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Acupuncture Stimulation to HT8 Enhances Cell Proliferation in Hippocampus on an Epilepsy Mouse Model

Seung-Tae Kim[1] · Hae-Jeong Park[2] · Mee-Sook Hong[2] · Seung-Nam Kim[3,4]
Ah-Reum Doo[3,4] · Chang-Shik Yin[3] · Hye-Jung Lee[3,4]
Joo-Ho Chung[2] · Hi-Joon Park[3,4]

[1]Division of meridian and structural medicine, School of Korean medicine, Pusan National University
[2]Kohwang Medical Research Institute, Kyung Hee University
[3]Acupuncture & Meridian Science Research Center, Kyung Hee University
[4]Dept. of meridian & acupoint, College of Korean Medicine, Kyung Hee University

마우스 간질 동물모델에서 소부혈 자침이 해마 치상회의 신경세포증식에 미치는 영향

김승태[1], 박해정[2], 홍미숙[2], 김승남[3,4], 두아름[3,4], 인창식[3], 이혜정[3,4], 정주호[2], 박히준[3,4]

[1]부산대학교 한의학전문대학원 경락구조의학부, [2]경희대학교 고황의학연구소
[3]경희대학교 침구경락과학연구센터, [4]경희대학교 한의과대학 경혈학교실

Abstract

목적 : 뇌의 신경세포 증식은 해마 치상회와 뇌실하영역에서만 나타나는 현상이다. Kainic acid(KA)를 이용한 간질 동물모델을 연구하던 중 침이 해마 치상회의 신경세포증식을 촉진하는 현상을 발견하여 이를 보고하고자 한다.

방법 : 수컷 ICR계 생쥐를 Saline(n=8), KA(n=8), KA+Acu(n=8)의 세 군으로 나누고, 모든 생쥐들에게 KA 주입 3일 전부터 1일 1회씩 5'-bromodeoxyuridine(BrdU)을 3일간 주입하였다. Saline군에는 멸균된 생리식염수를 뇌실 내에 주입하였고, KA군 및 KA+Acu군에는 0.1μg의 KA를 뇌실 내에 주입하였으며, KA+Acu군에 속한 쥐들에게는 KA 주입 2일전, 1일전, 주입 직후에 양쪽 少府(HT8)에 자침하였다. KA 주입 3시간 후 쥐의 뇌를 적출하고 해마 치상회부위의 BrdU 및 neuropeptide Y (NPY)의 발현을 측정하였다.

결과 : 少府 자침이 KA의 독성으로 인한 신경세포의 파괴를 줄여주었으며, BrdU 양성 세포 및 NPY를 유의하게 증가시켰다. KA 주입시 세포증식이 일어나긴 하나, 3시간 안에는 거의 일어나지 않는다.

결론 : 少府 자침이 해마 치상회의 신경세포증식을 촉진하며, 이는 KA의 효과가 아닌 KA 투여 전 少府 자침으로 인한 것으로 사료된다.

Key words : acupuncture, bromodeoxyuridine, cell proliferation, neuropeptide Y, hippocampus, dentate gyrus

Ⅰ. Introduction

Acupuncture, a technique of needling into specific locations in the body, has been used for therapeutic purposes including pain, neurological disorders and gastroenteric

· 교신저자 : 박히준, 서울시 동대문구 회기동 1번지
경희대학교 한의과대학 경혈학교실,
Tel. 02-959-9435, Fax. 02-966-4237,
E-mail: acufind@khu.ac.kr
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disorders in East Asia over 2000 years. Over the past two decades, the acupuncture therapy has also been used in Europe and the United States to help various types of patients[1].

Neurogenesis, the process of new neuronal birth, consists of cell proliferation, survival, migration and neuronal differentiation. In adult brain, it occurs in only subventricular zone and dentate gyrus (DG) of hippocampus[2]. DG is known to have a crucial role in learning, memory, stress and some neurological disorders[3]. Various physiological, pathological and pharmacological methods have been studied to enhance the adult neurogenesis in the DG[4].

Acupoints in the Heart Meridian (HT) have been used to treat psychopathic or neurological disorders such as epilepsy. Among them, HT8 (Sobu) is traditionally known as a representative acupoint for balancing homeostasis by regulating the excitatory or inhibitory functions in the body.

