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Electroacupuncture regulates TRPM7 expression through the trkA/PI3K pathway after cerebral ischemia–reperfusion in rats

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Abstract

Recently, it was demonstrated that TRPM7 is an essential mediator of anoxia-induced neuronal death. Meanwhile, nerve growth factor (NGF) is known to have survival and neuroprotective effects by interacting with the high affinity neurotrophin receptor, tropomyosin-related kinase A (trkA). In the present study, we found that electroacupuncture (EA) treatment could up-regulate trkA expression after focal cerebral ischemia in rats. At the same time, EA therapy obviously decreased the high expression of TRPM7 induced by ischemia. Using K252a to inhibit trkA, we found that the EA-mediated down-regulation of TRPM7 was significantly suppressed in rats subjected to cerebral ischemia. TrkA can utilize two distinct signaling pathways: the phosphatidylinositol 3-kinase (PI3K) pathway and the extracellular signal-related kinase (ERK) pathway. We found that the effect of EA on TRPM7 was also inhibited by a PI3K inhibitor, while an ERK inhibitor had no effect. Taken together, our findings suggest that EA can reverse the ischemia-induced increase of TRPM7 levels through the trkA–PI3K pathway.

Keywords: Cerebral ischemia; Electroacupuncture; TRPM7; trkA; PI3K

Introduction

Stroke is one of the leading causes of death in the world, and many people are currently affected by this disease. Well-documented experimental evidence strongly supports that Ca^{2+} influx through *N*-methyl-D-aspartate glutamate receptors (NMDARs) is the major pathway leading to the excitotoxic cell death associated with the ischemic periods of stroke (Lipton, 1999). However, NMDA receptor blockers are effective only during a narrow window of one hour post-stroke, and anti-excitotoxic therapy has failed to reduce brain damage in humans (Hoyte et al., 2004; Yang et al., 1997). These observations raise questions about our understanding of ischemic neurodegeneration. Recently, Aarts and colleagues discovered that a special cell surface channel, TRPM7, is activated when brain cells are deprived of oxygen and vital nutrients, triggering a lethal chain reaction (Aarts et al., 2003). TRPM7 is a member of the transient receptor potential (TRP) cation channel superfamily. TRPM7, a calcium-permeable non-selective ion channel, has been proposed as a requirement for cell survival (Nadler et al., 2001). In the present study, we further explored whether TRPM7 expression was increased in rats subjected to cerebral ischemia.

Nerve growth factor (NGF) is known to have survival and differentiation effects on neuronal cells (Kaplan and Miller, 1997; Segal and Greenberg, 1996). Neuroprotective effects of NGF in hippocampal and cortical neurons have been demonstrated both in vitro and in vivo (Culmsee et al., 1999; Kume et al., 2000). To mediate these actions, NGF binds to the high affinity neurotrophin receptor tropomyosin-related kinase A (trkA) to trigger the intracellular signaling cascades (Kaplan and Miller, 2000). Two kinases whose activities mediate these processes are phosphatidylinositol 3-kinase (PI3K) and extracellular signal-related kinase (ERK) (Hetman et al., 1999). An ischemia-induced increase in trkA in ischemia-resistant regions is important for neuronal survival and functioning in these regions. Also, it has been found that both PI3K and ERK are related to neuronal survival.

Electroacupuncture (EA) has been shown to produce beneficial effects in stroke patients, including reduced speech

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retardation (Liu et al., 2000; Zhang and Ni, 1998; Zhao et al., 1998), improved locomotion (Liu et al., 1999), and enhanced memory (Li and Shen, 1998). Experimental studies reported that EA could effectively attenuate cerebral infarction and inhibit apoptosis (OuYang et al., 1999; Shi et al., 1998). EA can reduce the expression of bax protein, which promotes apoptosis, and increase the expression of bcl-2 protein, which resists apoptosis, showing that EA has an anti-apoptotic effect (Guo et al., 2002). EA can enhance NGF expression for at least five weeks to protect the cerebral tissue in ischemia (Yi et al., 2006). However, the possible biochemical and molecular mechanisms through which EA exerts its effect are not yet completely clear. We investigated the effects of EA on the expression of TRPM7 and trkA in the present study. Furthermore, we sought to explore whether trkA was necessary for the effect of EA on TRPM7 expression and possible signaling pathways mediated by trkA. On the basis of the Xingnao Kaiqiao acupuncture method, Renzhong (DU26) and Chengjiang (RN24) were selected for this study (Han et al., 1998; Zou et al., 2005). The Xingnao Kaiqiao acupuncture method is an effective method for curing cerebral diseases and has significant effects on improving cerebral function (Ding and Shi, 2004). EA applied at Renzhong and Chengjiang could significantly protect neurons from apoptosis and reduce the area of cerebral infarction (Shi et al., 1998).

