Comparing the Effects of Acupuncture and Electroacupuncture at Zusanli and Baihui on Cell Proliferation and Neuroblast Differentiation in the Rat Hippocampus

In Koo HWANG1), Jin Young CHUNG2), Dae Young YOO1), Sun Shin YI1,3), Hwa Young YOUN2), Je Kyung SEONG1) and Yeong Sung YOON1)*

1) Department of Anatomy and Cell Biology, College of Veterinary Medicine and BK21 Program for Veterinary Science, 2) Department of Veterinary Internal Medicine, College of Veterinary Medicine, Seoul National University, Seoul 151–742, South Korea and 3) Department of Biomedical Sciences, College of Health Sciences, Marquette University, Milwaukee, WI 53233, U.S.A.

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ABSTRACT. We compared the effects of acupuncture and electroacupuncture on cell proliferation and neuroblast differentiation using specific markers, Ki67 and doublecortin (DCX), in the subgranular zone of the dentate gyrus (SZDG) in 13-week old Wistar rats. Acupuncture and electroacupuncture were applied simultaneously in the acu-points, ST36 (Zusanli) and GV20 (Baihui), once a day for 3 weeks. Acupuncture and electroacupuncture at these acu-points significantly increased the number of Ki67-positive cells and DCX-immunoreactive neuroblasts compared to the control or sham acupuncture group. Electroacupuncture treatment significantly increased the number of well-developed (tertiary) dendrites in the SZDG compared to acupuncture treatment. These results suggest that both acupuncture and electroacupuncture increase neurogenesis in the normal, but that electroacupuncture has greater effects on neuroblast plasticity than acupuncture in the dentate gyrus.

KEY WORDS: acupuncture, cell proliferation, electroacupuncture, neuroblast differentiation, Wistar rat.

ACU and EA enhance neurogenesis in several disease models. For example, decreased cell proliferation in the dentate gyrus (DG) of SAMP8 was improved by Yiqitiao xue and Fubenpei yuan acupuncture [1]. In addition, EA at GV20 and An-Mian (EX17) increased hippocampal progenitor cell proliferation in adult rats exposed to chronic unpredictable stress [20]. In ischemic models [17] and streptozotocin-induced diabetic models [16], acupuncture at ST36 increased cell proliferation in the DG. In a spontaneous recurrent seizure model, EA at ST36 significantly reduced the number of spontaneous recurrent seizures and significantly elevated the expression of GAD67 mRNA in the DG [9]. ACU at Tanzhong (CV17), Zhongwan (CV12), Qihai (CV6), ST36, and Xuehai (SP10) improve spatial memory impairment using the Morris water maze [26], maintain oxidant-antioxidant balance, and regulate cell proliferation in a rodent dementia model [1, 19].

Multi-potent neural stem cells/progenitors exist in some brain regions such as the subventricular zone of the lateral ventricle and the subgranular zone of the DG (SZDG) [24]. In particular, the SZDG of the adult hippocampus contains undifferentiated and rapidly proliferating progenitor cells that differentiate into granule cells in the DG throughout life as they fully integrate into the hippocampal network [25]. Damage to the granule cells produces cognitive impairment [22] as the hippocampus regulates learning and memory.

There are several markers to detect cell proliferation, migration and differentiation in the SZDG. Doublecortin (DCX) is generally used as a marker for neuronal precursors and neurogenesis [7, 8, 15] because DCX is specifically expressed in neuronal precursors in the developing and adult CNS [14]. Ki67 antigen is a prototypic cell cycle related nuclear protein which is expressed in proliferating cells in all phases of the active cell cycle (G1, S, G2 and M phase). In this aspect, Ki67 is routinely used as a marker of cell cycling and differentiation [4]. In the present study, therefore, we investigated the effects of ACU and EA on cell proliferation and neuroblast differentiation using Ki67 and DCX, respectively in the SZDG in healthy rats.

MATERIALS AND METHODS

Experimental animals: Male Wistar rats were purchased from Orient Bio Inc. (Seongnam, South Korea) and mated each other. They were housed in a conventional state under adequate temperature (23°C) and humidity (60%) control with a 12-hr light/12-hr dark cycle, and free access to food.
and water. The procedures for handling and caring for the animals adhered to the guidelines that are in compliance with the current international laws and policies (NIH Guide for the Care and Use of Laboratory Animals, NIH Publication No. 85–23, 1985, revised 1996). All of the experiments were conducted to minimize the number of animals used and the suffering caused by the procedures used in the present study.

