The effect of acupuncture on the development of epileptic focus formation in kainic acid-treated rat

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中文摘要

用針灸來治療癲癇在中國已有很久的歷史，但根據我們所知，尚未有用針灸來預防癲癇病灶形成的報告。因此本研究的目的是觀察針灸對於預防癲癇病灶形成的效用。我們將 kainic acid (12 mg/kg, KA) 於 Sprague-Dawley (SD) 大鼠的腹腔注射，六星期後建立一個慢性癲癇模式。KA 注射一星期後將大鼠隨機分組，並於相當人類的百會 (GV20) 和風府 (GV16) 穴處分別做施予手捻針 (manual acupuncture, MA)、2 Hz 電針 (electroacupuncture, EA) 和 100 Hz 電針刺激。KA 注射後六星期將大鼠犧牲取腦，並做成病理切片。用 Timm’s staining 觀察 mossy fiber sprouting，NeuN 免疫化學組織染色觀察神經細胞 (neuron)，用 glial fibrillary acidic protein (GFAP) 免疫化學組織染色觀察 astrogliosis。結果顯示 MA、2 Hz EA 和 100 Hz EA 都能減少海馬區域齒狀回 (dentate gyrus) Timm’s staining 的 optical density difference; 2 Hz EA 和 100 Hz EA 能減少海馬 CA1 區域的 NeuN 免疫反應細胞; MA 和 100 Hz EA 減少海馬 hilus 區域的 GFAP 免疫反應細胞; 2 Hz EA 和 100 Hz EA 能減少海馬 striatum oriens 和 striatum radiate 區域 GFAP 免疫反應細胞。

我們的結果顯示 MA、2 Hz 電針和 100 Hz 電針在百會和風府穴治療五星期能夠減少 SD 大鼠 KA 注射後六星期的 mossy fiber sprouting 和 astrogliosis，推測 MA、2 Hz 電針和 100 Hz 電針能預防癲癇病灶的形成。
發展（the development of epileptic focus formation），即有anti-epileptogenesis的作用。2 Hz 電針能夠減少 neuron 的喪失，否定 2 Hz電針對於保護神經細胞有較大的作用，需要更進一步的研究。

關鍵詞：手捻針、電針、kainic acid、Mossy fiber sprouting、Astrogliosis
Chapter 1: Introduction

Epilepsy is a chronic disabling neurological dysfunction characterized with repetitive excessive electric discharge of cerebral neuron. Hippocampus sclerosis is a common pathology change observed in the patients with temporal lobe epilepsy (TLE), which is characterized with hilar, CA3 and CA1 neuron loss, astrocytes proliferation and activation (astrogliosis) and mossy fiber sprouting. The epileptogenesis has been associated with an initial precipitating event during early childhood, such as an episode of febrile seizure or encephalitis. Kainic acid (KA) is a glutamate receptor agonist and commonly used to establish an animal model that is similar to TLE in human, because KA cause excessive excitation, excitotoxic injury in the inhibitory interneurons of hippocampus, and development of hippocampus sclerosis.

The neuron death of hippocampus in rat cause aberrant neuronal reorganization of dentate granular cells, i.e. mossy fiber sprouting. The aberrant mossy fibers sprout and terminate in the inner molecular layer (IML) of hippocampus. This reorganization was speculated to reduce inhibition.
and form a functional recurrent excitatory circuit \(^8\). Therefore, mossy fiber sprouting is regarded as a mark of epileptic focus formation, i.e. epileptogenesis. Timm’s staining can visualize the zinc cation (Zn\(^{+}\)) locates along the mossy fibers of granular cells, thus is used for detecting mossy fiber sprouting.

Glia also plays a critical role in the development of epileptic focus formation. Glial fibrillary acidic protein (GFAP) is a member of the intermediate filament family, and that binds together to form the main intermediate filament found in astrocytes \(^9\). Traditionally, up-regulation of GFAP in astrocytes was regarded as a reaction to neuron injury and death. However, early study suggested that astrocytes are not only responded to neuron injury but also to neuronal activity \(^10\). Moreover, several evidence revealed that astrocyte plays a determinant role in epilepsy \(^11, 12\). For example, astrocyte can regulate the excellular glutamate and GABA concentrations in the central nervous system (CNS) and therefore closed related to the abnormal glutamate cycle in patients with TLE \(^13\).
The antiepileptic drugs (AEDs) are used to prevent the development of epileptic focus formation; however, the effect is not satisfying in clinical trials \(^{14}\). Researchers also tried some new ADEs to prevent the development of epileptic formation, but the effect is still controversial \(^{15,16}\). Therefore, some clinicians and patients will seek for alternative treatments \(^{17}\).