We previously reported that acupuncture to the acupoint HT8 has neuroprotective effects against kainic acid (KA)-induced cell death and seizure behavior by decreasing the KA-induced FBJ osteosarcoma oncogene (Fos) and Jun expressions and enhancing glutamate decarboxylase 67 in the hippocampus[5,6]. In the present study, we examined whether acupuncture stimulation may enhance the cell proliferation in mouse hippocampus.

II. Materials and Methods

1. Animals and Grouping

This study was approved by the ethics committee of Acupuncture and Meridian Science Research Center and all efforts were taken to minimize the number of animals and their suffering in accordance with current guidelines for animal research, the NIH Guide for the Care and Use of Laboratory Animals (NIH Publication No. 85-23, 1985). Male ICR mice (20-25 g, Orientbio Inc., Gyeonggi-do, Korea) were housed at room temperature (22 ± 3℃) under a standard 12 hour light/dark cycle (lights on at 07:00 a.m.) and were given unlimited access to food and water. After 1-week-adaptation, the mice were randomly assigned to one of three groups (n=8 at each group); saline, KA or KA+Acu. Mice in the saline group would be injected with normal saline and not receive acupuncture stimulation, Those in the KA group (n=8), which would be injected with KA and not receive acupuncture stimulation, and those in KA+Acu group would be injected with KA and receive acupuncture stimulation bilaterally to acupuncture point HT8.

2. Bromodeoxyuridine Injection

Three days before KA injection, we began to give 5-bromo-2′-deoxyuridine (BrdU; 50 mg/kg in normal saline; Sigma, MO, USA) to all mice by intraperitoneally injection. The mice underwent the injection once a day (total 3 times).

3. Acupuncture Stimulation

From 10:00 to 10:30 a.m., the mice in the KA+Acu group were lightly immobilized, and acupuncture needles (0.18 × 8 mm, Dongbang Acupuncture Inc., Gyeonggi-do, Korea) were inserted to the bilateral acupuncture point HT8. HT8 have been used to treat not only balancing homeostasis by regulating the excitatory and inhibitory functions in the body but also psychopathic and neurological disorders such as epilepsy. The point was located on the palmar surface of the forelimbs, between the fourth and fifth metacarpal bones[7], and the point in mice corresponded anatomically to the point in humans. The depth of needle insertion was 1 mm. The needles were turned at a rate of two spins per second for 15 seconds and removed immediately afterward. The entire stimulation lasted for 30 seconds, and repeated 3 times (2 days before, 1 days before and immediately after the KA injection). The mice of the saline and the KA groups were also lightly immobilized as those in the KA+Acu group for 30 seconds, but they didn't have the acupuncture stimulation.

4. Kainic Acid Injection

KA (Sigma, MO, USA) was injected intracerebroventricularly at bregma with a 50 μl Hamilton microsyringe fitted with a 26-gauge needle, which was inserted to a depth of 2.4 mm according to Laursen and Belknap's method[8]. The injection volume was 5μl (0.02 μg/μl) in the KA and KA + Acu groups. The mice in the saline group underwent the same procedure, except that normal saline was injected intracerebroventricularly instead of KA.

5. Immunohistochemistry

Three hours after KA injection, the animals were perfused with 4% paraformaldehyde dissolved in 0.1 M phosphate buffer. The brains were sectioned coronally (40 μm) on a freezing microtome. Hippocampal sections (1.82 to 2.06 mm from the bregma) from each animal were stained. The sections were incubated with antibody against neuropeptide Y (NPY) (1:2000, Zymed Laboratories Inc., CA, USA) staining and then with biotinylated secondary antibody (Vector Laboratories, CA, USA). After incubation with a Vector Elite ABC Kit (Vector Laboratories, CA, USA), the antibody biotinavidinperoxidase complex was visualized with diaminobenzidine (DAB). After the DAB reaction, the tissues were rinsed with PBS, mounted on gelatin

coated slides, air-dried, dehydrated and coverslipped. For BrdU staining, sections incubated in 0.5% Triton X-100 at room temperature, pre-treated in 50% formamide 2 × standard saline citrate at 65℃ for 2 hours, denatured in 2 N HCl for 30 minutes at 37℃, and washed. Next procedures were same as above-mentioned immunostaining, but mouse antiBrdU antibody (Roche, Germany) was used as primary antibody. The histological pictures were taken using a bright field BX51 microscope (Olympus, Japan) and DP70 camera (Olympus, Japan). The number of BrdU or NPY positive cells in the DG was manually counted from each section exhaustively at 200 × magnification, and all counts were done in a blinded fashion. For Nissl staining, the DG tissues were mounted on gelatin-coated slides, dried for 1 hour at room temperature, and stained with 0.5% cresyl violet.

