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Electroacupuncture prevents cognitive deficits in pilocarpine-epileptic rats

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Abstract

Here we investigated the effects of electroacupuncture over the cognitive deficits in the pilocarpine model of epilepsy in rats. Acupuncture stimulation was provided at acupoints located in either the midline of the back and of the head [HD]: Gv-20 (*Baihui*), Gv-14 (*Dazhui*), Gv-2 (*Yaoshu*) and M-HN-3 (*Yin Tang*); or acupoints located in the limbs [LB]: St-36 (*Zusanli*) and Sp-6 (*Sanyinjiao*). In the elevated T-maze test, electroacupuncture at HD and LB acupoints produced an improvement in the acquisition and retention parameters. Retention in the inhibitory avoidance test was seen only in short-term retention and only for animals stimulated at HD. At histology it was found that electroacupuncture at HD acupoints abolished tissue shrinkage in dorsal hippocampus, basolateral nucleus of the amygdala, substantia nigra and perirhinal cortex, whereas stimulation of LB acupoints prevented tissue shrinkage in all of the above structures except dorsal hippocampus. Administration of *p*-chlorophenylalanine, a serotonergic releaser, abolished both behavioral and part of the histological changes in these animals. We conclude that electroacupuncture at HD and LB acupoints prevents atrophy of some limbic structures and improves cognitive deficits in pilocarpine-epileptic rats and that this effect is dependent on the serotonergic system.

Keywords: Electroacupuncture; Temporal lobe epilepsy; Learning; Memory; Rats

A number of structural abnormalities have been associated with temporal lobe epilepsy, including neuronal loss and mesial temporal sclerosis [4]. Neuronal injury, namely in hippocampus and amygdala, are likely to contribute to the cognitive deficits seen in both humans and laboratory animals with temporal lobe epilepsy [18,20], given the major role of these structures in learning and memory [1,11,26]. After injury, structural changes occur in the central nervous system. This post-lesional synaptic plasticity is associated with several of the behavioral consequences that take place thereafter [9,19].

It has been suggested that electroacupuncture (EA) applied at specific acupoints increases the extracellular serotonin levels. Serotonin is known to affect synaptic plasticity in various levels [2,24]. EA could thus affect post-lesional synaptic plasticity and cognitive deficits that ensue after pilocarpine-induced status epilepticus (SE).

Pilocarpine (320 mg/kg, i.p.) SE was induced in male adult Wistar rats (for detail of the protocol see [22]). Thirty minutes after SE induction, the animals were submitted to EA procedures. The following experimental groups were formed: Control (animals that were not injected with pilocarpine and not subjected to EA), Pilo (injected with pilocarpine and not submitted to EA), Sham (injected with pilocarpine and subjected to EA at 4 non-acupoints located in close vicinity to the acupoints), HD (injected with pilocarpine and subjected to EA at Gv-20 (*Baihui*), Gv-14 (*Dazhui*), Gv-2 (*Yaoshu*) and M-HN-3 (*Yin Tang*) acupoints) and LB (injected with pilocarpine and subjected to EA at St-36 (*Zusanli*) and Sp-6 (*Sanyinjiao*)

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acupoints). The localization of the acupoints was based on rat anatomical references [28]. A number of papers on different animal species have clearly evidenced that the location of acupuncture points follows a similar distribution in different mammals (for a review see [42]). The needle used for stimulation was 0.25 mm in diameter and 2 cm long, made of stainless steel stem and copper handle. For bilateral points, the positive output lead from the EA apparatus was connected to the left acupoint and the negative terminal was connected to the right acupoint and the polarity between these points was continuously alternated. For midline points the pairs were constituted between adjacent points. The LB group received acupuncture in points located on the hind limbs. In the rat, St-36 (*Zusanli*) is located approximately 1 mm lateral to the tibial tuberosity, whereas Sp-6 (*Sanyinjiao*) is located in the medial border of the tibia, 0.5 mm above the medial maleolus, both of which are easily located by manual inspection. The needles were bilaterally inserted at a depth of approximately 0.5 mm at the ST-36, and just above the skin at SP-6 (due to the lack of evident muscular mass under this area) and then subjected to EA (2 Hz/1 V, Plexus AP585, VWV Biotherapy/Lautz, Brazil), for a period of 20 min. The HD group received acupuncture in points located in the dorsal [Gv-14 (*Dazhui*), Gv-2 (*Yaoshu*)] and anterior [Gv-20 (*Baihui*), M-HN-3 (*Yin Tang*)], middle line of the body and the needles were inserted at a depth of approximately 0.5 mm in each acupoint and then subjected to EA (2 Hz/1 V), for a period of 20 min. Stimulation for the acupuncture and Sham groups was performed daily over a 7-day period.

