Acupuncture improves cognitive deficits and increases neuron density of the hippocampus in middle-aged SAMP8 mice

Guomin Li,1,2 Xuezhu Zhang,2 Haiyan Cheng,2,3 Xuemei Shang,2 Hui Xie,1 Xin Zhang,2 Jianchun Yu,2 Jingxian Han2

Abstract

Objectives To examine whether acupuncture could improve cognitive deficits and reduce the loss of neurons in mice models of ageing.

Methods Male 7.5-month-old senescence-accelerated mouse prone 8 (SAMP8) and age-matched senescence-resistant inbred strains 1 (SAMR1) were divided into four groups (n=15 per group): SAMP8 acupuncture group (Pa), SAMP8 non-acupuncture point control group (Pn), SAMP8 control group (Pc) and SAMR1 normal control group (Rc). The behaviours were examined by the Morris water maze test and the neuron density in the hippocampus was estimated by the optical fractionator technique.

Results The Morris water maze test demonstrated that the cognitive deficits of SAMP8 mice were improved by acupuncture treatment. Neuronal loss was found in hippocampal regions CA1 (~24%), CA3 (~18%) and DG (~28%) of Pc compared with Rc. The neuron number in hippocampal CA3 and DG of the Pa group was significantly increased by therapeutic acupuncture compared with the Pc group.

Conclusions Acupuncture improved the cognitive impairment of middle-aged SAMP8 mice which could be attributed to the reduced neuron loss in hippocampal regions CA3 and DG. These results suggest that reducing neuron loss in the hippocampus by acupuncture is a potential therapeutic approach for the treatment of Alzheimer’s disease and cognitive impairment diseases.

INTRODUCTION

The senescence-prone inbred strains senescence-accelerated mouse prone (SAMP) and senescence-resistant inbred strains (SAMR) are important mouse models of ageing.1–3 SAMP8 is an excellent model of Alzheimer’s disease (AD)4 as it exhibits age-related learning and memory impairment at an early age.5 A variety of neuropathological defects of SAMP8 have been documented, such as the deposition of Aβ, hyperphosphorylation of tau, impaired development of dendritic spines and sponge formation.6–9 Neuron loss in the hippocampus is related to memory impairment,9–12 but little attention has been paid to the neuron density of the hippocampus of SAMP8 mice.

Acupuncture is known to be an effective treatment for many chronic diseases. Clinical research has suggested that acupuncture has therapeutic effects on improving cognitive function and self-managing ability of vascular dementia.13 Meanwhile, experimental studies have shown that acupuncture provides neuroprotection with anti-oxidation and anti-apoptosis effects.14–17 However, it remains unclear whether acupuncture can reduce the loss of neurons.

In this study we aimed to reveal new mechanisms of the neuroprotective effects of acupuncture. SAMP8 and SAMR1 strains were used as the experimental models. We examined neuron density in the hippocampus of SAMP8 and SAMR1 left hemisphere by the optical fractionator technique and investigated whether acupuncture reduces the loss of neurons or increases the number of neurons in these strains compared with untreated control animals.

METHODS

Animals

SAMP8 and SAMR1 breeding pairs were kindly provided by Professor Takeda at Kyoto University, Japan.18 The animals were housed in a barrier facility of the Experimental Animal Centre of First Teaching Hospital of Tianjin University of Traditional Chinese Medicine and bred by brother–sister mating under live conditions of controlled temperature (24±2°C), a 12 h/12 h dark/light cycle, sterile drinking water and standard pellet diet ad libitum. All experiments were performed according to the National Institute of Health Guide for the Care and Use of Laboratory Animals (NIH Publications No. 80–23). Sixty 7.5-month-old SAM male mice were divided into four groups (n=15 per group): SAMR1 normal control group (Rc), SAMP8 control group (Pc), SAMP8 acupuncture group (Pa) and SAMP8 non-acupuncture point control group (Pn).
Acupuncture manipulation

In the SAMP8 acupuncture group (Pa), acupuncture treatment was performed once a day for 15 days (no treatment on the eighth day). The prescription of acupuncture points included CV17 Tanzhong, CV12 Zhongwan, CV6 Qihai, SP10 bilateral Xuehai and ST36 bilateral Zusanli. The locations of these points and acupuncture manipulation have been described previously. The needles were rotated at the rate of twice a second for 30 s at each point. For the non-acupuncture group (Pn), points at the hypochondrium on both sides of the body were needled and stimulated for 105 s at each point to observe the specificity of the acupuncture point effects. The other two groups were controlled with the same method for 210 s. The locations of the points are shown in table 1, which is in accordance with the Atlas of Animal Points from the Institute of Experimental Acupuncture Research of China.

