Electroacupuncture Attenuates Both Glutamate Release and Hyperemia After Transient Ischemia in Gerbils

Jinming Pang,* Toshifumi Itano,* Kazunori Sumitani,† Tetsuro Negi† and Osamu Miyamoto*

Departments of *Neurobiology and †Basic Sports Medicine, Kagawa Medical University
Ikenobe, Miki-cho, Kagawa 761-0793, Japan

Abstract: Although many studies have indicated that electroacupuncture (EA) provides a neuroprotective effect against ischemic brain damage, the protective mechanism is not fully understood. Glutamate release and hippocampal blood flow in ischemia with EA were investigated to elucidate the neuroprotective mechanism of EA. Transient 5-minute ischemia was induced in gerbils. EA (7 Hz, 0.6 mA, for 30 minutes) delivered to the points called Fengfu (GV16) and Shendao (GV11) was administered pre-, intra- or post-ischemia. The procedure rescued hippocampal neurons from ischemic insult and significantly attenuated both ischemia-induced glutamate release and transient increase of cerebral blood flow (CBF) during reperfusion (hyperemia). Hyperemia as well as excessive glutamate after ischemia are regarded as important factors in brain damage as they lead to reperfusion injury. These results suggest that EA protects neurons by suppressing both glutamate release and reperfusion injury after ischemia.

Keywords: Global Ischemia; Electroacupuncture; Neuronal Density; Glutamate; Cerebral Blood Flow; Reperfusion Injury; Gerbil Hippocampus.

Introduction

Acupuncture may decrease ischemic brain damage in clinical (Zhang et al., 1997; Si et al., 1998) and laboratory (Ying and Cheng, 1994; Zhao and Cheng, 1997; Si et al., 1998; Yang et al., 1999) settings. Si et al. (1998) reported that the neurological functions of acute ischemic stroke patients are significantly recovered by electroacupuncture (EA). In the laboratory, ischemic brain damage is decreased by EA in rats (Zhao and Cheng, 1997; Si et al., 1998; Yang et al., 1999) and gerbils (Ying and Cheng, 1994). Somatosensory potentials which are useful for monitoring ischemic damage recover more quickly with, than without EA in rats.
with middle cerebral artery occlusion (MCAO) (Si et al., 1998). Several mechanisms for the protective effects of EA have been suggested. Zhao and Cheng (1997) demonstrated that EA affects extracellular excitatory and inhibitory amino acid levels in MCAO rats. Electroacupuncture also enhances the expression of basic fibroblast growth factor, which may protect neurons from ischemic insult (Mattson and Scheff, 1994), in the striatum and cortex of MCAO rats (Yang et al., 1999). Moreover, EA attenuated nitric oxide release, which is neurotoxic when overproduced, from the MCAO rat striatum (Zhao et al., 2000).

However, the precise mechanism of EA function remains controversial.

Excessive release of glutamate during ischemia is widely regarded as an important factor in the pathogenesis of ischemic neuronal injury (Siesjö, 1988; Mitani et al., 1990). Glutamate levels abruptly increase, then slowly return to pre-ischemic levels upon reperfusion (Mitani and Kataoka, 1991; Asai et al., 1996). On the other hand, cerebral blood flow (CBF) frequently increases transiently during reperfusion after ischemia (hyperemia). Reactive oxygen species, such as hydroxyl radicals and superoxide radicals (Dugan and Choi, 1994), are produced during ischemia, especially during reperfusion, and hyperemia exacerbates ischemic neuronal damage (Aronowski et al., 1997). Thus, extracellular glutamate and CBF are significantly changed by ischemic insult and these events are considered as the main cause of neuronal cell death in ischemia. The present study investigates the mechanism of the protective effects of EA, focusing on ischemia-induced glutamate release and hyperemia.