In a second experiment, to investigate the relevance of the serotonergic system in the effects of EA, *p*-chlorophenylalanine (PCPA) at the dose of 100 mg/kg, i.p. was administered daily, for 3 consecutive days, previous to SE induction, resulting in the following groups: PCPA (injected with pilocarpine that received *p*-chlorophenylalanine and were not subjected to EA), Phd (injected with pilocarpine that received *p*-chlorophenylalanine and were subjected to EA at Gv-20 (*Baihui*), Gv-14 (*Dazhui*), Gv-2 (*Yaoshu*) and M-HN-3 (*Yin Tang*) acupoints) and Plb (injected with pilocarpine that received *p*-chlorophenylalanine and were subjected to EA at St-36 (*Zusanli*) and Sp-6 (*Sanyinjiao*) acupoints). This scheme of PCPA administration has been extensively validated as an effective means for serotonin depletion [3,36]. Stimulation for these acupuncture groups was performed with the same above-mentioned parameters, daily over a 7-day period. Eight and 9 days after SE induction, animals were evaluated in the elevated T-maze [7] and inhibitory avoidance [26] paradigms and were thereafter deeply anaesthetized (thionembutal 50 mg/kg, i.p.) and perfused. The brains were removed and processed with Nissl staining [22]. The following histological parameters were evaluated: number of neurons in the hilus, area of dorsal and ventral hippocampus, as well as, area of basolateral, lateral and central nuclei of the amygdala. Cell counts in the hilus region were performed at mid levels of the dorsal hippocampus (corresponding to paxinos Plate 30), at a selected area of 250 pixels^2 (1500 μm^2). The results attributed to each animal represent the mean value (left and right hemispheres of three adjacent sections). The area measurement of the above-mentioned structures was performed at coordinates corresponding to paxinos Plate 28 (for nuclei of the amygdala), 30 (for the dorsal hippocampus, perirhinal and entorhinal cortex), 40 (for the entorhinal cortex and substantia nigra), 42 (for the ventral hippocampus). The software package NIH-Image 1.62 was utilized to calculate the selected area. The data was obtained in pixels^2 and then converted into μm^2. The result attributed to each animal represents the mean value (left and right hemispheres of three adjacent sections). Two-way ANOVA followed by Tukey post hoc was used for comparison among groups. Student's *t* test for dependent measures was utilized for analysis of values within each group. Significance was set at $P < 0.05$.

As shown in Table 1, the Pilo and Sham groups had a poor performance in the elevated T-maze. Animals in those groups required significantly more trials to achieve the learning criterion (remain in the closed arm for over 298 s) when compared to controls ($F_{(7,49)} = 6.24$; $P < 0.05$) as well as showed a poor retention when tested 24 h latter ($F_{(7,49)} = 4.65$; $P < 0.01$). In contrast, animals subjected to EA in HD and LB showed a good performance both in acquisition and retention, and did not differ from controls. Furthermore, HD (but not LB) animals achieved a learning curve that was similar to that seen for controls, as confirmed by the significant improvement of the avoidance in the second and third trials as compared to the avoidance in the first trial. As expected, previous administration of *p*-chlorophenylalanine in the animals subjected to EA in HD and LB acupoints minimized the cognitive improvement of EA procedure, given that greater number of trials needed to achieve the learning criterion and the impairment of its retention performed 24 h latter.

In the short-term retention of the inhibitory avoidance paradigm, animals that received EA at HD acupoints, but not those stimulated at LB, showed a performance similar to that of controls. Again, as expected, this effect was abolished by previous treatment with *p*-chlorophenylalanine as the results were then similar to that observed in the other experimental groups injected with pilocarpine. In the long-term retention, all experimental groups showed a significant functional impairment ($F_{(7,49)} = 23.16$; $P < 0.01$). The background baseline latency for the animals to cross to the shock-receiving compartment was similar for all tested groups ($F_{(7,49)} = 0.40$) (see Fig. 1).

