Study of Effects of Electroacupuncture on the Hippocampal Cholinergic Neuronal Activity

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The purpose of this report is to investigate the electroacupuncture effect on the cholinergic neuronal activation in the hippocampal CA1 section. The electroacupuncture was performed on S36 of white rats and its consequencies were investigated by immunohistochemical method. Hippocampal CA1 sections of Sprague Dawley white male rats electroacupunctured on S36 at 20Hz and 100Hz are stained by cresyl violet to to show that the values of 100Hz and 20Hz group increased significantly compared to sham group's one. Especially, 100Hz group shows stronger neuronal activation compared to 20Hz group. Induction of AChE, a neurotransmitter, in hippocampal CA1 is increased significantly in 100Hz and 20HZ group compared to sham group. Especially 20Hz group shows higher AChE immunoreaction than 100Hz does, although it wasn't significant enough. Induction of NGF(Nerve Growth Factor) in hippocampal CA1 sections was observed higher in 20Hz and 100Hz group than in sham group. Especially, 20Hz group shows higher NGF immunoreaction compared to 100Hz. The facts above indicate that the eletroacupucture is effect to the cholinergic neuronal activation of hippocampus induced by focal ischemia.

Key words : Hippocampal CA1 neuron, Cresyl violet stain, AChE, NGF

Introduction

The cholinergic system is one of the most important neurotransmitter system in the brain and control activities that depend on selective attention, which is thought to play an important role in learning and memory through modulating long-term potentiation (LTP) in the CA1 and dentate gyrus[1-3]. In degenerative diseases of the brain, alterations in consciousness and memory are associated with regional deficits in cholinergic system[4-7]. According to recent research findings, it is indicated that cortical and hippocampal cholinergic synaptic systems and trophic factors can either reduce or accelerate pathogenesis and progression of Alzheimer's disease(AD) and Down's syndrome(DS) pathology by effects on amyloid precursor protein (APP) levels, metabolism and processing[8-11]. The early discovery of ACh deficiency singled out the loss of this neurotransmitter as one reason for the cognitive dysfunction in AD[8,12-13].

Acupuncture has been used to treat various diseases as a core method in oriental medical area and is becoming recognized as an effective mode of treatment for many chronic ailments including pain, nausea, addiction and stroke.

This study was performed to verify that acupuncture modulate cholinergic activities in hippocampal CA1 neuron related with memory function in neurodegenerative disease such Alzheimer's disease. In the present study, we investigated neuronal activities, Acetylcholinesterase(AChE) release, and Nerve Growth Factor (NGF) release in hippocampal CA1 by immunohistochemical method after acupuncture stimulating based on various frequency; 20Hz and 100Hz. Ⅱ.

Materials and Methods

1. The experimental animals.

The animals are Sprague Dawley white male rats (260g~300g) purchased from Sam-yuk animal center adapted to the laboratory condition(temperature 22±3C, humidity 50±10%) through 1 week. 3~4 rats are accepted in each cage and free to feed water and food(solid food; producted for experimental mice, Sam-yang Corp.,).

2. Experimental groups

The rats were divided into three groups- control group,

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low frequency electroacupuncture group(20Hz), high frequency electroacupuncture group(100Hz). Each group were acupunctured on S36 for 4 times(once a day/ 2 days/ on both S36/ 10AM) prior to biopsy.

3. Acupuncture point and treatment

Both S36 were chosen for the experiment. These points are on between the head of fibula and the tibial tuberosity of the rat which correspond to S26 on human body. The frequency was 20Hz in low frequency electronic acupuncture group and 100Hz high frequency electronic acupuncture group, while other conditions *Pulse duration & width is 1m.secs*, current was 3mA, time duration was 3 mins - were same.

