression of DCX, NeuN, PCNA, and the hippocampal p-tau protein, as well as the behavioral performance. We aimed to explore the changes of neurogenesis in the hippocampal DG among AD mice with the treatment of acupuncture-moxibustion.

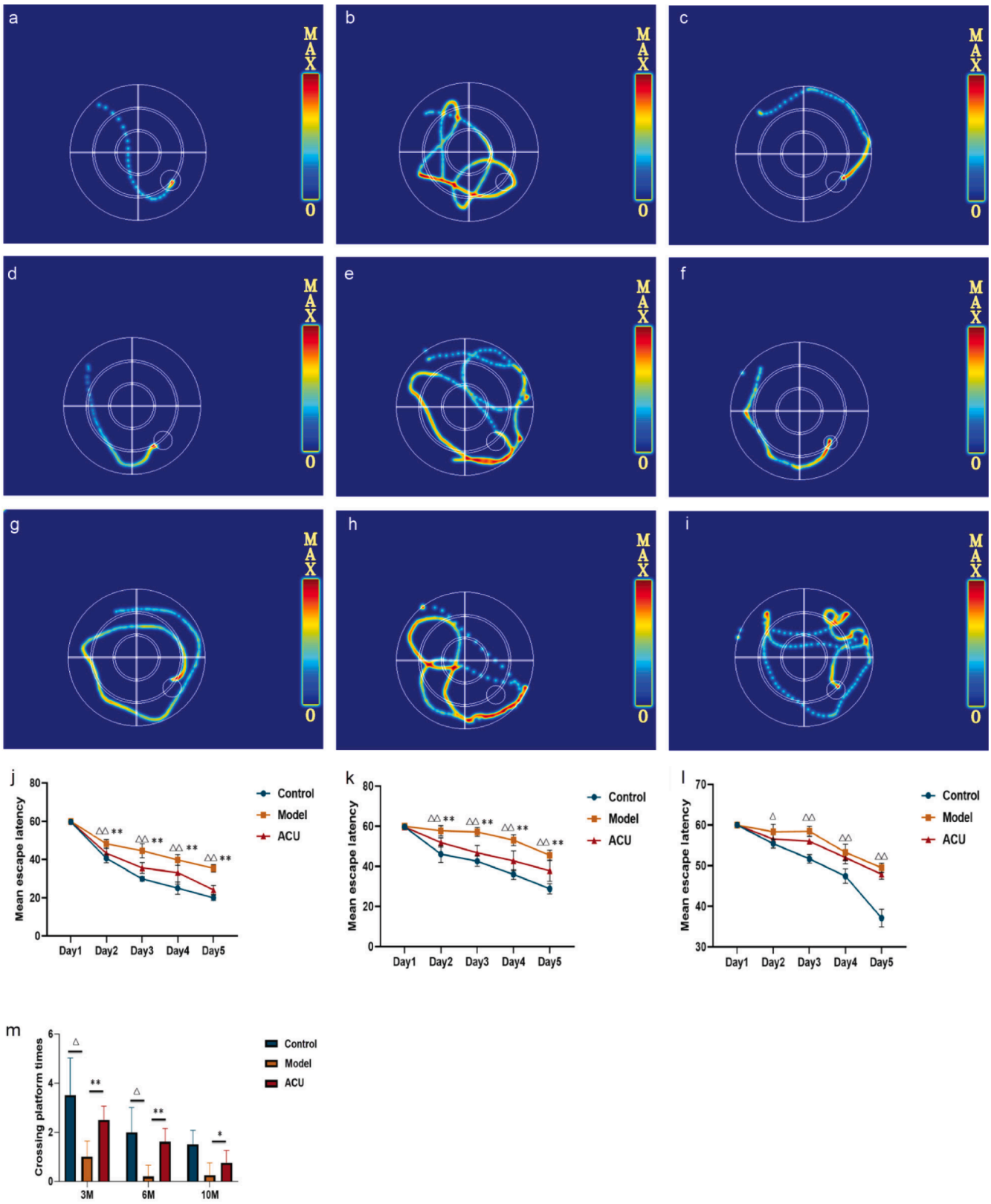

Fig. 2. Acupuncture and moxibustion could obviously improve the learning and memory ability of SAMP8 mice aged 3 months and 6 months, but they could not effectively improve the learning and memory ability of 10-month-old SAMP8 mice. (a–i) The representative swimming track of mice in (a) 3 M−Control group, (b) 3 M−Model group, (c) 3 M−ACU group, (d) 6 M−Control group, (e) 6 M−Model group, (f) 6 M−ACU group, (g) 10 M−Control group, (h) 10 M−Model group, and (i) 10 M−ACU group. The comparisons between Control, Model, and ACU groups in the average latency of escape among (j) 3-month-old mice, (k) 6-month-old mice, and (l) 10-month-old mice, and (m) the comparisons between these three groups in the number of times of crossing the original platform within 1 min. All data were expressed as mean ± standard deviation (n = 6 per group, △ means Control group vs. Model group, * means ACU group vs. Model group, $^{*}P < 0.05$, $^{\triangle}P < 0.05$, $^{**}P < 0.01$, $^{\triangle\triangle}P < 0.01$).