Materials and Methods

Mongolian gerbils weighing 60–70 g were obtained from Seiwa Experimental Animals (Fukuoka, Japan), and housed under a 12:12-hour light-dark cycle at 24°C with food and water ad libitum. The gerbils were divided into three groups; sham control (Sham), ischemia only (Isch) and EA treatment (EA) groups. The EA group was further divided into four subgroups: sham operation with EA (EA-sham), EA pre-ischemia (EA-pre), EA intra-ischemia (EA-intra), and EA post-ischemia (EA-post). All animal experiments proceeded in accordance with the guidelines described in the Guiding Principles for the Care and Use of Animals approved by the Council of the Physiological Society of Japan. The animals were anesthetized with barbiturate (30 mg/kg), then the common carotid arteries were exposed bilaterally and 3-0 silk was threaded around the carotid arteries. Transient global ischemia was induced for 5 minutes by occluding the bilateral common carotid arteries with micro-aneurysm clips (Sugita Clip, Mizuho, Nagoya, Japan). Rectal and tympanic temperatures were maintained at around 37°C using a feedback-controlled heating pad (CMA, Stockholm, Sweden) and an overhead lamp during the operation. Neurons of CA1 subfield in the hippocampus start to die 2 or 3 days after ischemic insult in this ischemic model [delayed neuronal cell death (Kirino, 1982)]. Sham control animals underwent the same procedure except for occlusion of the carotid arteries. The points Fengfu (GV16) and Shendao (GV11), which are considered very important for brain functions, were stimulated using an EA apparatus (Model G 6805-2 Multi-purpose Health Device, China) for 30 minutes with electrical pulses at a frequency of 7 Hz and an intensity of 6 mA. EA was started at 30 or 15 minutes before, or immediately after ischemia (EA-pre, EA-intra and EA-post, respectively).
For histological examination, the animals (n = 5 per group) were anesthetized with sodium pentobarbital (50 mg/kg i.p.) 1 week after ischemia and transcardially perfused with 4% phosphate-buffered paraformaldehyde after a flush of 0.1 M phosphate-buffered saline. The brains were removed, post-fixed at 4°C in the same fixative overnight and embedded in paraffin. Each brain was serially sectioned in the coronal plane at a thickness of 8 µm using a microtome, mounted on a gelatin-coated glass slide, then stained with hematoxylin-eosin (HE). Neurons were examined under a light microscope at ×400 magnification by a person who was blinded to the study. The number of normal neurons in the bilateral CA1 subfield was counted on one histological section corresponding to roughly 1.7 mm posterior to the bregma from each animal, and is expressed as the number of cells per 1 mm of the pyramidal cell layer [neuronal density (Lee et al., 1994)].

Extracellular glutamate was measured as described previously (Yamagami et al., 1998). Briefly, a dialysis electrode (Microdialysis Biosensor, Sycopel International Ltd., London, UK) was filled with a perfusate of 10 mM phosphate-buffered saline (PBS, pH 7.4) containing glutamate oxidase (100 U/ml, Yamasa Co. Ltd., Chiba, Japan). The perfusate entered the fluid inlet tube at a rate of 0.2 µl/min via a perfusion pump (IP-2, Bio Research Center Co Ltd., Nagoya, Japan). The dialysis electrode was connected to an ESP-800 potentiostat (Eicom, Kyoto, Japan) and a voltage clamp was controlled at +650 mV for 30 minutes to induce electropolymerization. After placing of the gerbils in a stereotaxic apparatus, the dialysis electrode was directed to the dorsal hippocampus (1.7 mm posterior to the bregma, 2 mm to the right of the midline and 1.5 mm-depth from the dura surface). Glutamate was monitored in the Isch (n = 12) and EA groups (n = 6 for EA-pre and EA-intra; n = 5 for EA-post) before, during and after ischemia. Body temperature was maintained at around 37°C during measurement. After measuring glutamate, the brains were fixed and cut to confirm the position of the electrode. Data from areas outside the dorsal hippocampus were excluded from the study.

We measured regional cerebral blood flow (rCBF) as already described (Yamagami et al., 1998). Briefly, rCBF in the dorsal hippocampus was monitored in all groups (n = 4, 10, 5, 9, 13 and 5 in Sham, Isch, EA-sham, EA-pre, EA-intra and EA-post, respectively) using a laser Doppler flowmeter (Bio Research Center Co., Ltd. Nagoya, Japan) with a needle-type probe. A laser Doppler probe of 0.5 mm-diameter was directed to the dorsal hippocampus as described for glutamate measurement. Regional CBF is expressed as a percentage of the average baseline, since values displayed by the laser Doppler flowmeter are not absolute. The influence of EA on arterial blood pressure (BP) was also investigated. The femoral artery of the anesthetized gerbils was cannulated (PE-10 tubing) and BP readings were digitized, displayed and recorded every second by computer before and during EA (n = 6).