Table 2 summarizes the histological results. Both experimental groups injected with pilocarpine, including those subjected to EA, showed a strong diminution of the number of hilar cells ($F_{(7,49)} = 8.16$; $P < 0.01$), shrinkage of the central nucleus of the amygdala ($F_{(7,49)} = 6.12$; $P < 0.05$), entorhinal ($F_{(7,49)} = 8.62$; $P < 0.01$) and piriform cortex ($F_{(7,49)} = 8.79$; $P < 0.01$). Otherwise, the areas of ventral hippocampus and lateral nucleus of the amygdala in these experimental groups was similar to controls values ($F_{(7,49)} = 0.61$ and $F_{(7,49)} = 0.42$, respectively). In contrast,

Table 1
Effects of electroacupuncture at Gv-20 (*Baihui*), Gv-14 (*Dazhui*), Gv-2 (*Yaoshu*) and M-HN-3 (*Yin Tang*) as well as St-36 (*Zusanli*) and Sp-6 (Sanyinjiao) acupoints in pilocarpine-epileptic rats in the elevated T-maze test

Groups	Avoidance 1	Avoidance 2	Avoidance 3	Avoidance 24 h	Trials
Control	173.6 ± 39.3	299.5 ± 0.3$^{\#\#}$	299.5 ± 0.33$^{\#\#}$	300 ± 0	1.6 ± 0.2
Pilo	55.8 ± 34.8	158.8 ± 53.2	156.6 ± 54	123.5 ± 51.4**	10.8 ± 3.5*
Sham	22.6 ± 7.4**	10.8 ± 2.4**	11.8 ± 1.8**	18.4 ± 5.2**	17.4 ± 1.7**
HD	26.9 ± 7.1**	136.2 ± 48.1$^{\#}$	200.4 ± 48.7$^{\#\#}$	194.1 ± 41.2	3.4 ± 0.8
LB	51.8 ± 35.3	156.1 ± 46.3	167.6 ± 46.8	240.4 ± 38.8	6.1 ± 2
PCPA	40.3 ± 7.5	33 ± 8.7*	27.7 ± 5.6**	32.3 ± 13.5**	20 ± 0**
Phd	56.9 ± 19.6	102 ± 51.3*	105.7 ± 50.8*	69.6 ± 38.8**	12.7 ± 3.5*
Plb	76 ± 39.8	123.3 ± 49.7	110.9 ± 51.1*	91.6 ± 43.8**	8.6 ± 3.1*

Data expressed as mean ± S.E.M. Two-way ANOVA followed by Tukey post hoc. Student's *t* test for dependent measures. Control: animals that were not injected with pilocarpine and not submitted to EA; Pilo: injected with pilocarpine and not submitted to EA; Sham: injected with pilocarpine and submitted to EA at a non-acupoint; HD: injected with pilocarpine and submitted to EA at Gv-20 (*Baihui*), Gv-14 (*Dazhui*), Gv-2 (*Yaoshu*) and M-HN-3 (*Yin Tang*) acupoints; LB: injected with pilocarpine and submitted to EA at St-36 (*Zusanli*) and Sp-6 (*Sanyinjiao*) acupoints; PCPA: injected with pilocarpine that received *p*-chlorophenylalanine and were not submitted to EA; Phd: injected with pilocarpine that received *p*-chlorophenylalanine and were submitted to EA at Gv-20 (*Baihui*), Gv-14 (*Dazhui*), Gv-2 (*Yaoshu*) and M-HN-3 (*Yin Tang*) acupoints; Plb: injected with pilocarpine that received *p*-chlorophenylalanine and were submitted to EA at St-36 (*Zusanli*) and Sp-6 (*Sanyinjiao*) acupoints.

* $P < 0.05$, as compared to controls.

** $P < 0.01$, as compared to controls.

$^{\#}$ $P < 0.05$ as compared to Avoidance 1 value, within each group.

$^{\#\#}$ $P < 0.01$ as compared to Avoidance 1 value, within each group.

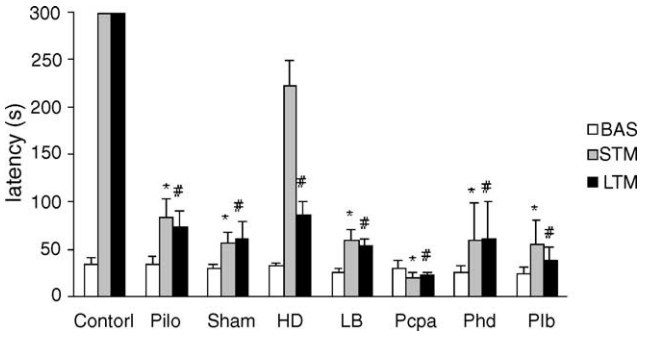

Fig. 1. Effects of EA at Gv-20 (*Baihui*), Gv-14 (*Dazhui*), Gv-2 (*Yaoshu*) and M-HN-3 (*Yin Tang*) as well as St-36 (*Zusanli*) and Sp-6 (*Sanyinjiao*) acupoints in pilocarpine-epileptic rats in the inhibitory avoidance test. Data expressed as mean ± S.E.M. Two-way ANOVA followed by Tukey post hoc. $^{*}P < 0.01$ as compared to Control and HD groups; $^{\#}P < 0.01$ as compared to Control group. Group division as in Table 1.