Acupuncture has been used to treat diseases for a long period in China, especially for analgesia \(^{18}\). A number of studies consider that acupuncture analgesia is via actions of some peptides, such as serotonin, enkephalin and dynorphine \(^{19,20}\). Some of these peptides were also reported to benefit for the treatment of epilepsy. For instance, serotonin, which is considered to be closely related to the analgesia of acupuncture \(^{21}\), also plays an important anti-epileptic role in the limbic systems \(^{22}\). In epileptic animal model, dyorphin may mediate via kappa-opioid receptor to regulate neuronal excitation in hippocampus \(^{23,24}\). Therefore, we hypothesized that acupuncture may be a potential alternative treatment for epilepsy. In fact, acupuncture has been used for epilepsy treatment in China for centuries. In animal models, the inhibitory neurotransmitters can be induced immediately after short-term auricular \(^{25,26}\) and scalp \(^{27}\) electroacupuncture (EA). Furthermore, long-term EA can enhance the expression of the mRNA of
glutamic acid decarboxylase (GAD) in the dentate granular cells\textsuperscript{28}. GAD is a rate-limiting enzyme for GABA synthesis. GABA, a strong inhibitory neurotransmitter, and its related receptors have been shown to be able to prevent the development of epileptic focus formation\textsuperscript{29}. To our knowledge, the study in acupuncture on epileptogenesis has not been reported yet until now.

Therefore, the aim of the present was to investigate the effect of acupuncture on the epileptic focus formation, i.e. epileptogenesis. We established a chronic epilepsy animal model by using intra-peritoneal administration of kainic acid in Spragruce-Dawley (SD) rat. Acupuncture or EA was applied to Baihui (GV20) and Fengfu (GV16) acupoints, and mossy fiber sprouting by Timm’s stain was used as a marker of epileptic focus formation.
Chapter 2: Literatures review

2.1 Mechanism of acupuncture

The mechanism of acupuncture, especially for analgesia, has been widely explored. Early studies investigated relationship between acupuncture and endogenous opiates (β-endorphin\(^{30}\), enkephalin\(^{31}\), endomorphin\(^{32}\) and dynorphin\(^{33}\)). Before the 1990s, most experts agreed on the concept that lower frequency EA stimulates the release of β-endorphin, enkephalin and endomorphin, which in turn activates the μ- and δ-opioid receptors, and that higher frequency EA stimulates dynorphin which activates the κ-opioid receptor\(^{20}\). Aside from endogenous opiates, the serotonergic descending inhibitory pathway is suggested as an important mechanism of acupuncture analgesic collaborated with endogenous opiates\(^{19,34}\). Recently, researches on the autonomic nervous system (ANS) seem to indicate its connection with acupuncture\(^{35}\). Indeed, studies had suggested that acupuncture can regulate the functions of ANS\(^{36-38}\). The inflammatory reflex via the ANS can elucidate why acupuncture has diversified therapeutic effects.
2.2 Relationship of acupuncture and epilepsy in Traditional Chinese Medicine (TCM)

It is believed that the earliest description of the epileptic attack (seizure) in ancient China can be traced back at least to the Warring States (B.C. 403-221). In the Fifty-two Prescriptions (五十二病方) from the Tomb 3 at Mawangdui (馬王堆三號墓)\textsuperscript{39}, symptoms and the treatment of febrile convulsion of infants were depicted. The ancient Chinese had noticed a kind of disease characterized with abnormal motor convulsion following a precipitated event (febrile, or fever). Later on, there were many sections discussing the seizure conditions in the Yellow Emperor’s Canon\textsuperscript{40, 41}. However, there were many terminology problems in the Yellow Emperor’s Canon. For example, a same character (癲) can stand not only for the epilepsy but also for a kind of psychosis. Therefore, the descendant scholars and literatures about epilepsy or seizure had lots of arguments and discordances. Sometimes, the treatment is for epilepsy in one book and for psychosis in another. This condition makes it difficult to review the relationship of epilepsy and acupuncture in the ancient TCM literatures. However, there is a general agreement that acupuncture treatment is a part of the traditional channel (meridian) theory in TCM. There are many different
manifestations of a diseased channel. Among the 14 common channels, Governor (GV) and Bladder channels connect to the brain \(^{41, 42}\). If the functions of these two channels are abnormal, opisthotonus \(^{41}\) and seizure \(^{40}\) will occur. Therefore, in ancient China, the acupoints locating along these two channels are the most preferring choices for treating patients with epilepsy. Indeed, GV 20 and GV 16 are commonly used in the ancient TCM literatures \(^{43-46}\).
2.3 Function of GV 20 and GV 16

As mentioned previously, GV channel connected to the brain according to the channel theory in TCM. The anatomy location of GV 20 is at the midpoint of the line connecting two ear apex in humans and rats, just at the sagittal suture. GV 16 is at the surface below the external occipital protuberance near the atlanto-occipital joint. The acupoints GV 20 and GV 16 locating along GV channel are therefore used for treating CNS dysfunction. Epilepsy is one of their indication\textsuperscript{47}, especially GV 20. GV 20 has been proved to regulate CNS functions\textsuperscript{48-51}, and a report has shown that acupuncture at GV 20 and GV 16 has a short-term benefit to epileptic animal model\textsuperscript{27}. They found that acupuncture can induce exogenous taurine and further decrease the severity of seizure attack in the penicillin-induced epileptic rat model. Taurine, a semi-essential amino acid, has similar function to GABA in the mammalian brain.
2.4 Kainic acid-induced epilepsy