4. Immunohistochemistry

1) Cresyl violet stain

Mice were anesthetized by sodium pentobarbital (100mg/kg, i.p.) soon after acupuncturing. 0.9% saline 200ml are flow through the body via heart for 2 mins rapidly, followed by 4% formalin(fixative) 800ml manipulated by phosphate buffer for 25 mins slowly. Brains were then taken outside and fixed again in the fixing fluid above for 2 hrs and kept in phosphate buffered saline(PBS) containing 20% sucrose for 24hrs(at 4 degrees). Next day brains were quick-frozen and the hippocampus was cut by 30 micrometer. The samples were washed in PBS for several times then fat-removed and dehydrated in following fluid in order; xylene(5mins), 100% alcohol(2mins), 95% alcohol (1min), 70% alcohol(1min), D.W(2mins). Lastly, the samples were stained by cresyl violet buffer(5mins). After these processing the density of neurons were counted by the optic microscope(x 400), using Scion image program(Scion Corp. MD, USA).

2) Acetylcholinesterase(AChE) stain

Brain samples washed about 3 times in PBS show light green a few seconds after being put in mixed fluid(0.1M sodium hydrogen phosphate(NaH_2PO_4 H_20, PH 6.0) 325ml, acetylcholine iodide 250mg, 0.1M sodium citrate 25ml, 30Mm copper sulfate 50ml, 5Mmpotassium ferricyanide 50ml). These samples were cultured for 1~2 hrs in room temperature and investigated its density of AChE neurons of hippocampus, magnified 100 times by a 200x200 micrometer rectangle grid, using Scion image program(Scion Corp. MD, USA).

3) Nerve growth factor(NGF) stain

Anti-NGF (1:500, poly-clonal, Santa Cruz Biltechnolgy Inc., CA, USA), which is the most popular stuff in NGF gene inducing study, was applied on brain samples washed about 3 times in PBS. Primary antibody was prepared from PBST(PBS added 0.3% Triton X-100) diluted 2000 times with 0.1% sodium acid and 2% rabbit serum. Brain samples were shaken continuously to be cultured in primary antiserum for 72 hrs. After being washed in PBST more than three times, samples were reacted with biotinylated anti-sheep serum(Vector Laboratories, Burlingame, CA, USA) diluted 200 times from PBST(including 2% rabbit serum) for 2 hrs in room temperature. Samples washed in PBST for 3 times were put in Vectastain EliteABC reagent(Vector). After being washed by PBS for several times, samples were hardened by nickel chloride and induced by diaminobensidine(DAB), a coloring agent. In control group brain samples, the primary antibody was skipped or substituted by nonimmuno sheep serum and both cases didn't show the certain sign. After all the processing, the brain samples were fixed on gelatine-coated slide and covered with a cover glass. These samples were magnified 100 times to be countered the density of NGF- immunoreactive neurons of hippocampus using Scion image program(Scion Corp. MD, USA).

5. Statistical analysis

All the results are presented as the mean±SE and the statistical analysis is performed by SPSS(for Window). Repeated ANOVA test is applied for comparing the values of the groups, one-way ANOVA is for values countered by immunohistochemical methodand Tukey test is for post examination. Statistical evaluation of the results is considered significant at a value of $P<0.05$.

RESULTS

1. Brain neuron activation

1) *Histological observation*

After electroacupuncturing on S36 with 20Hz and 100Hz, the pyramidal neurons in the hippocampus CA1 section were observed more activated than those of sham group. Especially the density of neurons of 100Hz group was higher than that of 20Hz group.

2) Quantitative analysis

The hippocampal CA1 sections got stained by cresyl violet to be observed their neuroprotective effect. Sham group showed 94.0±5.19, 20Hz group showed 104.5± 2.12 and 100Hz group showed 116.75± 5.92; the value of 100Hz group was increased significantly in statistic($P<0.05$).(Fig. 2)

2. AChE(a brain neurotransmitter) induction

1) Histological observation

After acupuncturing on S36 with 20Hz and 100Hz, the density of induced AChE in the hippocampus CA1 section observed by immunohistochemical method is higher than that

of sham group. Especially the immune reaction of AChE in 100Hz group turned out stronger than that of 20Hz group.