for EA animals neither HD nor LB groups presented tissue shrinkage in the basolateral nucleus of the amygdala, substantia nigra and perirhinal cortex, whereas animals of the HD group also presented a preservation of the dorsal hippocampus, which thus did not differ from controls. Previous administration of *p*-chlorophenylalanine was able to block the above described neuroprotective effect in both the hippocampus and basolateral nucleus of the amygdala. In contrast, previous administration of *p*-chlorophenylalanine did not alter the neuroprotective effect of acupuncture that was seen for the substantia nigra and perirhinal coretx.

Using two different cognitive tests we have shown that stimulation of Gv-20 (*Baihui*), Gv-14 (*Dazhui*), Gv-2 (*Yaoshu*) and M-HN-3 (*Yin Tang*) acupoints as well as St-36 (*Zusanli*) and Sp-6 (*Sanyinjiao*) acupoints promotes a functional recovery in the pilocarpine model of epilepsy in rats.

Table 2
Histological findings from brains of pilocarpine-epileptic rats submitted to EA at Gv-20 (*Baihui*), Gv-14 (*Dazhui*), Gv-2 (*Yaoshu*) and M-HN-3 (*Yin Tang*) as well as St-36 (*Zusanli*) and Sp-6 (*Sanyinjiao*) acupoints

	Control	Pilo	Sham	Hd	Hl	Pcpa	Phd	Plb
Number of hilar cells	15.9 ± 0.7	3.4 ± 0.3**	4.6 ± 0.6**	4.6 ± 0.4**	3 ± 0.9**	4.1 ± 0.8**	4.4 ± 0.6**	3.9 ± 0.4**
Area of dHpc (10^3 μm^2)	757 ± 15	642 ± 24*	632 ± 50*	736 ± 52	646 ± 24*	630 ± 34*	638 ± 32*	644 ± 22*
Area of vHpc (10^3 μm^2)	1046 ± 16	988 ± 25	1078 ± 37	1074 ± 35	977 ± 48	1038 ± 36	1019 ± 40	1054 ± 44
Area of LAmg (10^3 μm^2)	30 ± 4	21 ± 4	23 ± 4	26 ± 2	27 ± 4	28 ± 2	24 ± 3	29 ± 5
Area of BlAmg (10^3 μm^2)	90 ± 9	64 ± 8*	67 ± 9*	95 ± 6	100 ± 11	63 ± 9*	65 ± 5*	71 ± 8*
Area of CeAmg (10^3 μm^2)	81 ± 4	47 ± 5**	59 ± 8**	63 ± 7*	55 ± 4**	58 ± 6**	52 ± 4**	58 ± 8**
Area of SN (10^3 μm^2)	157 ± 7	106 ± 8**	102 ± 9**	121 ± 9	128 ± 10	108 ± 3**	148 ± 15	146 ± 8
Area of Peri (10^3 μm^2)	126 ± 4	94 ± 5*	89 ± 2**	138 ± 5	128 ± 7	90 ± 3*	138 ± 7	142 ± 11
Area of Pir (10^3 μm^2)	64 ± 3	39 ± 3**	32 ± 4**	28 ± 3**	29 ± 2**	26 ± 2**	34 ± 6**	31 ± 6**
Area of Ent (10^3 μm^2)	32 ± 2	17 ± 2**	22 ± 2**	22 ± 2**	19 ± 2**	16 ± 1**	21 ± 3**	20 ± 2**

Data expressed as mean ± S.E.M. Two-way ANOVA followed by Tukey post hoc. dHpc: area of dorsal hippocampus; vHpc: area of ventral hippocampus; LAmg: area of lateral nucleus of amygdala; BlAmg: area of basolateral nucleus of amygdala; CeAmg: area of central nucleus of amygdala; SN: substantia nigra; Ent: entorhinal cortex, layer 2; Peri: perirrinal cortex; Pir: piriform cortex, layer 2. Abbreviations for the different animal groups are the same as in Table 1.