Several kinds of classification of epilepsy are used. In addition to the major International League Against Epilepsy (ILAE) classification, some experts prefer localization-related classification. Four common epilepsies are included, temporal, frontal, parietal and occipital lobe epilepsy. TLE also called complex partial seizure that the clinical characterization is oral or behavioral automatism or even secondary general tonic-clonic (GTC) seizure. Intrahippocampal or systemic injection of KA can induce epileptic seizure that is similar to TLE in humans. In KA-induced epileptic seizure model, status epilepticus (SE) is observed sometimes. Followed by SE, spontaneous seizure develops. From the initial insult (KA-induced SE) to the spontaneous seizure, an epileptic focus, i.e. epileptogenesis, develops during this period. However, the processes are still not elucidated completely until now. However, common pathology changes in patients with TLE are also observed in rats with KA-induced TLE model.
2.5 The effect of acupuncture-related neuropeptides on epilepsy

Both acupuncture and EA can induce the release of some important neuropeptides. There are several kinds of endogenous opiates and β-endorphin, enkephalin, endomorphin and dynorphin have been shown strongly related to acupuncture \(^{18}\). They are the explanation why acupuncture can induce convincing analgesic effect. Some of them have also been studied for their effect on epilepsy. For example, dynorphin acting on kappa opioid receptor can prolong hippocampal neurons depolarization by augmenting potassium current \(^{23}\) and prevent the behavioral and electroencephalographic seizure in an animal model \(^{24}\). Serotonin (5-hydroxytryptamine, 5-HT) is released in the CNS after acupuncture \(^{19}\). There are several kinds of serotonin receptors and 5-HT\(_{1A}\) subtype is reduced in the limbic system in the patient with TLE \(^{54,55}\). Activation of 5-HT\(_{1A}\) can prevent the epileptic focus formation in a pilocarpine-induced epileptic rat model \(^{56}\). Previous study has revealed that acupuncture can activate the serotoninergic raphe nucleus in the brain stem \(^{57}\), and serotonin binding to its receptors (specifically, 5-HT\(_{1A}\) subtype) can elicit potent analgesia \(^{19,58}\). Therefore, through dynorphin and serotonin, acupuncture might have anticonvulsant effect.
Chapter 3: Material and method

3.1 Animal

Adult male SD rats, weighing 250~350 g, were purchased from BioLASCO Taiwan Co., Ltd. The rats were housed in animal center of China Medical University. The center maintained on a 12-h light-dark cycle at 25°C. All animal experiments were undertaken in accordance with the Guideline Principles for the Care and Use of Laboratory Animals.

3.2. The establishment of chronic epileptic model.

3.2.1 The selection of SD rats

The rat was intra-peritoneal injection of KA (12 mg/kg, Sigma, St. Louis, MO) following by the behavior of the rats was observed in a transparent plastic cage for 3 hrs. KA-induced behavior included wet dog shakes, facial myoclonus, ton-clonic seizures and loss of postural tone. The rats which had class IV-V behavior seizures according to Racine 59 were selected into the present study.
3.2.2. Grouping

The rats were divided randomly into five groups as follows: 1) normal group, the rats without KA administration; 2) control group, the rats were without acupuncture or EA treatment; 3) manual acupuncture (MA) group, 7 days after KA administration the rats were treated with manual acupuncture. The stainless acupuncture needle (25mm, 32G, Yu Kuang, Taiwan) was inserted into Baihui (GV20) and Fengfu (GV16) acupoints, and then twisted the needle by thumb and index finger in the GV 20, 15 min in duration, 1 Hz in frequency and 3 days per week for 5 weeks; 4) 2 Hz EA (EA2) group, the method was identical to MA but the rats were treated with 2 Hz EA with Trio 300 electric stimulator (Ito, Japan), 1 mA in intensity for 5 min, followed by 2 mA for 5 min and finally 3 mA for 5 min; 5) 100 Hz EA (EA100) group, the method was identical to EA2, but the rats were treated with 100 Hz EA (Fig. 1).
3.3. Preparation of brain tissue

The rats were anesthetized with chloral hydrate (400 mg/kg) i.p. 6 weeks after KA administration. The rat brains were perfused with 0.37% sodium sulfide solution (1.17 g of Na\textsubscript{2}S · 9H\textsubscript{2}O, 1.19g of NaH\textsubscript{2}PO\textsubscript{4} · H\textsubscript{2}O per 100 ml) and 4% paraformaldehyde transcardially as described previously\textsuperscript{60}. The rats were sacrificed and the brain were then removed. The brain tissues were postfixed in 4% paraformaldehyde. Two days prior to the sectioning, the brain tissues were immersed in a 30% sucrose solution dilated in the phosphate buffer solution (PBS). After sinking, the brain tissues were removed from the sucrose solution and were embedded in the tissue freezing medium (OCT) under -80-degree Celsius. The embedded brains were cut by the Cryostat and were stored under -20-degree Celsius. Coronal brain sections of 40 μm were made for Timm and sections of 16 μm for IHC staining. The sections of 40 and 16 μm were processed alternatively.
3.3.1 Timm staining