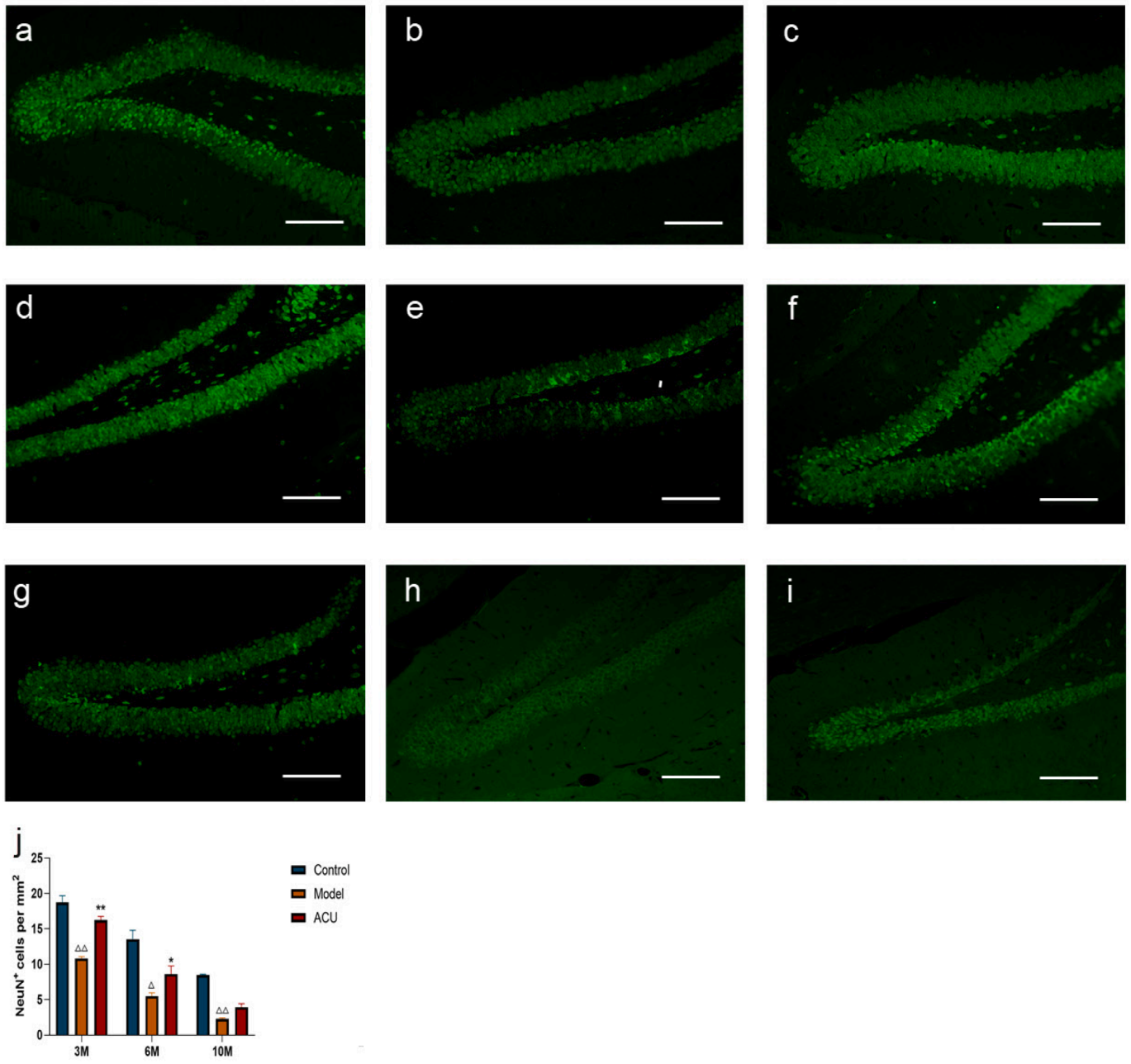

Fig. 3. Acupuncture and moxibustion could increase the number of NeuN$^+$ cells in the hippocampal DG of SAMP8 mice. (a–i) The number of NeuN$^+$ cells of mice in (a) 3 M−Control group, (b) 3 M−Model group, (c) 3 M−ACU group, (d) 6 M−Control group, (e) 6 M−Model group, (f) 6 M−ACU group, (g) 10 M−Control group, (h) 10 M−Model group, (i) 10 M−ACU group. (j) The comparisons between the Control, Model, and ACU groups in the number of NeuN$^+$ cells among 3-month-old mice, 6-month-old mice, and 10-month-old mice. All data were expressed as mean ± standard deviation (n = 6 per group, $^*P < 0.05$, $^{\triangle}P < 0.05$, $^{**}P < 0.01$, $^{\triangle\triangle}P < 0.01$). Scale bars = 100 μm.

Behavioral performance of SAMP8 mice at various month ages varied and a better therapeutic effect of acupuncture-moxibustion was achieved when starting the treatment among the mice at a younger month age. This suggests that early intervention with these two therapies can significantly improve the spatial learning and memory abilities and object recognition ability of SAMP8 mice. Taken together, these results indicate that acupuncture-moxibustion could help SAMP8 mice resist early cognitive decline to a certain extent. Since the development of age-associated deficits in learning and memory abilities and various age-related neuropathological changes in SAMP8 mice, the 3-month, 6-month, and 10-month mice were roughly assigned to mildly, moderately, and severely senescence-accelerated ones, respectively. Consistently, the behavioral changes of the 4-month, 7-month, and 11-month SAMP8 mice without acupuncture-moxibustion intervention corresponded to a behavioral presentation of mild, moderate, and severe dementia patients staged by pathological features among the SAMP8 mice, respectively (Liu et al., 2020).

Although the pathogenesis of AD is still unclear, its pathological features have been dominated by abnormal Aβ deposition, NFTs, and neuronal injury (Yook et al., 2016). Aβ peptides are generated from amyloid precursor protein through sequential proteolytic cleavage of β- and γ -secretases, and their production exists in a dynamic balance with its clearance under normal conditions (De Strooper and Karran, 2016). An imbalance in the metabolism of Aβ-peptide causes $A\beta_{1\text{-}42}$ deposition