Morris water maze behavioural test

The water maze consisted of a circular tank (90 cm in diameter, 50 cm in height) filled with water to a depth of 29 cm maintained at 24±1°C and rendered opaque with powdered milk (1 kg). A removable circular platform (9.5 cm diameter, 28 cm height) with its top surface 1 cm below the water was located inside the pool. The area of the pool was conceptually divided into four quadrants (NE, NW, SW and SE) of equal size. Data were collected by a video camera (TOTA-450III, Japan) which was fixed to the ceiling of the room and connected to a video recorder and an automated tracking system (China Daheng Group, Beijing, China).

Ten mice were randomly selected from each group and trained for a total of 10 days, including:

(1) A 5-day hidden platform trial: the hidden platform (submerged) was located in the same position (in the middle of quadrant NE) during the procedure. Once the mouse reached the platform the time taken was recorded and it was allowed to remain there for 10 s. If it failed to find the platform in 90 s, then the time was recorded as 90 s. Each mouse was given two trials with an inter-trial interval of 2 h per day.

(2) A 1-day probe trial: the platform was removed from the pool and the time spent swimming in the quadrant where the platform had been was recorded over 60 s.

(3) A 3-day reversal trial: in this phase the hidden platform was located in a different quadrant (in the middle of quadrant SW) in the pool, diagonal to its previous location. Each animal was given two trials per day.

(4) A 1-day visible platform trial: in this phase the platform, which was 2 cm above the surface of the water, was marked by yellow adhesive tape.

All these methods were based on former research in our laboratory. To acclimatise to the test conditions, 24 h prior to the start of training the mice were allowed to swim for 90 s in the tank without the escape platform.

<table>
<thead>
<tr>
<th>Table 1</th>
<th>Acupuncture points, anatomical position and innervation</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Therapeutic method</strong></td>
<td><strong>Points</strong></td>
</tr>
<tr>
<td>Acupuncture points</td>
<td>CV17</td>
</tr>
<tr>
<td></td>
<td>CV12</td>
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<tr>
<td></td>
<td>CV6</td>
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<tr>
<td></td>
<td>SP10</td>
</tr>
<tr>
<td></td>
<td>ST36</td>
</tr>
<tr>
<td>Non-acupuncture points</td>
<td>On the hypochondrium, 3 mm above the iliac crest</td>
</tr>
</tbody>
</table>

Tissue preparation and histochemical staining

The mice were anaesthetised with 10% chloral hydrate and perfused with 0.9% NaCl via the left cardiac ventricle. The left brain halves of the transcardially perfused mice were post-fixed in ice cold 4% (w/v) paraformaldehyde (PFA) fixative in 0.1 M phosphate buffer sodium (PBS, pH=7.4) at 4°C and then cryoprotected by immersing in 30% sucrose solution in phosphate buffer at 4°C overnight. Post-fixed hemi-brains were cut into series of 40 µm thick frontal sections under a freezing microtome (Leica CM1900, Germany). All serial sections were kept in consecutive order in a 96-well tissue culture tray with 400 µl 4% PFA fixative. One series of every fourth section per animal was stained with 0.05% toluidine blue solution in PBS, then mounted onto glass slides and immediately cover-slipped with water-soluble mounting media (AR1018, Boster, China) for cell counting.

Stereological evaluation

The total number of neurons in the hippocampal subregions was estimated using the optical fractionator method which combines an efficient and unbiased stereological counting method (optical dissector) with an...
unbiased sampling scheme. Cell counting was performed on a stereological workstation consisting of a modified light microscope (Nikon Eclipse 80i, Japan), a motorised specimen stage for automatic sampling (MicroBrightField, Williston, Vermont, USA), a CCD colour video camera (Qimaging Fast1394, Canada) and stereology software (MicroBrightField). The total number of neurons in different subregions of the hippocampus (N) was estimated using the following formula:

\[ N = \sum Q \times \frac{1}{ssf} \times \frac{1}{asf} \times \frac{1}{tsf} \]

where \( \Sigma Q \) represents the total dissector number of cells counted in all optically sampled fields of the hippocampus, \( ssf \) is the section sampling fraction, \( asf \) is the area sampling fraction and \( tsf \) is the thickness of the sampling fraction. Details of the counting scheme are summarised in table 2.