* $P < 0.05$ as compared to control values.

** $P < 0.01$ as compared to control values.

Stimulation of these acupoints minimized tissue shrinkage in some of the structures damaged in the pilocarpine model. This improvement behavioral aspect was blocked by previous *p*-chlorophenylalanine administration, indicating a role for the serotonergic system in the effects of acupuncture.

The similar behavioral findings observed for the HD and LB groups with regard to the T-maze are apparently at odds with the anatomical findings. Indeed, whereas the LB group displayed pronounced cell damage in the dorsal hippocampus, this was not seen for the HD group. It has been suggested that the dorsal hippocampus might be critical for the T-maze test [8,23]. Therefore, our current data support the notion that other structures, such as the basolateral amygdala are more fundamental for this test. However, in the literature, in contrast to several works that associated the amygdala to anxiety levels measured in the T-maze test [35,41], there are no reports with regard to the importance of the amygdala concerning the cognitive aspects evaluated by this model. Therefore, currently we have no good basis to explain the above discrepancy.

Only animals of the HD group presented an inhibitory avoidance response similar to controls. This result, however, was seen only in short-term memory. Indeed, the mechanisms underlying short- and long-term memory have long been known to have different basis [1,26]. Accordingly, our results suggest that stimulation of the specific set of acupoints performed for the HD group generates a functional selective effect over short-term memory in this test. We speculate that the better performance of the HD group is associated with the lesser damage in the dorsal hippocampus.

The positive consequences of EA over learning and memory have already been reported before [5,10,39]. Our findings of a blockade of these effects by *p*-chlrophenylalanine suggest an involvement of serotonin in these effects. Matsumoto et al. verified that administration of 5-HT_4 receptor agonist SC 53116 had an ameliorative effect on scopolamine-induced impairment of learning [25]. Indeed, Chang et al. verified that acupuncture at Gv-20 (*Baihui*) prevented the cycloheximide-induced impairment of passive avoidance response and this effect was reduced by a serotonergic releasing agent [5]. In contrast, various works also report on the negative effects of serotonin in learning and memory. It has been described that administration of 5-HT_{1A} agonist [11,12,26] and 5-HT_2 agonist [11] impair retention performance in the inhibitory avoidance-learning task. Thus, the improvement of cognitive function seen in both HD and LB groups might not be a simple reflection of a general increase in serotonin levels, as this would probably yield opposite effects.

The functional recovery shown here could be explained by both neuroprotective effects and altered synaptic plasticity triggered by EA. In fact, acupuncture suppresses the apoptosis induced by schema in the hippocampal CA1 region [13], as well as by hemorrhage [6] and by 6-hydroxydopamine [31] in the striatum. In agreement, here we report significant neuronal protection provided by acupuncture in a rat model of epilepsy. Whereas we have not addressed the effects of EA over synaptic plasticity; those have been reported elsewhere [14,15,21,34,40] and might have contributed to the observed functional results.

It has been shown that serotonin mediates neuroprotective effects against neuronal damage [17,38]. Vermetten et al. described that long-term treatment with paroxetine (a serotonin uptake inhibitor) abolished hippocampal atrophy in patients with posttraumatic stress disorder [38]. Indeed, Klisch et al. described a neuroprotective effect of 5-HT_{1A} agonist and 5-HT_{2A} antagonist against transient forebrain schema in gerbils [17]. Thus, it can be speculated that augmented extracellular serotonin levels associated with electroacupuncture, can activate 5-HT_{1A} receptors thus promoting neuroprotection against SE-induced neuronal damage. In fact, stimulation of 5-HT_{1A} receptor promotes neuroprotection in different animal models of central nervous system injury, such as schema [34,37], NMDA excitotoxicity [29,30] and traumatic brain injury [16]. The relevance of serotonin-mediated damage does not seem to apply for all brain areas given that previous administration of *p*-chlorophenylalanine resulted in only a partial blockade of the neuroprotective action provided by acupuncture. Further studies are needed to investigate this interesting finding.

To summarize, EA at Gv-20 (*Baihui*), Gv-14 (*Dazhui*), Gv-2 (*Yaoshu*), M-HN-3 (*Yin Tang*) acupoints as well as at St-36 (*Zusanli*) and Sp-6 (*Sanyinjiao*) acupoints prevents the tissue shrinkage at some limbic structures and improves cognitive deficits in pilocarpine-epileptic rats. The functional recovery is probably due to neuroprotective effects and increment of synaptic plasticity related to serotonergic pathways.

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