The sections of 40 μm were then immersed in the developing solution in the darkness (5.1 g of citric acid, 4.7 g of sodium citrate, and 3.4 g of hydroquinone were dissolved in 80 ml of water and added to 180 ml of 50 % arabic gum, supplemented by 1 ml of 17 % silver nitrate solution, just before the developing process should start). The sections were checked occasionally until proper staining and then were washed in running water. After staining, the sections were dehydrated with graded ethanol for 15 minutes (75% for 5 minutes, then 95% for 5 minutes, and finally 99% for another 5 minutes) and xylene for 5 minutes. The stained sections were then coverslipped.
3.3.1.1 Timm’s Quantification

To objectively quantify mossy fiber spourting in the supragranular molecular layer of the dentate gyrus, we analyzed the digitalized picture of the Timm stained sections modified from previous report 61. Briefly, horizontal stained sections corresponding to 3.64 - 4.10 mm to bregma were mounted on Zeiss microscope, and the pictures were captured by Nikon digital camera. Utilizing image analysis software (Image-Pro Plus, Media Cybernetics, Inc), the pictures were converted to 8-bits gray scale. Optic density (OD) measurements based on an average gray scale value (0 – 255 pixels unit) were obtained for two parallel lines, along the inner and outer edges of inner molecular layer (IML). A lower value of OD represents a darker image. The inner edge of IML is the place where aberrant mossy fibers terminate and the outer edge of IML was regarded as a background value. The difference of OD between inner and outer edge of IML of each section was defined by:

\[(OD_o-OD_i)/[(OD_o+OD_i)/2]\]

\(OD_o\) is the OD value of the outer edge of IML (background value) and \(OD_i\) is the OD value of the inner edge of IML (sprouting mossy fiber terminals). The difference of OD therefore represents the degrees of mossy fiber
sprouting in the IML. The greater the difference of OD is, the denser the sprouting mossy fiber terminals are.
3.3.2 *Immunohistochemistry staining*

Light microscope was used for NeuN and GFAP-stained section. The rat brain tissue located between 3.64 to 4.10 mm posterior to bregma were analyzed. Cell count were conducted separately in the dentate hilar, CA3c, CA1 regions, and the numbers of immunoreactive cells were represented as mean ± SD (standard deviation).

3.3.2.1 *NeuN staining*

The sections of 16 μm were used for IHC staining. Initially the sections were washed twice (5 minutes each) in 0.1 M TRIS buffer (pH 7.6) and treated with 1% H₂O₂ made in 0.1 M TRIS buffer for 30 minutes to inhibit the endogenous peroxidase activity. Sections were then washed in 0.1 M TRIS buffer (pH 7.6; 5 minutes) and then treated with TRIS A for 10 minutes (TRIS A: 0.1% Triton X-100 dissolved in 0.1 M TRIS buffer), followed by TRIS B for another 10 minutes (TRIS B: 0.1% Triton X-100 and 0.005% bovine serum albumin (BSA) in 0.1 M TRIS buffer). Sections were then blocked in normal goat serum. Forty-five minutes after blocking, sections were subsequently washed in TRIS A and then TRIS B (10 minutes each) and incubated in antisera (NeuN 1:1000, diluted in TRIS B) over night
at 4°C. On the following day, sections were washed with TRIS A (10 minutes) followed by TRIS B (10 minutes) and then incubated for 45 minutes with a biotinylated secondary antibody against rabbit immunoglobulin (Ig) G made in goat. Sections were washed in TRIS A (10 minutes), then TRIS D (0.1% Triton X-100 and 0.005% BSA in 0.5 M TRIS buffer) for 10 minute, and finally incubated for 1 hour in avidin-biotin horseradish peroxidase complex. Sections were washed three times in 0.1 M TRIS buffer (5 minutes each), developed in diaminobenzidine tetrahydrochloride (DAB) for 1-2 minutes and then washed three times (5 minutes each) in 0.1 M TRIS buffer. The reaction was stopped in 0.1 M TRIS buffer and all sections were counterstained with hematoxylin solution. After washing 3 times in 0.1 M TRIS buffer, the sections were dried and coverslipped. The NeuN-immunoreactive (NeuN-IR) cells were counted all over the CA3c region under microscope manually. In CA1 region, the NeuN-IR cells were measured as average number of three random 400X high power field (HPF).
3.3.2.2 GFAP staining

The sections of 16 μm were washed briefly in PBS and then treated with 3% H₂O₂ in methanol for 30 min at room temperature. The sections were then blocked for one hour with normal goat serum. After two rinses in PBS (3 minutes each), the sections were incubated for 1 hour at 37°C with anti-sera (GFAP 1:500 diluted in PBS). Sections were washed 3 times in PBS (3 minutes each) and then incubated for 1 hour at room temperature with biotinylated secondary antibody. Sections were further washed 3 times in PBS and then incubated for 1 hour with avidin-biotin horseradish peroxidase complex. Sections were washed three times in PBS (3 minutes each), and then developed in diaminobenzidine tetrahydrochloride (DAB). The reaction was stopped in running water and all sections were counterstained with hematoxylin solution. After washing in running water, the sections were dried and coverslipped. The GFAP positive cells were counted separately in dentate hilus, striatum radiate (SR) and striatum oriens (SO).
3.4. Statistical analysis