Statistical Analysis

All the data were expressed as the mean ± SEM, and the data were analyzed by one-way analysis of variance with the Newman-Keuls post hoc multiple comparison test. In all the analyses, differences were considered statistically significant at $p<0.05$.

III. Results

1. Acupuncture Prevents KA-induced Cell Death in the Dentate Gyrus

To observe the KA-induced cell death in the DG, we stained hippocampal sections with cresyl violet. The KA + Acu group showed a substantial protective effect against the KA-induced cell death in the DG compared to the KA group(Fig. 1).

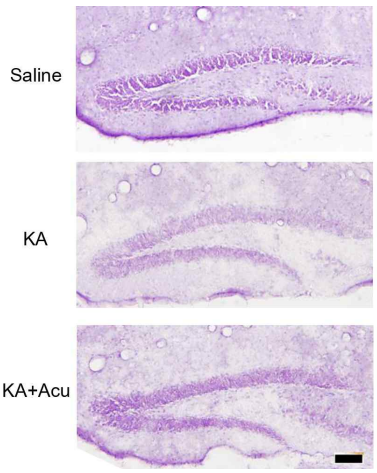

Fig. 1. Nissl staining in the dentate gyrus (DG) of hippocampus.
Saline, saline-injected group; KA, kainic acid (KA)-injected group; KA+Acu; KA-injected and acupuncture stimulated group. Scale bar represents 100 μm.

2. The Increase of 5′-bromo-2′deoxyuridine -positive Cells in the Dentate Gyrus after Acupuncture Stimulation

The number of BrdU-positive cells of DG in the KA + Acu group (19.4 ± 1.6) was significantly increased comparing to both the saline group (10.7 ± 1.5, $p<0.05$) and the KA group (13.2 ± 1.7, $p<0.05$). However, the number of BrdU-positive cells in the KA group was not significantly different

compared to the Saline group(Fig. 2).

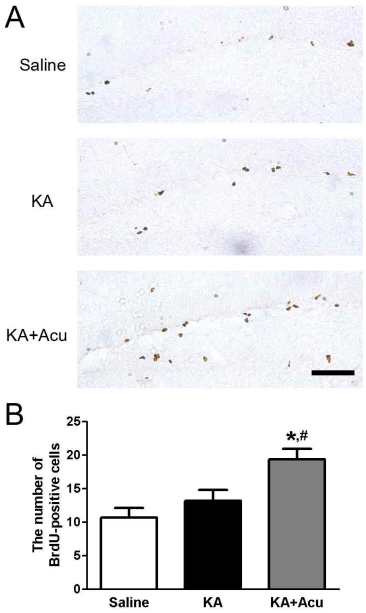

Fig. 2. Photographs (A) and changes (B) of 5'-bromo -2'deoxyuridine (BrdU)-positive cells in the dentate gyrus (DG) of hippocampus.
Saline, saline-injected group; KA, kainic acid (KA)-injected group; KA+Acu; KA-injected and acupuncture stimulated group. The number of BrdU-positive cells in the DG. Data in the graph are presented as means ± SEM. * $p<0.05$, compared to the KA group; # $p<0.05$ compared to the Saline group. Scale bar represents 100 μm.

3. The Increase of NPY-positive Cells in the Dentate Gyrus after Acupuncture Stimulation

Acupuncture stimulation to HT8 induced significantly to be increase the number of NPY-positive cells (55.4 ± 2.5) in the DG compared to the KA group (46.3 ± 2.3, $p<0.01$). However, there was no significant difference on the number of NPY-positive cells between the Saline (43.3 ± 1.9) and KA group(Fig. 3).