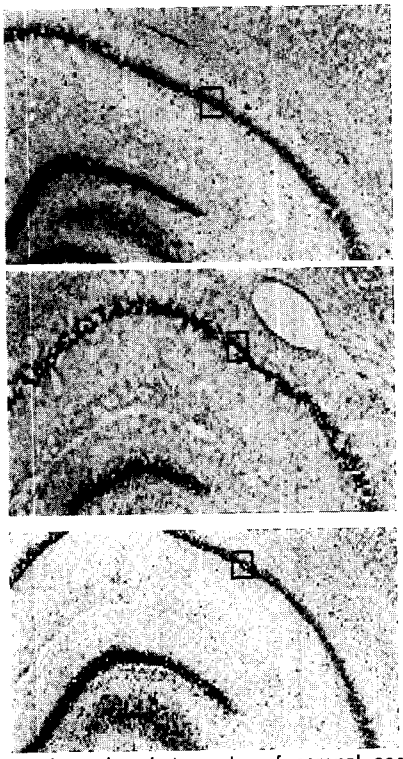

Fig.1. Representive microphotographs of coronal sections in the hippocampal CA1. As compared with density of Sham group, that of The density of 100Hz groups was increased(P<0.05) A:Sham, B:20Hz, C:100Hz. cresyl violet-stain. ×40. Scale box is 50.0㎛^{2}

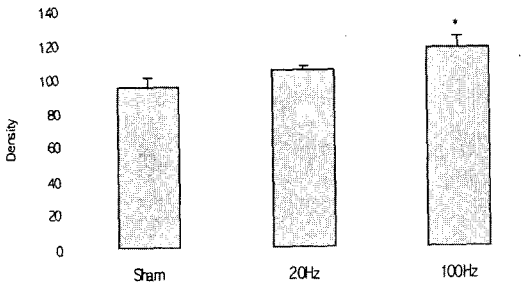

Fig. 2. The values of density of cresyl violet-stained sections in the hippocampal CA1 are shown. Measures of one-way ANOVA(Tukey test) of density among the groups. Results are shown as mean±S.E. *, P<0.05, as compared with the corresponding data of Sham group.

2) Quantative analysis

The density of induced AChE in the hippocampal CA1 section was 10.75±0.39 in sham group, 15.5± 4.52 in 20Hz group, 14.2±0.93 in 100Hz group; the value of 100Hz group was increased significantly in statistic(P<0.05).(Fig.4)

3. NGF inducing

1) Histological observation

After electroacupuncturing on S36 with 20Hz and 100Hz, the density of induced NGF in the hippocampus CA1 section observed by immunohistochemical method was higher than that of sham group. Especially the immunoreaction of NGF in 20Hz group turned out stronger than that of 100Hz group.

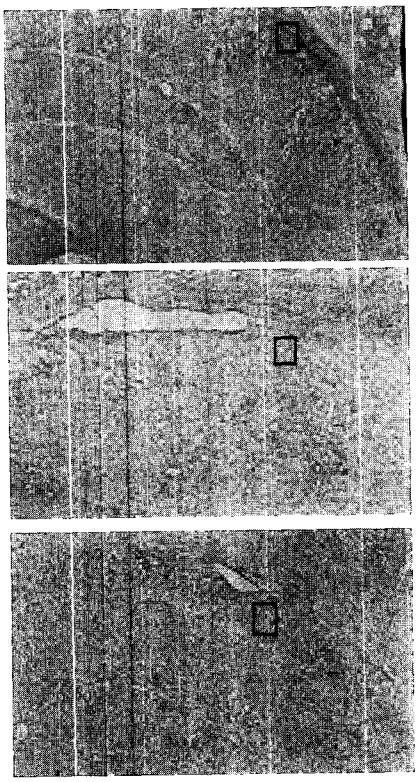

Fig.3. Representive microphotographs showing density of AchE in coronal sections at the hippocampal CA1. As compared with density of Sham group, that of The density of 100Hz groups was increased(P<0.05) A:Sham, B:20Hz, C:100Hz. ×40. Scale box is 50.0㎛^{2}

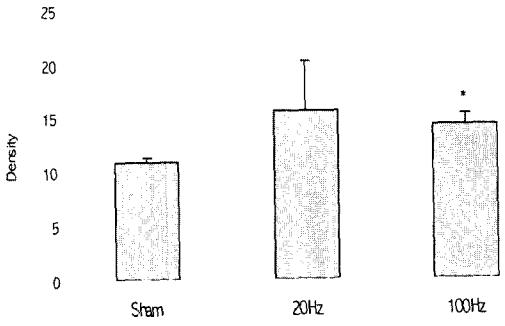

Fig. 4. The values of density of acetylcholine esterase(AchE) stained nuclei in the hippocampal CA1 are shown. Measures of one-way ANOVA(Tukey test) of density among the groups. Results are shown as mean±S.E.*, P<0.05, as compared with the corresponding data of Sham group.