Data expressed as means ± standard deviation (SD) were analyzed by the one-way analysis of variance for neuronal density and the two-way analysis of variance for glutamate and CBF, followed by Scheffé’s F post-hoc test. A probability level of < 0.05 was considered to be statistically significant. All statistics were generated using Statview software (Abacus concept, Berkeley, USA).
Results

Histological results are illustrated in Figs. 1 and 2. Neuronal density between Sham (230 ± 15 cells/mm) and EA-sham (220 ± 4 cells/mm) groups in the CA1 subfield did not significantly differ. Ischemia for 5 minutes reduced neuronal density to 5% of that of the Sham group (20 ± 11 cells/mm in the Isch group). Hippocampal neurons were significantly rescued from ischemic insult in all EA groups (121 ± 12 cells/mm, 203 ± 24 cells/mm, 192 ± 19 cells/mm in EA-pre, EA-intra and EA-post, respectively).

The time-course of glutamate release in the CA1 subfield during 5-minute ischemia and reperfusion in the four groups is shown in Fig. 3. Glutamate levels were significantly increased by ischemia, reached a maximum 15 minutes after reperfusion (six-fold increase) that remained higher than the pre-ischemic levels even 60 minutes after reperfusion (Isch group). EA intra-ischemia significantly inhibited glutamate release during and after ischemia (EA-intra), and EA post-ischemia also depressed glutamate release after reperfusion (EA-post). Pre-ischemic EA treatment did not affect glutamate release during ischemia.

The effect of EA on hippocampal rCBF pre-, intra- and post-ischemia is shown in Figs. 4 and 5. Electroacupuncture tended to increase rCBF in the EA-sham group (data not shown), while EA did not affect BP (83.5 ± 14.4 mmHg pre-EA and 80.6 ± 7.4 mmHg during-EA, n.s.). Ischemia for 5 minutes decreased hippocampal CBF to around 40% of the pre-ischemic level in all groups except EA-intra. Increase of rCBF during ischemia was observed with intra-ischemic EA stimulation (EA-intra, Figs. 4c and 5a). Regional CBF started to increase immediately after reperfusion, and lasted for 45 minutes in the Isch group (hyperemia, Fig. 4a). On the other hand, hyperemia was suppressed in all EA groups (Figs. 4b to d). The time taken to return to baseline was decreased in all EA groups and the time to peak level of hyperemia was also significantly depressed in the EA-post group compared with Isch group (Fig. 5b).

Figure 1. Representative hematoxylin-eosin-stained sections from CA1 subfield of the hippocampus. Almost all neurons have been killed by ischemia (c), while many viable neurons are present in EA groups (d–f). a, Sham; b, EA-sham; c, Isch; d, EA-pre; e, EA-intra; and f, EA-post (bar = 100 µm).
Figure 2. Bar graph of neuronal density of the CA1 subfield 1 week after ischemia or sham operation. Values are means ± SD. *p < 0.001 compared with Isch group. †p < 0.01; ††p < 0.001 compared with Sham group.

Figure 3. Effect of EA on hippocampal glutamate release by ischemia. Glutamate was significantly increased by ischemia, while EA intra- and post-ischemia significantly depressed glutamate release during and after ischemia. Values are means ± SD. * or †p < 0.05; ** or ††p < 0.01 compared with Isch group.
Figure 4. Effect of EA on hippocampal blood flow during and after ischemia. Values are means ± SD. Ischemia was induced from −5 minutes to 0 minutes, and all groups except Isch were stimulated by EA for 30 minutes. a, Isch; b, EA-pre; c, EA-intra; and d, EA-post. * p < 0.05; ** p < 0.001 compared with Isch group.