The efficiency of sampling for estimating the total number of neurons was examined by estimation of the coefficient of error (CE).23 24 All the parameters for mice hippocampuses were determined by a pilot study so that the CE for individual estimates was less than 10%.

**Definitions and divisions of the hippocampus**

The definitions of the hippocampus are based on the descriptions of Paxinos and Franklin.25 The granular cells of dentate gyrus (DG) are composed of the smallest and densely packed cells. The pyramidal cells of cornu ammonis 3 (CA3) form loosely packed cells, and their cell bodies and nuclei are larger than those of CA1. The neuronal cells of CA1 are tightly packed next to CA2 and towards the subiculum. We included CA2 in the CA3 region because of the difficulty in distinguishing its borders from CA3. All delineations were made using a 4× objective lens (NA=0.2, Nikon, Japan) and the subsequent counting was performed using a 40× objective lens (NA=0.95, Nikon, Japan).

**Statistical analysis**

All data were expressed as mean±SD for each group. For the Morris water maze test the escape latency time of the hidden platform trial and reversal trial were analysed with two-way ANOVA of repeated measures, while one-way ANOVA was conducted on the data obtained from the probe trial, the visible platform trial and the number of neurons in the hippocampal subregions. Mean values were considered to be significantly different when \( p<0.05 \). All statistical analysis was performed using the SPSS software V13.0.

**RESULTS**

**Behavioural tests**

During training in the hidden platform trial (figure 1A), two-way ANOVA showed a significant effect of training on escape latency time measure (F(3, 33)=4.81, \( p<0.01 \)) and a significant interaction between groups and training days (F(12, 132)=2.07, \( p<0.05 \)). The mice in the acupuncture treatment group (Pa) had a significantly shorter latency time to find the hidden platform on the training day than Pc mice (\( p<0.05 \)) and Pn mice (\( p<0.05 \)) but not than Rc mice.

In the probe trial (figure 1B), a significant effect of training on retention time was observed by one-way ANOVA (F(3, 33)=9.14, \( p<0.01 \)). Pa mice spent longer residence time than Pc (\( p<0.05 \)) and Pn mice (\( p<0.05 \)) but not than Rc mice.

During the reversal trial, two-way ANOVA showed a significant effect of training on escape latency time (F(3, 33)=4.18, \( p<0.05 \)) but no effect on the interaction between groups and training days (F(6, 66)=0.415, \( p>0.05 \)). However, Pa mice exhibited shorter latency time than Pc (\( p<0.05 \)) and Pn mice (\( p<0.05 \)) but not than Rc mice.

In the visible platform trial no significant difference in performance was observed among the groups by two-way ANOVA (F(3, 34)=0.53, \( p>0.05 \)), indicating that the deficits of SAMP8 in the hidden platform test were not the result of abnormalities in sensory processes, motivation or coordination.

**Stereology evaluation**

Neuron density was quantitatively assessed by high-precision design-based stereology (figure 2A,B). One-way ANOVA showed partial loss of neurons in hippocampal regions CA1, CA3 and DG of Pc mice compared with Rc mice (−24%, −18% and −28%, respectively; \( p<0.01 \)). The number of neurons was increased by acupuncture in hippocampal regions CA3 (+19%, \( p<0.01 \)) and DG (+19%, \( p<0.05 \)) of Pa mice compared with Pc mice. A significant decrease in neurons was observed in CA1 (\( p<0.01 \)) and DG (\( p<0.05 \)) of Pa mice compared with Rc mice. In contrast, more neurons were detected in CA3 of Pa mice than Pn mice, but not in CA1 and DG. Moreover, a significant loss of neurons was seen in CA1, CA3 and DG of Pn mice compared with Rc mice (\( p<0.01 \), \( p<0.05 \), \( p<0.01 \), respectively).