The data was represented as mean ± SD, a one-way ANOVA and with Schaffer’s *post-hoc* tests was used to analysis the difference among the groups, we definite p< 0.05 was significantly difference.
Chapter 4: Results

4.1 The effect of acupuncture on mossy fiber sprouting

In the Timm’s staining, the OD difference was 65.5±22.1% in the control group greater than 0.58±0.80% in the normal group (p < 0.001; Fig. 2-3). The OD difference in the control group was greater than 23.50±13.10% in the MA group, 13.30±7.70% in the EA2 group and 10.45±9.20% in the EA100 group (all p < 0.001; Fig. 2-3).
4.2 Effect of acupuncture on NeuN-immunoreactive (NeuN-IR) cells

4.2.1 Effect of acupuncture on NeuN-IR cell in the CA3c region of the hippocampus

In the CA3c region of the hippocampus, the count of NeuN-IR cells was 124.83±20.15 in the normal group greater than 53.5±23.34 in the control group (p < 0.001; Fig. 4-5). The count of NeuN-IR cells in the control group was similar to 44±28.63 in the MA group, 92.2±24.51 in the EA2 group, 67.33±24.97 in the EA100 group (all p > 0.05; Fig. 4-5).

4.2.2 The effect of acupuncture on NeuN-IR cell in the CA1 region of the hippocampus

In the CA1 region of the hippocampus, the count of NeuN-IR cells was 103.7±8.41 in the normal group greater than 13.2±13.57 in the control group (p < 0.001; Fig. 6-7). The count of NeuN-IR cells in the control group was similar to 39.8±21.22 in the MA group (p > 0.05; Fig. 6-7). The count of NeuN-IR cells was 61.75±7.39 in the EA2 and 42.7±9.36 in the EA100 were greater than in the control group (p < 0.001; Fig. 6-7).
4.3 The effect of acupuncture on GFAP-immunoreactive cells (GFAP-IR)
cells

4.3.1 The effect of acupuncture on GFAP-IR cells in dentate hilus of the hippocampus

In dentate hilus of the hippocampus, the count of the GFAP-IR was 158±33.97 in the control group greater than 97±20.12 in the MA group (p < 0.05; Fig. 8 and 10), 82±33.87 in the EA100 group (p < 0.01; Fig. 8 and 10). The count of the GFAP-IR in the control group was similar to 112.7±23.06 in the EA2 group (p > 0.05; Fig. 8 and 10). The count of the GFAP-IR also was greater than 23.25±11.87 in the normal group (p < 0.001; Fig. 8 and 10).

4.3.2 The effect of acupuncture on GFAP-IR cells in striatum oriens (SO) of the hippocampus

In SO of the hippocampus, the count of the GFAP-IR was 286.33±83.66 in the control group greater than 190.71±33.36 in the EA2 (p < 0.05; Fig. 9-10), 148.40±25.96 in the EA100 group (p < 0.01; Fig. 9-10). The count of the GFAP-IR in the control group was similar to 197.17±38.11 in the MA group (p > 0.05; Fig. 9-10). The count of the GFAP-IR also was greater than 67.40±25.75 in the normal group (p < 0.001; Fig. 9-10).
4.3.3 The effect of acupuncture on GFAP-IR cells in striatum radiate (SR) of the hippocampus

In SR of the hippocampus, the count of the GFAP-IR was 327.17±74.57 in the control group greater than 232.14±23.67 in the EA2 (p < 0.05; Fig. 9-10), 166.60±17.64 in the EA100 group (p < 0.001; Fig. 9-10). The count of the GFAP-IR in the control group was similar to 234.17±74.46 in the MA group (p > 0.05; Fig. 9-10). The count of the GFAP-IR also was greater than 49.8±21.11 in the normal group (p < 0.001; Fig. 9-10).
Chapter 5: Discussion

5.1 Acupuncture and EA may prevent the development of epileptic focus formation in KA-treated SD rats

Our results indicated that OD difference of Tmm’s staining increased in the hippocampus 6 weeks after KA administration, and these increase can be reduced by manual acupuncture, 2 Hz EA and 100 Hz EA treatment at GV16 and GV20, suggesting that acupuncture, 2 Hz or 100 Hz EA can reduce mossy fiber sprouting because Tmm’s staining is an indicator of mossy fiber sprouting. Mossy fiber sprouting has closely relationship to the development of epileptic focus formation. Therefore, the results of the present study demonstrated that acupuncture, 2 Hz EA and 100 Hz EA at GV16 and GV20 may prevent epileptogenesis induced by KA in sprague-Dawley rats.