2) Quantative analysis

The density of induced NGF in hippocampal CA1 section was 17.9±3.07 in sham group, 29.4 ±3.59 in 20Hz group, 21.0±4.86 in 100Hz group; the value of 20Hz group was increased significantly in statistic(P<0.05)(Fig.6).

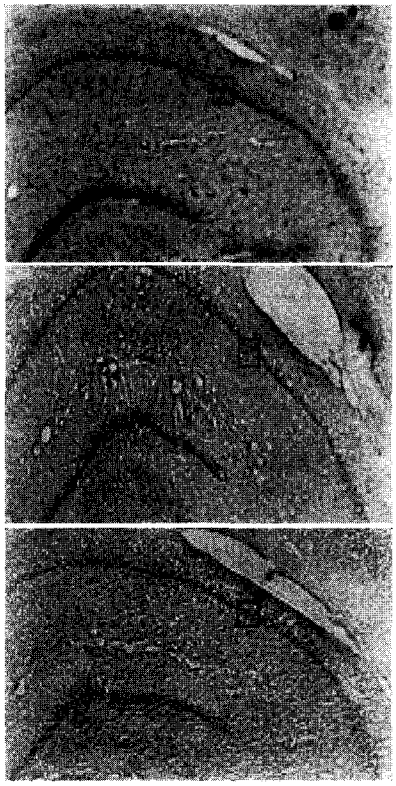

Fig. 5. Representive microphotographs showing density of NGF in coronal sections at the hippocampal CA1. As compared with density of Sham group, that of The density of 20Hz groups was increased($P<0.05$) A:Sham, b:20Hz, C:100Hz, ×40 Scale box is 50.0㎛

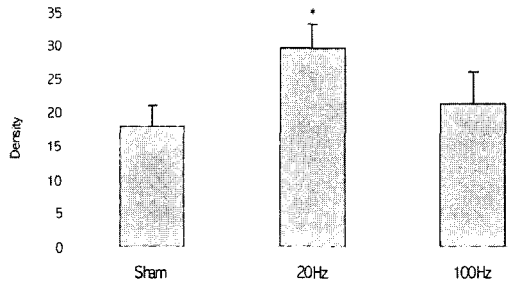

Fig.6 The values of density of Nerve growth factor(NGF) stained nuclei in the hippocampal CA1 are shown. Measures of one-way ANOVA(Tukey test) of density among the groups. Results are shown as mean±S.E.*, $P<0.05$, as compared with the corresponding data of Sham group.

Discussion

Acupuncture is becoming recognized as an effective mode of treatment for many chronic ailments including depression, pain, addiction and stroke. One recent Chinese matched controlled study of Vascular Dementia (VD) showed substantial and statistically significant improvements in cognition. Also, other studies show relief of depression or anxiety in cognitively intact adults. As yet, no published report exists of an experimental trial for any of the effects of acupuncture on Alzheimer's disease (AD), VD or other dementias.

Alzheimer's disease(AD) is an incurable neurodegenerative condition characterized by a progressive decline in cognitive function. The cognitive decline in Alzheimer's disease is defined by a loss of memory, decreased ability to learn, decreased attention span, and a severe compromise in thinking ability, judgement, and decision making[6,13-14]. Recent therapeutic investigations of Alzheimer's disease(AD) have been guided by two seemingly opposed hypothesis: the amyloid cascade theory, which favors the amyloid plaques as the cause of AD; and the cholinergic theory, which favors cholinergic neuron loss as the cause. A pathological cascade in AD-related memory loss could be triggered by alterations in amyloid precursor protein(APP) processing or ACh-mediated neuronal function, or both, which in turn trigger the overexpression of amyloid ß, synaptic malfunction and trophic factor loss in hippocampus or cortical region.