**Application of acupuncture and electroacupuncture:** Male Wistar rats were randomly divided into 4 groups: control (n=7), sham-acupuncture (sham-ACU, at bilateral nearby nonacupoints in the hamstring muscles) (n=12), acupuncture (ACU, n=12) and electroacupuncture (EA, n=12) groups. At 13 weeks of age, the animals were anesthetized with chloral hydrate (30 mg/kg) and acupuncture and electroacupuncture was administered one a day for 3 weeks. The prescription of acupuncture includes ST36 (Zusanli, 5 mm below head of fibula under knee joint, and 2 mm lateral to the anterior tubercle of the tibia) and GV20 (Baihui, located at the midmost point of parietal bone) at a depth of 5 mm into the skin with stainless needle measuring 0.25 × 20 mm of length with guide-tube for 20 min (Wujiang Shenli Medical & Health Material Co., Ltd., Wujiang, China). Electric stimulation was generated by an EA apparatus (Model G-6805) for 20 min and the stimulation parameter were disperse-dense waves of 5/20 Hz (28.5 ms/15 ms pulse duration) of frequency and current density of 2–4 mA. Controls were not experienced with any ACU.

**Tissue processing for histology:** For immunohistochemical analysis, animals in each group (n=7) were anesthetized with chloral hydrate at 16 weeks of ages and perfused transcardially with 0.1 M phosphate-buffered saline (PBS, pH 7.4) followed by 4% paraformaldehyde in 0.1 M phosphate-buffer (PB, pH 7.4). The brains were removed and post-fixed in the same fixative for 12 hr. The brain tissues were cryoprotected by infiltration with 30% sucrose overnight. Thereafter the frozen tissues were serially sectioned on a cryostat (Leica, Wetzlar, Germany) into 30-μm coronal sections, and they were then collected into six-well plates containing PBS.

**Immunohistochemistry for Ki67 and DCX:** For immunohistochemistry, the free-floating sections were processed under the same conditions as previous mentioned method [12]. The sections were sequentially treated with 0.3% hydrogen peroxide (H₂O₂) in PBS and 10% normal horse serum in 0.05 M PBS. They were next incubated with diluted goat anti-DCX antibody (1:50, SantaCruz Biotechnology, Santa Cruz, CA, U.S.A.) and rabbit anti-Ki67 antibody (1:1,000, Abcam, Cambridge, UK) overnight and subsequently exposed to biotinylated rabbit anti-goat or goat anti-rabbit IgG (diluted 1:200, Vector, Burlingame, CA, U.S.A.) and streptavidin peroxidase complex (diluted 1:200, Vector). Then, the sections were visualized by staining with 3,3’-diaminobenzidine tetrahydrochloride.

In order to establish the specificity of primary antibody, the procedure included the omission of the primary antibody, substitution of normal goat or rabbit serum for the primary antibody. As a result, immunoreactivity disappeared completely in tissues (data not shown).

To elucidate the effects of ACU and EA on cell proliferation and neuroblast differentiation in the DG, the corresponding areas of the DG were measured from 20 sections per animal. The studied tissue sections were selected corresponding to Bregma −3.00 to −4.08 mm of the rat brain atlas [23]. The measurement of the number of Ki67- and DCX-immunoreactive cells in each group was performed using an image analyzing system equipped with a computer-based CCD camera (software: Optimas 6.5, CyberMetrics, Scottsdale, AZ, U.S.A.). The number of the cells was counted as a mean number in the DG per each section. On the other hand, images of all DCX-immunoreactive structures were taken from the DG through a BX51 light microscope (Olympus, Tokyo, Japan) equipped with a digital camera (DP71, Olympus) connected to a PC monitor. Dendritic complexity of DCX positive cells was dendritic complexity of DCX positive cells was traced using camera lucida at 100 × magnification (Neurolucida; MicroBrightField, Williston, VT, U.S.A.).