KA is a glutamate receptor agonist, and it is an excitotoxicity substance resulting in dentate hilar mossy neuron death. The hilar mossy neurons receive the signal from the mossy fiber of granular neurons and hilar mossy cells further project inhibitory axon back to the dendrites of granular neurons.
This synaptic pattern is from granular neuron followed by mossy neuron and terminates to granular neuron. Thus, an inhibitory circuit in the dentate gyrus of hippocampus is established. However, the granular neurons are spared by KA, and the mossy fibers (axons) of the survived granular neurons which originally contacted with these died mossy neurons will aberrantly sprout and the axons will terminate at the dendrites of granular cells in the IML \(^8,\,^62\). Therefore, this synaptic reorganization leads a granular-to-granular circuit in the dentate gyrus of the hippocampus. In addition, these sprouting mossy fibers alter their synaptic property compared with the normal mossy fibers \(^63\). There were increased excitatory glutamate and zinc release in these sprouting mossy fibers. Indeed, previous studies have suggested that mossy fiber sprouting can cause excitatory recurrent within the granular layer in the dentate gyrus \(^8\) and this recurrent might further become the epileptic focus, namely, epileptogenesis \(^64\). The process of this reorganization often needs several weeks to establish which is corresponding to the development of spontaneous recurrent seizure.
5.2 EA may reduce neuron loss of hippocampus in KA-treated SD rats

Our results indicated that NeuN-IR cells decreased in the CA3c and CA1 region of hippocampus 6 weeks after KA administration. Because NeuN-IR cells represent the neurons in the hippocampus, the reduction of NeuN-IR cells indicated neuron loss in the hippocampus. This reduction can be reversed by 2 Hz and 100 Hz EA treatment in the CA1, suggesting that 2 Hz and 100 Hz EA may reduce neuron loss induced by KA in SD rats. The present study and other study has shown that KA can induce neuron loss, astrogliosis and mossy fiber sprouting in the hippocampus. Traditionally, the effects of EA are frequency-dependent. Different frequencies can result in different responses, especially in analgesia studies. Previous studies have shown that both lower and higher frequency EA have positive effects in the epileptic animal models. Especially in a chronic epileptic animal, long-term lower frequency (4-20 Hz alternatively) EA can increase the expression of GAD mRNA in the dentate gyrus. GAD is the key enzyme of inhibitory GABA synthesis in the CNS. Increased GABA can inhibit the excitatory circuit in the hippocampus. As regard to our results indicated that 2 Hz and 100 EA cannot reduce the neuron loss in the CA3c need further study.
5.3 Acupuncture, 2 Hz EA and 100 Hz EA may reduce atrogliosis of hippocampus in KA-treated SD rats

Our results indicated that GFAP-IR cells increased in the hippocampus 6 weeks after kainic acid administration and these increased can be reduced by manual acupuncture, 2 Hz EA and 100 Hz EA treatment at GV16 and GV20, suggesting that 2 Hz EA and 100 Hz EA treatment can decrease astrogliosis of hippocampus induced by KA due to GFAP-IR is an indicator of astrocyte (Barres and Barde, 2000). Previous study has suggested that the astrocyte number in CA2 and CA3 regions is correlated with the seizure duration in human TLE subject. This astrogliosis cannot be solely regarded as a reactive phenomenon to neuron death. In contrast, the altered functions of reactive astrocyte may contribute to epileptic focus formation. The major functions of astrocyte in the hippocampus are to regulate the extracellular milieu (concentration of potassium and calcium) and to uptake excitatory neurotransmitter, glutamate. In an animal model, the chemical induced seizure can cause not only neuron death but also astrocyte death in the hippocampus, and then astrocyte regeneration follows weeks later. The regenerated astrocytes were shown to be functional impairment. One of these impairments is glutamate transporters dysfunction. Impairment of
these transporters altered the metabolism of glutamate and GABA. These alterations are though to be a causation of epilepsy. Indeed, there were increased glutamate level and decreased GABA level in the specimens from patient with complex partial seizure \(^70\), and the increased glutamate level is suggested originated from reactive astrocyte in patients with TLE \(^71\). For example, extracellular glutamate should be absorbed by astrocyte and further convert into glutamine \(^13\). Dysfunctional astrocyte thus might lead an accumulation of extracellular glutamate and even can be a source of glutamate \(^71\). Our study suggests that acupuncture can reduce the number of reactive astrocyte in both dentate hilar, CA2 and CA3 regions. Previous studies have shown that electroacupuncture can increase the inhibitory neurotransmitter such as taurine, glycine and GABA, and decrease the excitatory somatostatin, aspartate and glutamate \(^25, 27\) in epileptic animal models. Some of these neurotransmitters are regulated by astrocyte. Therefore, reversed astrogliosis response by acupuncture treatments in our study could prevent the epileptic focus formation. Indeed, previous study has suggested that somatic EA can reduce the glutamate release in the CNS in an ischemic animal model \(^50\). Together above-mentioned results, although the severity of mossy fiber sprouting is not correlated to the severity of seizure
attack in some studies\(^72, 73\), and preventing neuron loss and the formation of mossy fiber sprouting cannot reverse the development of epilepsy in a animal model \(^74\), our consider that manual acupuncture, 2 Hz and 100 Hz EA are advantage to prevent the development of epileptic focus formation.