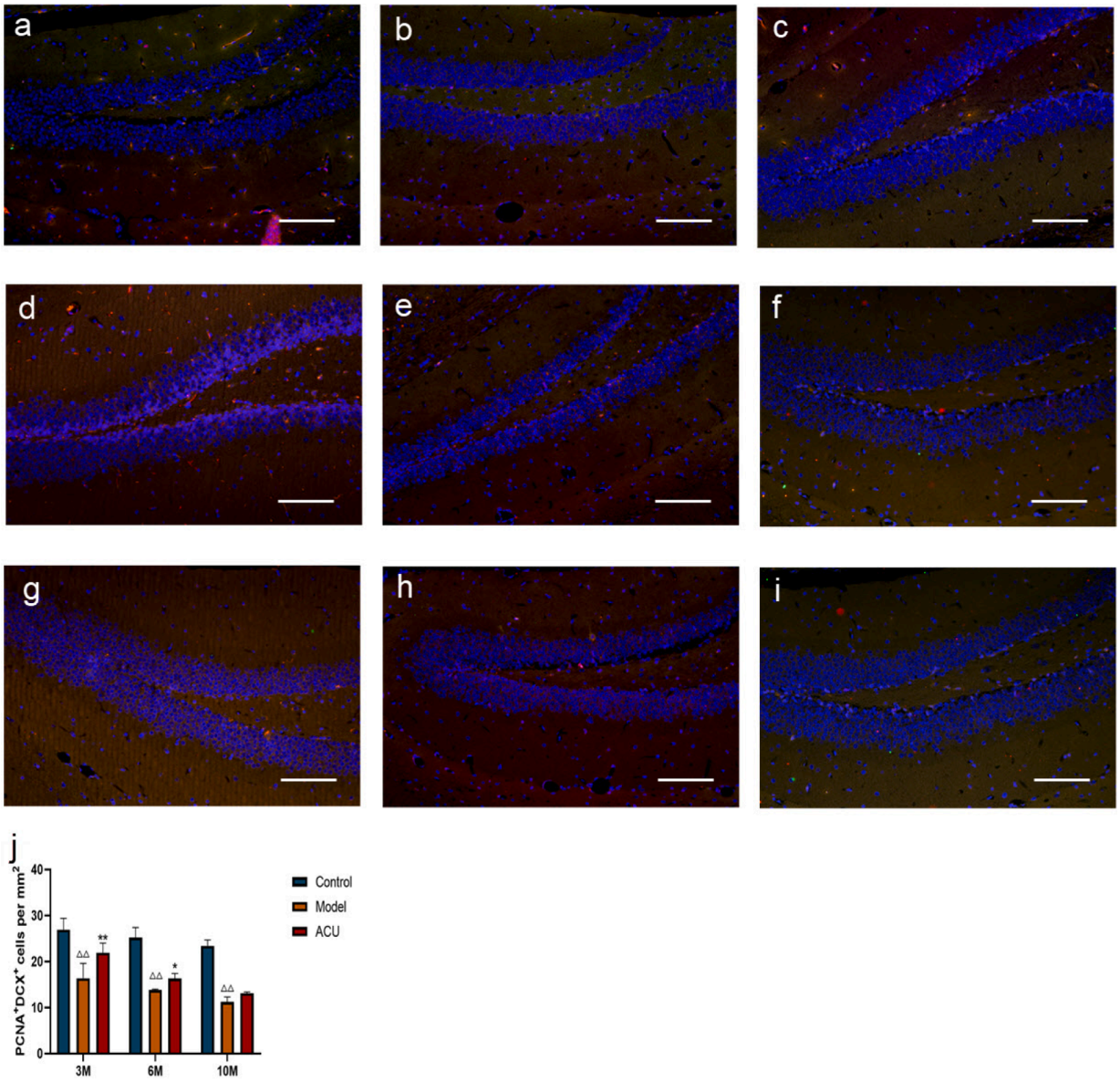

Fig. 4. Acupuncture and moxibustion could increase the number of $PCNA^{+}DCX^{+}$ cells in the hippocampal DG of SAMP8 mice. (a–i) The number of $PCNA^{+}DCX^{+}$ cells of mice in (a) 3 M−Control group, (b) 3 M−Model group, (c) 3 M−ACU group, (d) 6 M−Control group, (e) 6 M−Model group, (f) 6 M−ACU group, (g) 10 M−Control group, (h) 10 M−Model group, and (i) 10 M−ACU group. (j) The comparisons between Control, Model, and ACU groups in the number of $PCNA^{+}$ DCX^{+} cells among 3-month-old mice, 6-month-old mice, and 10-month-old mice. All data were expressed as mean ± standard deviation (n = 6 per group, $^{*}P < 0.05$, $^{\triangle}P < 0.05$, $^{**}P < 0.01$, $^{\triangle\triangle}P < 0.01$). Scale bars = 100 μm.

in a large quantity due to several intricate factors (Dyakin et al., 2020). $A\beta_{1\text{-}42}$ is a small neurotoxic peptide (4 kDa) (Rana and Sharma, 2019), and its excessive deposition alters the activity of kinases such as GSK3β, CDK5, and PKA and leads to hyperphosphorylation of tau protein (Resende et al., 2008; Liu et al., 2016; Du et al., 2014; Wu et al., 2020). $A\beta_{1\text{-}42}$-induced neurotoxicity directly or indirectly brings about neuronal cell death, synaptic dysfunction, and ultimately cognitive decline. NFTs are a commonly found neuropathological hallmark in the brain of AD patients at the early stage. In this stage, the presence of insoluble NFTs inhibits the role of