In this study, we hypothesized that acupuncture may regulate cholinergic system in hippocampus related neurodegenerative diseases as Alzheimer. For the purpose, we investigated activities of hippocampal neuron in CA1, AChE, and NGF after performing electroacupuncture depend on different frequency. Before all, in the staining using cresyl violet of hippocampal CA1 neuron after acupuncture, the increased density of the pyramidal neuron in the hippocampal CA1 was observed in acupuncture groups. Cresyl violet is a synthetic dye that is widely utilized to stain neuronal tissues. It readily binds to the acidic components of the neuronal cytoplasm such as corpus nissl, as well as the nuclei and nucleoli of the nerve cells.

Therefore, the above result of increased cholinergic activity indicate that cholinergic neuron in hippocampal CA1 was activated electroacupuncture. The Cholinergic system which is thought to play an important role in learning and memory through modulating long term potentiation in the CA1 and dentate gyrus may be controlled by electroacupuncture depend on frequency. Cholinesterase are enzymes that hydrolyze acetylcholine, and the type of cholinesterase found in tissue often reflects the type of tissue; neural tissue contains AChE, while non-neural tissue contains butyrycholinesterase.

The enzyme can be secreted from the cell as a soluble protein, attached to the membrane by a hydrophobic peptide or associated with the extracellular matrix. The acetylcholinesterase enzyme (AChE) catalyses the catabolism of the acetylcholine (ACh) neurotransmitter to its constituent components of acetic acid and choline. In addition to a role in modulating synaptic acetylcholine levels, AChE appears to have non-cholinergic functions, some of which may involve proteinprotein

interaction rather than enzymatic catalysis.

Thus, studies on neuronal development suggest that AChE promotes neurite outgrowth, possibly through adhesive interactions[15-21]. In this study, AChE was increased in experimental group controlled by electroacupuncture(20Hz, 100Hz) rather than sham group. Thus, the result of AChE increased by electroacupuncture has significance in that electroacupuncture may be a treatment method of neurodegenerative disease induced by cholinergic degeneration through modulating AChE releasing. But, in the sight of Studies in vitro have suggested that acetylcholinesterase (AChE) may interact with beta-amyloid to promote deposition of amyloid plaques in the brain of patients with Alzheimer's disease, this result is controversial.

Pathological processes operating in age-related cognitive impairment, idiopathic AD and DS might be related to physiological changes in several systems, including synaptic complexes associated with cholinergic nerve terminal. Such synapse depend on the trophic action of NGF for their function[8,22-25]. NGF is synthesized by neurons in the target regions and is related in an activity dependant manner[8,26-28]. And these peptides are transported from the terminals to the cell body retrograssively through binding to trkA receptor. According to recent report, after exposure to NGF, primary cortical neuronal cultures showed increased levels of membrane phospholipidsthat might promote APP expression and secretion of the soluble form of APP(sAPP), which has been shown to have neurotrophic activities. Also, the other study report that some types of cholinergic neuron lesions can increase hippocampal NGF levels in the normal adult rodent brain. The result of this study is similar to above report, therefore, thus indicate that acupuncture can modulate neurotrophic activities in hippocal CA1 synapses.

As we have seen, acupuncture affected on cholinergic system in hippocampal CA1 neuron by means of increased density of neuronal activity, increased AChE release, and increased NGF release. Accordingly, these results suggest that There is a possibility of modulating cholinergic system in hippocampal CA1 neuron, But these results are not sufficient to show that acupuncture is a entire method established scientific evidence to treat neurodegenerative disease such as Alzheimer or DS.

Conclusions

The author has obtained the following results through the study of low & high frequency electronic acupuncture effect on S36 of white mice to the activation of brain neurons, the induction of AChE a brain neurotransmitter and NGF.

In observation of the neuron activation in hippocampal CA1 section using cresyl violet, the activation of 100Hz group is significantly stronger than that of sham group. Especially neurons of the high frequency(100Hz) group get more activated as compared with those of the 20Hz group. Induction of AChE in hippocampal CA1 section significantly increased in 100Hz group than in sham group. Especially, induction in 20Hz group is higher than that in 100Hz, which is not significant enough, though. Induction of NGF in hippocampal CA1 sectión significantly increased in 20Hz group than in sham group. Especially, higher immunoreaction of NGF is observed in 20Hz group than in 100Hz group.

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