Critics might doubt the effect of acupuncture is resulted from electric stimulation rather than acupuncture or acupoints themselves. However, our study suggested that there is no different between manual acupuncture and electroacupuncture groups on the astrogliosis and mossy fiber sprouting. Therefore the therapeutic effect of scalp acupuncture is owing to the specific effects of the acupoints GV20 and GV16. Indeed, GV channel and its related acupoints have been used for treating some CNS disorders \(^48, 49, 51\), including epilepsy \(^27\).
Chapter 6: Conclusion

Our results indicated that long term treatment of manual acupuncture, 2 Hz and 100 Hz at GV20 and GV16 can reduce mossy fiber sprouting and also can reduce astrogliosis 6 weeks after KA administration in SD rats, suggesting manual acupuncture, 2 Hz and 100 Hz may prevent the development of epileptic focus formation, i.e. anti-epileptogenesis. In addition, 2 Hz EA may reduce neuron loss of hippocampus, whether or not 2 Hz may produce a greater effect in protection of neuron, further study is needed.
Figure 1. Flow chart.

The epileptic seizures were induced by intra-peritoneal administration of kainic acid in the Sprague-Dawley Rats. The epileptic seizure rats were divided randomly into control group (Control) without acupuncture treatment, MA group (MA) with manual acupuncture treatment, EA2 group (EA2) with 2 Hz EA treatment, EA100 group (EA100) with 100 Hz EA treatment, whereas normal group (Normal) with phosphate buffer saline (PBS) treatment only (without kainic acid treatment). Acupuncture or electroacupuncture treatment (three times per week) begins one week after KA administration, and the rats were sacrificed at 6 weeks after kainic acid administration.
Figure 2. The effect of acupuncture on mossy fiber sprouting. The optical density difference of Timm’s staining in hippocampus increased 6 weeks after kainic acid administration (Control) in the Sprague-Dawley rats, these increase was reduced by manual acupuncture (MA), 2 Hz electroacupuncture (EA2) and 100 Hz electroacupuncture (EA100) treatments. No prominent optical density difference of Timm’s staining was seen in the normal group (Normal) without kainic acid administration. Arrow: band of Timm’s staining positive fiber; Arrow head: Timm’s staining positive fiber.
Figure 3. The effect of acupuncture on the mossy fiber sprouting in the kainic acid treated rats.

The optic density difference increased 6 weeks after kainic acid administration. These increases were reduced by manual cupuncture, 2 Hz electroacupuncture and 100 Hz electroacupuncture treatments. Normal: normal group; Control: control group; MA: manual acupuncture group; EA2: 2 Hz electroacupuncture group; EA100: 100 Hz electroacupuncture group. ***p<0.001 compared with Normal; ###p<0.001 compared with Control.
Figure 4. The effect of acupuncture on the NeuN immunoreactive cells in the kainic acid treated rats.

The NeuN immunoreactive cells reduced in the CA3c region of hippocampus 6 weeks after kainic acid administration. Manual cupuncture, 2 Hz electroacupuncture and 100 Hz electroacupuncture treatments could not increased NeuN immunoreactive cells. Normal: normal group; Control: control group; MA: manual acupuncture group; EA2: 2 Hz electroacupuncture group; EA100: 100 Hz electroacupuncture group. Arrow: NeuN immunoreactive cells. 40X: microscopic field 40 amplifiers, 100X: microscopic field 100 amplifiers; 400X: 400 amplifiers.
Figure 5. The effect of acupuncture on the NeuN immunoreactive cells in the kainic acid treated rats.

The NeuN immunoreactive cells reduced in the CA3c region of hippocampus 6 weeks after kainic acid administration. Manual cupuncture, 2 Hz electroacupuncture and 100 Hz electroacupuncture treatment could not increased NeuN immunoreactive cells. Normal: normal group; Control: control group; MA: manual acupuncture group; EA2: 2 Hz electroacupuncture group; EA100: 100 Hz electroacupuncture group. Arrow: NeuN immunoreactive cells. ***p<0.001 compared with Normal.
Figure 6. The effect of acupuncture on the NeuN immunoreactive cells in the kainic acid treated rats.

The NeuN immunoreactive cells reduced in the CA1 region of hippocampus 6 weeks after kainic acid administration. 2 Hz electroacupuncture and 100 Hz electroacupuncture treatment increased NeuN immunoreactive cells. Normal: normal group; Control: control group; MA: manual acupuncture group; EA2: 2 Hz electroacupuncture group; EA100: 100 Hz electroacupuncture group. Arrow: NeuN immunoreactive cells; Arrow head: CA1 region of hippocampus. 40X: microscopic field 40 amplifiers, 100X: 100 amplifiers; 400X: 400 amplifiers
**Figure 7.** The effect of acupuncture on the NeuN immunoreactive cells in the kainic acid treated rats.