**Western blot analysis for DCX:** To confirm changes in DCX levels in the hippocampus at each age, 5 animals at sham-ACU, ACU and EA group were sacrificed and used for Western blot analysis. After sacrificing them and removing the brain, hippocampus were then dissected with a surgical blade. The tissues were homogenized in 50 mM PBS (pH 7.4) containing 0.1 mM ethylene glycol bis (2-aminoethyl Ether)-N,N,N’,N’ tetraacetic acid (EGTA) (pH 8.0), 0.2% Nonidet P-40, 10 mM ethylenediamine tetraacetic acid (EDTA) (pH 8.0), 15 mM sodium pyrophosphate, 100 mM β-glycerophosphate, 50 mM NaF, 150 mM NaCl, 2 mM sodium orthovanadate, 1 mM phenylmethysulfonyl fluoride (PMSF) and 1 mM diithiothreitol (DTT). After centrifugation, the protein level was determined in the supernatants using a Micro BCA protein assay kit with bovine serum albumin as the standard (Pierce Chemical, Rockford, IL, U.S.A.). Aliquots containing 20 μg of total protein were boiled in loading buffer containing 150 mM Tris (pH 6.8), 3 mM DTT, 6% SDS, 0.3% bromophenol blue and 30% glycerol. The aliquots were then loaded onto a 10% polyacrylamide gel. After electrophoresis, the gels were transferred to nitrocellulose transfer membranes (Pall Crop, East Hills, NY, U.S.A.). To reduce background staining, the membranes were incubated with 5% non-fat dry milk in PBS containing 0.1% Tween 20 for 45 min, followed by incubation with goat anti-DCX (1:100) and anti-β-actin (1:1,000, SantaCruz Biotechnology) for loading control, peroxidase-conjugated rabbit anti-goat IgG (Sigma, St. Louis, MO, U.S.A.) or horse anti-rabbit IgG (Sigma) and an ECL kit (Pierce Chemical).

The result of the Western blot analysis was scanned, and the quantification of the Western blotting was done using Scion Image software (Scion Corp., Frederick, MD, U.S.A.), which was used to count the relative optical density (ROD): ROD of DCX was calibrated into β-actin of each group, respectively and A ratio of the ROD was calibrated into a mean number in the DG per each section.
as %.

Statistical analysis: The data shown here represent the means of experiments performed for each experimental area. Differences among the means were statistically analyzed by one-way analysis of variance followed by Duncan's new multiple range method in order to elucidate differences between control and sham-ACU/ACU/EA group.

RESULTS

Effects of ACU and EA on cell proliferation: In the control group, Ki67-positive (⁎) nuclei were detected in clusters in the SZDG (Fig. 1A). In the sham-ACU group, Ki67+ nuclei were slightly decreased in the SZDG (Fig. 1B). ACU and EA increased the number of Ki67+ nuclei by 843% and 911%, respectively, compared to sham-ACU (Fig. 1C-1E).

Effects of ACU and EA on neuroblast differentiation: Control animals had DCX immunoreactive neuroblasts in the SZDG (Fig. 2A), some with well-developed dendrites (tertiary dendrites) that extended two-thirds of the way into the molecular layer of the DG (Fig. 2B); however, many neuroblasts had poorly-developed dendrites. In the sham-ACU group, the number of DCX immunoreactive neuroblasts without tertiary dendrites was similar to that in the control group (Fig. 2C, 2D and 2I). ACU increased the number of DCX immunoreactive neuroblasts without tertiary dendrites by 278% compared to sham-ACU (Fig. 2E and 2F), but decreased the number of neuroblasts with tertiary dendrites (Fig. 2I). EA also increased the number of DCX immunoreactive neuroblasts (Fig. 2G and 2H), particularly those with tertiary dendrites by 317% (Fig. 2I).

ACU and EA also increased DCX protein levels, with levels highest (showing the highest level) in the EA group (Fig. 3).