tau protein in maintaining neuronal skeleton and therefore promotes progressive decline in cognitive function and further progression of AD from the early stage (Baas and Qiang, 2019). Meanwhile, tau protein may contribute to Aβ-induced neurotoxicity and might present a higher correlation with AD lesions (Roberson et al., 2007). Therefore, a reduction in the excessive aggregation of tau protein in brain tissue could help inhibit the occurrence and development of AD. Mounting studies have confirmed that neuronal loss occurs in the whole process of AD, and lack of new neurons, overproduction of neurons, and abnormalities in neuronal functioning can cause neuronal damage (Sorrells et al., 2018; Spangenberg et al., 2016; Winkler et al., 2015). The formation of amyloid plaques due to abnormal Aβ deposition and the presence of NFTs owing to abnormal hyperphosphorylation of cytoskeletal tau protein can both damage neurons (Cataldo et al., 2004).

Our study showed that acupuncture-moxibustion intervention could down-regulate APP and p-tau protein levels in the hippocampal DG of SAMP8 mice, and up-regulate the expression of NeuN in the hippocampal DG of SAMP8 mice. The SAMP8 mice receiving acupuncture-moxibustion had a higher number of mature neurons compared with model mice at the same month age, which indicates the intervention could reduce neuronal loss in the hippocampal DG. According to the behavioral performance of SAMP8 mice, we concluded that early