The NeuN immunoreactive cells reduced in the CA1 region of hippocampus 6 weeks after kainic acid administration. 2 Hz electroacupuncture and 100 Hz electroacupuncture treatment increased NeuN immunoreactive cells. Normal: normal group; Control: control group; MA: manual acupuncture group; EA2: 2 Hz electroacupuncture group; EA100: 100 Hz electroacupuncture group. ***p < 0.001 compared with Normal; ###p < 0.001 compared with Control.
Figure 8. The effect of acupuncture on the glial fibrillary acid protein (GFAP) immunoreactive cells in the hilus in the kainic acid treated rats. The GFAP immunoreactive cells increased hippocampus 6 weeks after kainic acid administration. Manual acupuncture, 2 Hz electroacupuncture and 100 Hz electroacupuncture treatment reduced GFAP immunoreactive cells. Normal: normal group; Control: control group; MA: manual
acupuncture group; EA2: 2 Hz electroacupuncture group; EA100: 100 Hz electroacupuncture group. Arrow: GFAP immunoreactive cells; CA1 region of hippocampus; SO: striatum oriens region of hippocampus; SR: stratum radiate region of hippocampus; arrow: GFAP immunoreactive cells, less reactive; Arrow head: GFAP immunoreactive, reactive astrocytes; Asterisk: area with abundant GFAP immunoreactive cells. 40X; microscopic field 40 amplifiers; 100X: microscopic field 100 amplifiers; 400X microscopic field 400 amplifiers.
Figure 9. The effect of acupuncture on the glial fibrillary acid protein (GFAP) immunoreactive cells in the SO and SR in the kainic acid treated rats.

The GFAP immunoreactive cells increased hippocampus 6 weeks after kainic acid administration. Manual acupuncture, 2 Hz electroacupuncture and 100 Hz electroacupuncture treatment reduced GFAP immunoreactive cells. Normal: normal group; Control: control group; MA: manual acupuncture group; EA2: 2 Hz electroacupuncture group; EA100: 100 Hz electroacupuncture group. Arrow: GFAP immunoreactive cells; CA1 region of hippocampus; SO: striatum oriens region of hippocampus; SR: stratum
radiate region of hippocampus; arrow: GFAP immunoreactive cells, less reactive; Arrow head: GFAP immunoreactive, reactive astrocytes; Asterisk: area with abundant GFAP immunoreactive cells. 40X; microscopic field 40 amplifiers; 100X: microscopic field 100 amplifiers; 400X microscopic field 400 amplifiers.
Figure 10. The effect of acupuncture on the glial fibrillary acid protein (GFAP) immunoreactive cells in the kainic acid treated rats.

The GFAP immunoreactive cells increased hippocampus 6 weeks after kainic acid administration. Manual acupuncture; 2 Hz electroacupuncture and 100 Hz electroacupuncture treatment reduced GFAP immunoreactive cells. Normal: normal group; Control: control group; MA: manual acupuncture group; EA2: 2 Hz electroacupuncture group; EA100: 100 Hz electroacupuncture group. Hilus: hilus region of hippocampus; SO: stratum oriens region of hippocampus; SR: stratum radiate region of hippocampus.

***p < 0.001 compared with Normal; #p < 0.05, ##p < 0.01, ###p < 0.001 compared with Control
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Abstract

Acupuncture has been used for epilepsy treatment for a long period of time in China. However, to our knowledge, the efficacy of acupuncture for preventing the development of epileptic focus formation is not yet elucidated until now. Therefore, the purpose of the present study was to investigate the effect of acupuncture on the development of epileptic focus formation. We establish a chronic epileptic model by using intraperitoneal administration of kainic acid (12 mg/kg, KA) in Sprague-Dawley (SD) rats for 6 weeks. The rats were randomly divided into groups in which they receive the manual acupuncture (MA), 2 Hz electroacupuncture (EA), 100 Hz EA on Baihui acupoint (GV20) and Fengfu acupoint (GV16), respectively, one week after KA administration. The rats were sacrificed 6 weeks after KA administration and the brain tissue was removed for section. The Timm’s staining was done for mossy fiber sprouting, NeuN staining for neuron, and glial fibrillary acidic protein (GFAP) staining for astrogliosis. The results indicated that MA, 2 Hz EA and 100 Hz EA reduced optical density difference of Timm’s staining of hippocampal dentate gyrus; 2 Hz EA and 100 Hz EA reduced NeuN immnuoreactive cells in the CA1 regions of hippocampus; MA and 100 Hz EA reduced GFAP immunoreactive cells in the hilus region of
hippocampus, and 2 Hz EA and 100 Hz EA reduced GFAP immunoreactive cells in the striatum oriens and stratum radiate regions of hippocampus.

In conclusion, our results indicated that MA, 2 Hz EA and 100 Hz EA at GV20 and GV16 for 5 weeks can reduce mossy fiber sprouting and also can reduce astrogliosis 6 weeks after KA administration in SD rats, suggesting that MA, 2 Hz EA and 100 Hz EA may prevent the development of epileptic focus formation, i.e. anti-epileptogenesis. In addition, 2 Hz EA may reduce neuron loss of hippocampus, whether or not 2 Hz may produce a greater effect in protection of neuron, further study is needed.

**Key words:** Manual acupuncture; Electroacupuncture; Kainic acid; Mossy fiber sprouting; Astrogliosis
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