Fig. 1. Immunohistochemistry for Ki67 in the DG in control (A), sham-ACU (B), ACU (C), and EA (D) groups. In the control group, Ki67+ cells (arrows) are detected in the subgranular zone of the dentate gyrus. In the sham-ACU group, Ki67+ cells are slightly fewer in dentate gyrus than that in the control group. In the ACU and EA groups, Ki67+ nuclei are detected in clusters and are abundantly detected in the dentate gyrus. GCL, granule cell layer; ML, molecular layer; PoL, polymorphic layer. Bar=100 μm. E: Analysis of Ki67+ cells per a section in the control, sham-ACU, ACU, and EA groups (n=7 per group; * P<0.05, significantly different from control group, a P<0.05, significantly different from the sham-ACU group, b P<0.05, significantly different from the ACU group). The bars indicate the means ± SEM.
Fig. 2. Immunohistochemistry for DCX in DG in control (A and B), sham-ACU (C and D), ACU (E and F), and EA (G and H) groups. In the sham-ACU group, DCX+ neuroblasts are detected in the (SZDG). Some DCX+ neuroblasts have tertiary dendrites (arrows) that extend to the molecular layer (ML). In the ACU group, more DCX+ neuroblasts without tertiary dendrites are present (arrowheads), whereas the EA group has more cells with tertiary dendrites (arrows).

GCL, granule cell layer; PoL, polymorphic layer. Bar=100 μm (A, C, E and G), 25 μm (B, D, F and H).

I: Numbers of DCX+ cells with/without tertiary dendrites per a section in the control, sham-ACU, ACU, and EA groups (n=7 per group; a P<0.05, significantly different from control group, b P<0.05, significantly different from the sham-ACU group, c P<0.05, significantly different from the ACU group). The bars indicate the means ± SEM.
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One possible reason of the 

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after birth, a stage when the cells express DCX [3]. These 

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the first time, we show that both ACU and EA increased 

cell proliferation in the SZDG compared to sham-ACU. For 

[3, 19, 21], to induce cell proliferation and differentiation.

The mechanism whereby ACU or EA increases cell pro-

liferation and neuroblast differentiation has not been fully 

explained. In the next section, we discuss some of the 

possible mechanisms that may underlie the effects of 

acupuncture on neurogenesis.

Fig. 3. Western blot analysis of DCX in the hippocampi of sham-

ACU, ACU, and EA groups. The relative optical density of 

immunoblot bands are shown as percent values (n=5 per group; 

a P<0.05, significantly different from sham-ACU group, 

b P<0.05, significantly different from the ACU group). The bars 

indicate the means ± SE.

DISCUSSION

Acupuncture, an oriental medicine technique that can be 

traced back at least 2,500 years, is gaining popularity in the 

West as an alternative and complementary intervention [6]. 

Here, we compared the ability of ACU and EA stimulation 

at ST36 and GV20 together, which improve hippocampus-

related neuropathologies such as epilepsy [9] and ischemia 

[3, 19, 21], to induce cell proliferation and differentiation. 

ACU or EA at GV20 and ST36 significantly increased 

cell proliferation in the SZDG compared to sham-ACU. For 

the first time, we show that both ACU and EA increased 

neuroblast differentiation in the SZDG in normal rats, with 

EA also increasing the number of tertiary dendrites. The 

increase of tertiary dendrites showed that newborn neurons 

undergo an accelerated maturation after ACU and EA treat-

ment because the dendrites of adult-born granule cells 

become progressively more complex during the 4 weeks 

after birth, a stage when the cells express DCX [3]. These 

increases of cell proliferation and accelerated maturation 

may be associated with improvement of memory because 

reduced neural stem cell proliferation decreases spatial 

learning but not contextual fear conditioning [29], and ACU 

at ST36 improved cognitive impairment in the dementia rat 

[27].

The mechanism whereby ACU or EA increases cell prolif-

eration and neuroblast differentiation has not been fully 

explained. In addition, we observed EA significantly 

increased the DCX immunoreactive neuroblasts with ter-

tiary dendrites compared to that in the ACU group. This 

result suggests that EA significantly increases the matura-

tion of neuroblasts because adult-born granule cells become 

progressively more complex [3]. One possible reason of the 

differences is increased growth factors, as EA influences 

brain-derived neurotrophic factor expression in the hippoc-

ampus of the rat exposed to immobilization stress [28], and 

activates endogenous glial cell line-derived neurotrophic 

factor and its receptor system in rats with neuropathic pain 

[5].

In conclusion, ACU and EA enhanced cell proliferation 

and neuroblast differentiation in the SZDG with greater 

effects for EA than that in ACU. These effects may help to 

enhance neurogenesis in normal and diseased animals.

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