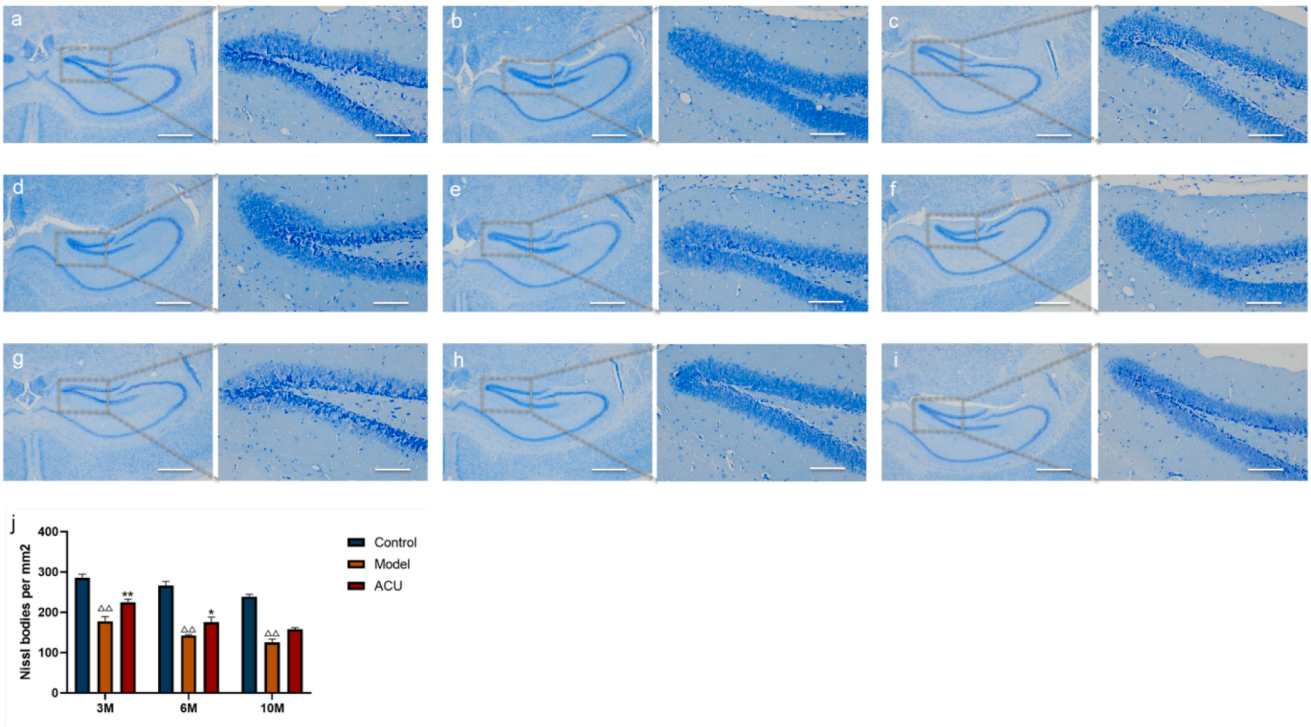

Fig. 5. Acupuncture and moxibustion could increase the number of Nissl bodies in the hippocampal DG of SAMP8 mice. (a–i) The number of Nissl bodies of mice in (a) 3 M−Control group, (b) 3 M−Model group, (c) 3 M−ACU group, (d) 6 M−Control group, (e) 6 M−Model group, (f) 6 M−ACU group, (g) 10 M−Control group, (h) 10 M−Model group, and (i) 10 M−ACU group. (j) The comparisons between Control, Model, and ACU groups in the number of Nissl bodies among 3-month-old mice, 6-month-old mice, and 10-month-old mice. All data were expressed as mean ± standard deviation (n = 6 per group, $^{*}P < 0.05$, $^{\triangle}P < 0.05$, $^{**}P < 0.01$, $^{\triangle\triangle}P < 0.01$). Scale bars (left panel) = 500 μm. Scale bars (right panel) = 100 μm.