Figure 5. Bar graphs of levels and time course of hippocampal blood flow. Hyperemia developed after ischemia in all groups, but time of hyperemia were significantly attenuated in all EA groups. a, rCBF level in reperfusion (left) and ischemia (right). b, Time required to return to pre-ischemic level (left) and time required to reach peak level of rCBF (right). Values are means ± SD. * p < 0.01; ** p < 0.001 compared with Isch group.
Discussion

The present study showed that EA significantly protected hippocampal neurons from delayed neuronal cell death. The effectiveness of EA against ischemic brain damage has been demonstrated by others (Ying and Cheng, 1994; Yang et al., 1999). Ying and Cheng (1994) reported that neuronal degeneration 24 hours after 10 minutes of ischemia in the gerbil hippocampus is attenuated by 30 minutes EA during and after ischemia. Yang et al. demonstrated that 60 minutes of EA not only intra- but also post-ischemia reduces cerebral infarction 24 hours after middle cerebral artery occlusion (MCAO) in the rat (Yang et al., 1999). Results from such studies, taken together with the present results, suggest that pre-, intra- and post-ischemic EA rescues neurons from acute and delayed neuronal cell death induced by ischemia.

EA affected both glutamate release and rCBF in ischemia. Real time monitoring of extracellular glutamate by electro-enzymatic microdialysis in the present study confirmed that glutamate rapidly increases during and after ischemia (Asai et al., 1996). Intra-ischemic EA attenuated glutamate release during and after ischemia, while post-ischemic EA depressed the peak level of glutamate during reperfusion. Zhao and Cheng (1997) reported that EA significantly decreases ischemia-induced aspartate, but not glutamate levels in the striatum of MCAO rats. This discrepancy may arise from differences in animal models (striatum of MCAO rats in Zhao’s study; gerbil hippocampus of global ischemia in the present study) and EA points (Fengfu and Jinsuo in Zhao’s study; Fengfu and Shendao in the present study). The inhibition of glutamate release might also be one mechanism of the neuroprotective effect of EA.

Uchida et al. reported that acupuncture-like stimulation increases cortical blood flow by activating cholinergic vasodilators and the increase of blood flow is independent of BP (Uchida et al., 2000). Kagitani et al. found an increase in hippocampal blood flow by activating nicotinic receptors with nicotine attenuating ischemia-induced delayed neuronal cell death in the hippocampus (Kagitani et al., 2000). Hippocampal CBF was also increased by EA stimulation and BP was not involved in the increase of CBF in the present study. The increase of rCBF may reflect the activation of nicotinic acetylcholine receptors caused by EA delivered to the points of Fengfu and Shendao. The increase of rCBF during ischemia in the EA-intra group may be one of the neuroprotective mechanisms of EA.

Several researchers have noted the contribution of reperfusion after ischemia to brain damage (Leff and Repine, 1990; Uyama et al., 1992; Strasser et al., 1994; Aronowski et al., 1997). CBF usually increases after ischemia (hyperemia) and a considerable number of reactive oxygen species are produced during reperfusion because of the increased oxygen supply and metabolism; this exacerbates ischemic brain damage (reperfusion injury) (Leff and Repine, 1990; Strasser et al., 1994; Aronowski et al., 1997). Seventy-two percent of ischemic brain damage is produced by reperfusion in transient focal ischemic rats (Aronowski et al., 1997). Hyperemia during reperfusion was inhibited in all EA groups in the present study. Zhao et al. (2000) reported that EA directly attenuates nitric oxide (NO) release, which is considered an important factor in hyperemia after ischemia (Greenberg et al., 1995), in MCAO rats. Attenuation of NO release by EA may suppress hyperemia, leading to neuroprotection during reperfusion after ischemic insult.
These results suggest that EA suppresses both hyperemia and excessive glutamate release during and after ischemia, and that such suppression contributes to the neuroprotective effect of EA. Therefore, EA may be a useful tool with which to treat stroke patients since post-ischemic as well as intra-ischemic EA is very effective against ischemic brain damage.

Acknowledgments

This work was supported by a Grant-in-Aid for Scientific Research from the Japanese Society for the Promotion of Science.

References