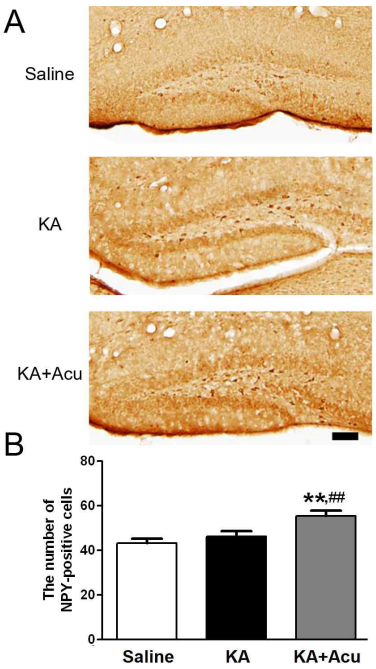

Fig. 3. Photographs (A) and changes (B) of neuropeptide Y (NPY)-positive cells in the dentate gyrus (DG) of hippocampus.
Saline, saline-injected group; KA, kainic acid (KA)-injected group; KA+Acu; KA-injected and acupuncture stimulated group. The number of NPY-positive cells in the DG. Data in the graph are presented as means ± SEM. ** $p<0.01$ compared to the KA group; ## $p<0.01$ compared to the Saline group. Scale bar represents 100 μm.

IV. Discussion

This study demonstrates that acupuncture stimulation to HT8 significantly increases BrdU and NPY expressions in the DG of KA-injected mouse hippocampus.

It has been known that cell proliferation in the DG was increased after KA injection[9,10]. However, since the duration of the cell

cycle of dividing cells in the DG of mice has been estimated to be around 20 hours[11], it is unlikely that the proliferation was increased within 3 hours after KA injection. Indeed, Ledergerber et al. showed that there were no differences between vehicle and KA injection in the hippocampus after 24 hours[12]. Consistently, in our result, the number of BrdU-positive cells in the DG of KA-injected mice without acupuncture stimulation was not increased. Therefore, the result indicated that KA didn't have influence on the increase of cell proliferation in the present study. Meanwhile, since acupuncture stimulations were performed 51 hours and 27 hours before the sample preparation for immunohistochemical staining, it is likely that the increase in the number of BrdU-positive cells in the DG of KA-injected mice may be associated with acupuncture stimulation to HT8.

There were several reports on the correlation between acupuncture and cell proliferation. Although some reports proposed that acupuncture decreased the cell proliferation in stroke rat[3] or gerbi[13] model, mounting evidence suggested that acupuncture stimulation can enhance cell proliferation in the DG of cognitive deficient mice[14], chronic unpnhaictable stressha[15], stereptozocin-induced diabetic[16] or maternally separated rats[17]. Moreover, it has been shown that ischemic gerbils with acupuncture stimulation had more BrdU-positive cells than sham-operated gerbils in the DG[18]. Altogether, these results may support that the increase in the number of BrdU-positive cells in the DG of KA-injected mice in this study could be due to acupuncture stimulation to HT8.

NPY, a widely expressed peptide in the central and peripheral nervous system, is involved in various brain diseases including seizure, depression, anxiety and drug addiction[19], and it is also important to promote cell proliferation in the DG of hippocampus[20,21]. In addition, it was reported that the substance was increased by acupuncture stimulation for a few days in the rat hippocampus against the NPY reduction by maternal separation[22] or streptozocin-injection[16]. Therefore, the increased NPY-positive cells in the DG by acupuncture stimulation to HT8 may possibly mediate the cell proliferation under the KA-injected condition. However, since the present study only demonstrates that acupuncture stimulation to HT8 increased the number of BrdU-positive cells and NPY-positive cells significantly under the KA-injected condition, it remains to be tested whether acupuncture stimulation to HT8 could exert similar effects on cell proliferation and NPY expression under normal conditions.

In conclusion, we show that acupuncture stimulation to HT8 increased the population of both BrdU-positive cells and NPY-positive cells in the DG of KA-injected mice. These

results may suggest that acupuncture stimulation to HT8 can enhance the cell proliferation, probably through upregulating NPY expression in the DG of KA-injected mice.

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