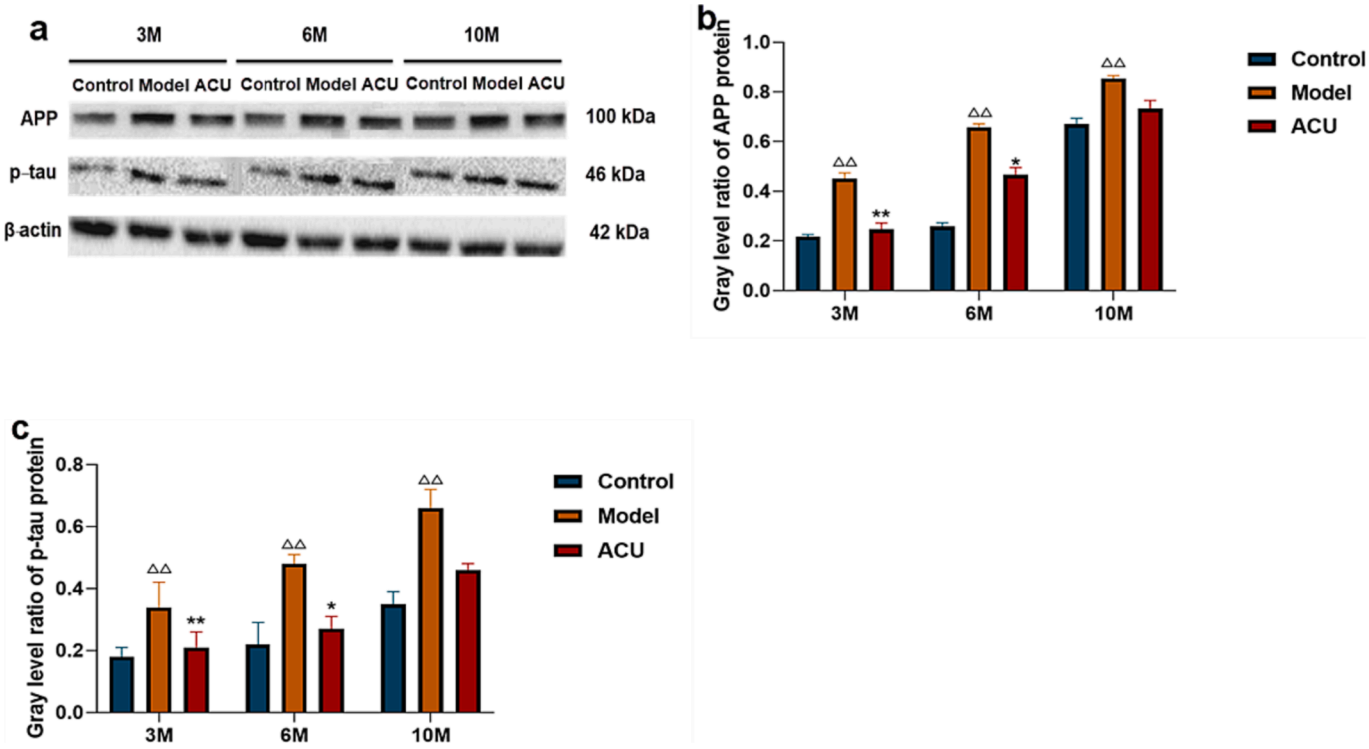

Fig. 6. Acupuncture and moxibustion could delay the AD-related pathological presentation of SAMP8 mice. (a) The levels of APP and p-tau protein of mice in each group. (b) The comparisons between Control, Model, and ACU groups in the number of Nissl bodies among 3-month-old mice, 6-month-old mice, and 10-month-old mice. All data were expressed as mean ± standard deviation (n = 6 per group, $^{*}P < 0.05$, $^{\triangle}P < 0.05$, $^{**}P < 0.01$, $^{\triangle\triangle}P < 0.01$).

intervention with acupuncture-moxibustion could significantly improve the cognitive function of SAMP8 mice. This could be explained by the reduction of APP and p-tau protein levels as well as the neuronal loss and the delay of the occurrence and progression of AD.

NeuN is a mature neuron marker for labeling most post-differentiated neurons (Duan et al., 2016). DCX is an immature neuron marker for labeling neuronal precursor cells and immature newborn neurons (La Rosa et al., 2018). PCNA is an endogenous nuclear protein for labeling mitotic divisions, is closely related to cell DNA synthesis, plays an important role in the initiation of cell proliferation, and is a good indicator of cell proliferation (Juríková et al., 2016). Therefore, we further in this study detected the expression of PCNA/DCX double-labeled cells with the immunofluorescence double-label method. The number of new neurons produced in the hippocampal DG of the SAMP8 mice was observed to analyze the effect of acupuncture-moxibustion on neurogenesis in the hippocampal DG. In the early stage of AD, the hippocampus suffers the most in the characteristic pathological changes including senile plaques, NFTs, or neuronal loss (Furcila et al., 2019). Substantial evidence supports that hippocampal neurogenesis changes in the whole process of AD. Decrease in the numbers of neural stem cells and new neurons in the hippocampal area in mice carrying targeted mutations in presenilin-1 and amyloid precursor protein (both led to Aβ deposition) (Zhang et al., 2007). Overexpression of neurogenesis markers in AD hippocampus was also found through autopsy (Jin et al., 2004). Consistently, in our study, the behavioral and pathological changes of untreated SAMP8 mice at the real-time age of 4 months, 7 months, and 11 months were detected, which correspond to the behavioral and pathological presentation of mild, moderate, and severe AD, respectively. This provides support for the use of SAMP8 mice as AD models (Liu et al., 2020).