The CE for measurements in an individual subject provided a measurement of the precision of the estimates. The CEs and the coefficient of variation (CV) for each estimate are shown in table 3. The ratio of \( CE^2/CV^2 \) was within an acceptable range (\( <0.50 \)).
DISCUSSION

This study employed, for the first time, the optical fractionator method to estimate the number of neurons in SAMP8 and SAMR1 hippocampus and found a significant loss of neurons in hippocampal CA1, CA3 and DG in middle-aged SAMP8 mice compared with SAMR1 mice. Neuron density in CA3 and DG of SAMP8 mice was increased after acupuncture treatment. In addition, behavioural tests on these mice showed that acupuncture could improve cognitive deficits in SAMP8 mice, consistent with a previous report that acupuncture has a therapeutic effect in middle-aged SAMP8 and younger mice.15

Stereological study of post-mortem tissue has shown that the most distinctive lesion of AD is the loss of neurons in the CA1 region.26 27 Our study showed that there was a significant loss of neurons in hippocampal CA1 of SAMP8 mice. These data suggest that SAMP8 manifests a pattern of neuron loss in the hippocampus similar to that in patients with AD. It has been proposed that the loss of neurons is induced by apoptosis due to Aβ toxicity. Aβ is known to induce cytotoxic effects including oxidative stress, changes in the activities of various kinases and hyperphosphorylation of tau, which could contribute to neuron loss.28–31

Interestingly, we found neuron loss in hippocampal CA3 and DG which addressed one of the paradoxes of the neuropathology of AD. As described above, neuron loss was only found in CA1 of the hippocampus and not in other hippocampal regions in patients with AD.26 27 32 However, in AD transgenic mice no or mild neuron loss was observed in the hippocampus of single transgenic mice. For example, no significant neuron loss was found in hippocampal CA1 in Tg2576 mice33 or PDAPP mice34 except in CA3.35 Mild neuron loss in CA1 has been reported in APP23 mice.36 By contrast, neuron loss in hippocampal CA1 was found in the majority of double transgenic lines such as Tg2576 ×PS1-M146L37 and Swedish and London mutations ×knock in PS1 (M146L).38 In addition, loss of neurons in the hippocampal pyramidal cell layer was shown in Swedish and London mutations ×PS1 (M146L).39 To our knowledge, no neuron loss has been found in DG in transgenic mice. Strain differences may explain the discrepancy in neuron loss in different regions of the hippocampus. The CA3

Figure 1  (A) Performance in training trials of the four groups of mice. Days 1–5: hidden platform trial; days 7–9: reversal trial; days 10: visible platform trial. The graph shows average latency time to find the platform (n=10). (B) Performance in probe trial of the four groups of mice showing retention time spent in the quadrant with the platform removed on the sixth day. Rc group: senescence-resistant inbred strain 1 (SAMR1) normal control group; Pc group: senescence-accelerated mouse prone 8 (SAMP8) control group; Pa group: SAMP8 acupuncture group; Pn group: SAMP8 non-acupuncture point control group. **p<0.01, Pc vs Rc groups; *p<0.05, Pa vs Pc groups; #p<0.05 Pa vs Pn groups.
region may support multiple mnemonic processes which are critical to the formation and subsequent retrieval of spatial memory. The neurons (mainly pyramidal cells) of CA3 receive perforant path inputs from the medial and lateral entorhinal cortex, and mossy fibre inputs from the dentate gyrus. Cognitive impairment may be a result of the neuron loss in CA3, leading to decreased inputs from these regions. DG is one of the few brain regions where neurogenesis continues into adulthood. Increasing evidence suggests that these newly generated neurons correlate with certain forms of brain function involving hippocampus-dependent learning and memory. In addition, computational hypotheses indicate that neuronal turnover is associated with neurogenesis in the DG, which increases memory capacity. Consequently, a reduction in hippocampal neurogenesis is likely to account for the loss of neurons in the DG.

Based on our previous finding that acupuncture regulates brain cell proliferation of SAMP8 mice, we hypothesised that acupuncture increases the number of neurons in the hippocampus. Our results support this hypothesis, as shown in Figure 2.