Based on the theories of acupuncture and moxibustion and previous relevant research, the administration of “Baihui” (GV20), “Shenshu” (BL23), and “Shenmen” (HT7) acupoints could in a holistic manner boost the functions of “Kidney” and regulate the conditions of the “Governing Vessel” to simultaneously treat the “Heart” and the “Brain” and thus restore the functions of the “Mind”. The SAMP8 mice receiving acupuncture-moxibustion had a higher labeling density of $PCNA^+$ DCX^+ cells in the hippocampal DG than the untreated model mice. Even in the presence of the up-regulatory effect of acupuncture-moxibustion, the older SAMR8 mice had a relatively lower labeling density of $PCNA^+$ DCX^+ cells in the hippocampal DG, namely, the earlier the month age of the SAMR8 mice, the more the double-labeled cells in the hippocampal DG. These results suggest that the administration of acupuncture and moxibustion on GV20, BL23, and HT7 acupoints could improve neuronal regeneration during aging and delay cognitive decline and the development of AD in pathological presentation. Early acupuncture-moxibustion intervention would bring more benefits to AD patients. In this study, we focused on the dysfunction of neurogenesis in the hippocampal DG during the whole pathological process of AD and hoped to provide some new experimental basis for the pathogenesis of AD and the mechanism of acupuncture-moxibustion in the prevention and treatment of AD. Therefore, SAMP8 and SAMR1 mice aged 3, 6, and 10 months were selected respectively to find the optimal intervention time for AD with acupuncture-moxibustion, and meanwhile to determine the dynamic changes of neurogenesis in the mouse hippocampal DG.

5. Conclusion

The pathological changes in SAMP8 mice including Aβ deposition, abnormal level of p-tau protein, neuronal loss, and dysfunction of neurogenesis in the hippocampal DG were aggravated with month age. Acupuncture and moxibustion can effectively delay the pathological process of AD and improve cognitive impairment by restoring the functions of neurogenesis to some extent. This provides support for the promising role of acupuncture-moxibustion in the prevention and treatment of AD.

Authors’ contributions

Xinyuan Liu and Jiangmin Chen drafted the manuscript. Yanjun Du conceived the study design and supervised the paper. Qing Tian gave many important suggestions about experimental design. Xinyuan Liu and Jiangmin Chen conducted the experiments. Li Wang and Xiaoni Deng contributed to the analysis and interpretation of data and graphs. Weixian Li, Guangya Liu, Qian Tan, and Jingzhi Wang assisted during the experimentation and manuscript preparation. All authors have read and approved the final version of the manuscript.

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CRediT authorship contribution statement

Xinyuan Liu: Writing – original draft, Methodology, Investigation, Formal analysis. **Jiangmin Chen:** Data curation. **Yanjun Du:** Writing – review & editing, Writing – original draft, Validation, Supervision, Funding acquisition, Conceptualization. **Qing Tian:** Conceptualization. **Li Wang:** Data curation. **Weixian Li:** Data curation. **Guangya Liu:** Data curation. **Qian Tan:** Formal analysis. **Jingzhi Wang:** Data curation. **Xiaoni Deng:** Data curation.

Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Data availability

Data will be made available on request.

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