Figure 2  (A) Images of toluidine blue staining in the same region of the hippocampus among the four groups. Images C–F represent the Rc group, G–J represent the Pc group, K–N represent the Pa group, O–R represent the Pn group. Original magnifications: C, G, K, O 40×; D–F, H–J, L–N, P–R 400×. (B) Neuron density in the four groups. The graph shows the number of neurons in the same region of the hippocampus in the four groups (n=5). Data are expressed as mean±SD. *p<0.05, **p<0.01 vs Rc group; Δp<0.05, ΔΔp<0.01 vs Pc group. Rc group: senescence-resistant inbred strains 1 (SAMR1) normal control group; Pc group: senescence-accelerated mouse prone 8 (SAMP8) control group; Pa group: SAMP8 acupuncture group; Pn group: SAMP8 non-acupuncture point control group.
neurons in the hippocampus. Our study demonstrates that acupuncture increases the number of neurons in hippocampal CA3 and DG in SAMP8 mice. Unexpectedly, in Pa there was an increase in the number of neurons in CA3 by acupuncture, approaching that in SAMR1 mice, which indicates that acupuncture has a more prominent effect on the number of neurons in hippocampal CA3. Meanwhile, cognitive deficits in SAMP8 mice were improved after acupuncture, suggesting that memory and learning disorders are ameliorated by increasing the number of neurons in hippocampal CA3 and DG.

What might account for the increased number of neurons in SAMP8 by acupuncture? A previous study using 5-bromo-2’-deoxyuridine (BrdU)-specific immuno-detection indicated that the newly generated neurons were enhanced by therapeutic acupuncture in SAMP8 mice. The change in mRNA level of Mdm-2, a kind of apoptosis depressed gene, was inhibited by acupuncture in SAMP10 mice in which neurons are lost with ageing. Furthermore, the expression of apoptosis-related genes Bcl-2 and Bax were regulated by acupuncture in the hippocampus of cerebral multi-infarction rats. These results suggest that acupuncture could decrease the loss of neurons in the hippocampus by inhibiting apoptosis. Acupuncture therapy has also been implicated in increasing various neuroprotective agents (such as brain-derived neurotrophic factor, glial cell line-derived neurotrophic factor and cyclophilin A) and attenuating oxidative stress in a neurodegenerative disease animal model.

Based on the above research, we hypothesise that the mechanism of acupuncture in increasing the number of neurons might be regulated by the interaction of three factors, as follows: (1) more neurons are generated during acupuncture; (2) cell death is decreased; or (3) the expression of various neuroprotective agents is increased.

In conclusion, our study provides statistical evidence for neuron loss in hippocampal CA1, CA3 and DG in SAMP8 mice. Acupuncture improves the cognitive impairment of middle-aged SAMP8 mice and reduces neuron loss in CA3 and DG. These results suggest that acupuncture may be a potential therapeutic approach for the treatment of AD and cognitive impairment diseases by reducing neuron loss in the hippocampus.

**Summary points**

- SAMP8 is a genetic strain of mouse that models Alzheimer’s disease.
- Daily manual acupuncture slowed the rate of cognitive decline and neuron loss.

**REFERENCES**


**Table 3**  Mean total number of neurons in mice hippocampal regions (×10⁶). CE and CV of stereological analysis

<table>
<thead>
<tr>
<th>Regions of hippocampus (n=5)</th>
<th>CA1</th>
<th>CA3</th>
<th>DG</th>
</tr>
</thead>
<tbody>
<tr>
<td>Rc</td>
<td>2.519 (0.038)</td>
<td>1.781 (0.030)</td>
<td>4.276 (0.042)</td>
</tr>
<tr>
<td>CV</td>
<td>0.085</td>
<td>0.067</td>
<td>0.093</td>
</tr>
<tr>
<td>Pc</td>
<td>1.918 (0.039)</td>
<td>1.452 (0.052)</td>
<td>3.083 (0.068)</td>
</tr>
<tr>
<td>CV</td>
<td>0.087</td>
<td>0.117</td>
<td>0.153</td>
</tr>
<tr>
<td>Pa</td>
<td>2.147 (0.022)</td>
<td>1.732 (0.030)</td>
<td>3.658 (0.066)</td>
</tr>
<tr>
<td>CV</td>
<td>0.049</td>
<td>0.068</td>
<td>0.147</td>
</tr>
<tr>
<td>Pn</td>
<td>1.991 (0.059)</td>
<td>1.542 (0.037)</td>
<td>3.383 (0.022)</td>
</tr>
<tr>
<td>CV</td>
<td>0.131</td>
<td>0.083</td>
<td>0.050</td>
</tr>
</tbody>
</table>

(CE) is the estimated intra-animal coefficient of error (SEM/mean). CV is the observed inter-animal cmean. The mean CE of an estimate is calculated as mean/CE. The mean CE of an estimate calculated as (mean/CE)^2.

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