Methods

Animals

Adult male Sprague–Dawley rats (Center of Experimental Animals, Tongji Medical College of Huazhong University of Science and Technology) weighing 200–250 g were divided into seven groups, namely sham group (no middle cerebral artery occlusion and EA treatment), ischemia group (ischemic reperfusion), ischemia-EA group (ischemic reperfusion with EA treatment), DMSO group (ischemic reperfusion with EA and DMSO treatments), K252a group (ischemic reperfusion with EA and K252a treatments), Wortmannin group (ischemic reperfusion with EA and Wortmannin treatments) and U0126 group (ischemic reperfusion with EA and U0126 treatments). The experimental protocol and procedures were approved by the Animal Committee of Tongji Medical College of Huazhong University of Science and Technology. The rats were housed with the dam in the home cage under a 12:12 h light/dark cycle, with food and water available ad libitum throughout the study.

Surgical procedures

The surgical procedures were described previously (Longa et al., 1989), with slight modification. Briefly, each rat was first anesthetized with 10% chloral hydrate (350 mg/kg). Then, the right common carotid artery was carefully exposed, and the right internal and external carotid arteries were dissected out. The origin of the right external carotid artery was ligated with 5-0 silk suture. A 60-mm-long 4-0 nylon surgical thread, heated to make the distal end round, was then inserted from the right carotid bifurcation. The thread was passed through the internal carotid artery to occlude the middle cerebral artery (MCA). When the blunted distal end met resistance, the proximal end of the thread was tightened at the carotid bifurcation. Approximately 18 mm of nylon surgical thread (depending on the weight of the rat) was inserted into the right internal carotid artery. After a 30 min occlusion of the right MCA, the nylon surgical thread was removed to allow complete reperfusion of the ischemic area. During ischemia, rectal temperature was monitored in all the animals and was maintained at approximately 37 °C with a heating pad and an overhead lamp. After restoration of cerebral blood flow (CBF), the animals were allowed to recover at ambient temperature (24 °C) until sampling. Sham control rats received the same surgical procedures except that the MCA was not occluded.

EA treatment

EA was applied at the anatomical points of Renzhong (DU26, anode) and Chengjiang (RN24, cathode) by using an EA stimulator (Model DM-A, Haidian Medical Electronic Instruments, Beijing, China) as soon as the nylon filament was removed. In humans, Renzhong (Du26) lies at the junction of the superior third and middle third of the philtrum, and Chengjiang (RN24) lies in the center of the mentolabial groove. In rats, Renzhong (DU26) is located on the midline, 1 mm inferior to the nose, and Chengjiang (RN24) is located on the midline, 1 mm inferior to the underlip (Li, 2003). Rats were subjected to EA for 30 min under the following conditions (Sun et al., 2003): alternating trains of dense–sparse frequencies (dense 16 Hz for 1.5 s and sparse 4 Hz for 1.5 s, alternately) were selected, and the initial tension of 1 V was increased at a rate of 1 V per 10 min, to a terminal tension of 3 V.

Drug treatment

Just before MCA occlusion, the rats received an intracerebroventricular injection of 5 μl K252a [200 nM (Gooney and Lynch, 2001), BioSource, California, USA], Wortmannin (0.1 nmol, Alexis, Lausen, Switzerland), or U0126 (1 nmol, Alexis, Lausen, Switzerland) in DMSO into the right hemisphere. Injections were performed with a Hamilton syringe with a 27-gauge needle. The location of each injection was 1 mm posterior to the bregma, 1.8 mm lateral to the midline, and 4.2 mm below the skull surface (Paxinos and Watson, 1998). The rats underwent MCA occlusion and EA treatment as described above.

Immunofluorescent labeling

At 24 h following reperfusion, the rats were deeply anesthetized and then perfused through the left ventricle with 4% paraformaldehyde in 0.1 M phosphate buffer saline (PBS, pH 7.4). Brains were then removed, post-fixed overnight at 4 °C in the same fixative, and immersed in 0.1 M PBS containing 30% sucrose for one day. Coronal sections (20 μm) through the forebrain were cut on a freezing sliding microtome. Then sections of the ischemic side were rinsed three times in 0.01 M PBS (pH 7.4).

To single-label TRPM7, 20 μm free-floating sections were blocked with 3% bovine serum albumin (BSA) in 0.01 M PBS for 1 h. Then the sections were incubated for 1 h at room temperature and over 48 h at 4 °C with a first antibody: a goat polyclonal antibody against TRPM7 (1:50, Santa Cruz, CA, USA) in orifice plate. After washes with 0.01 M PBS, donkey anti-goat antiserum

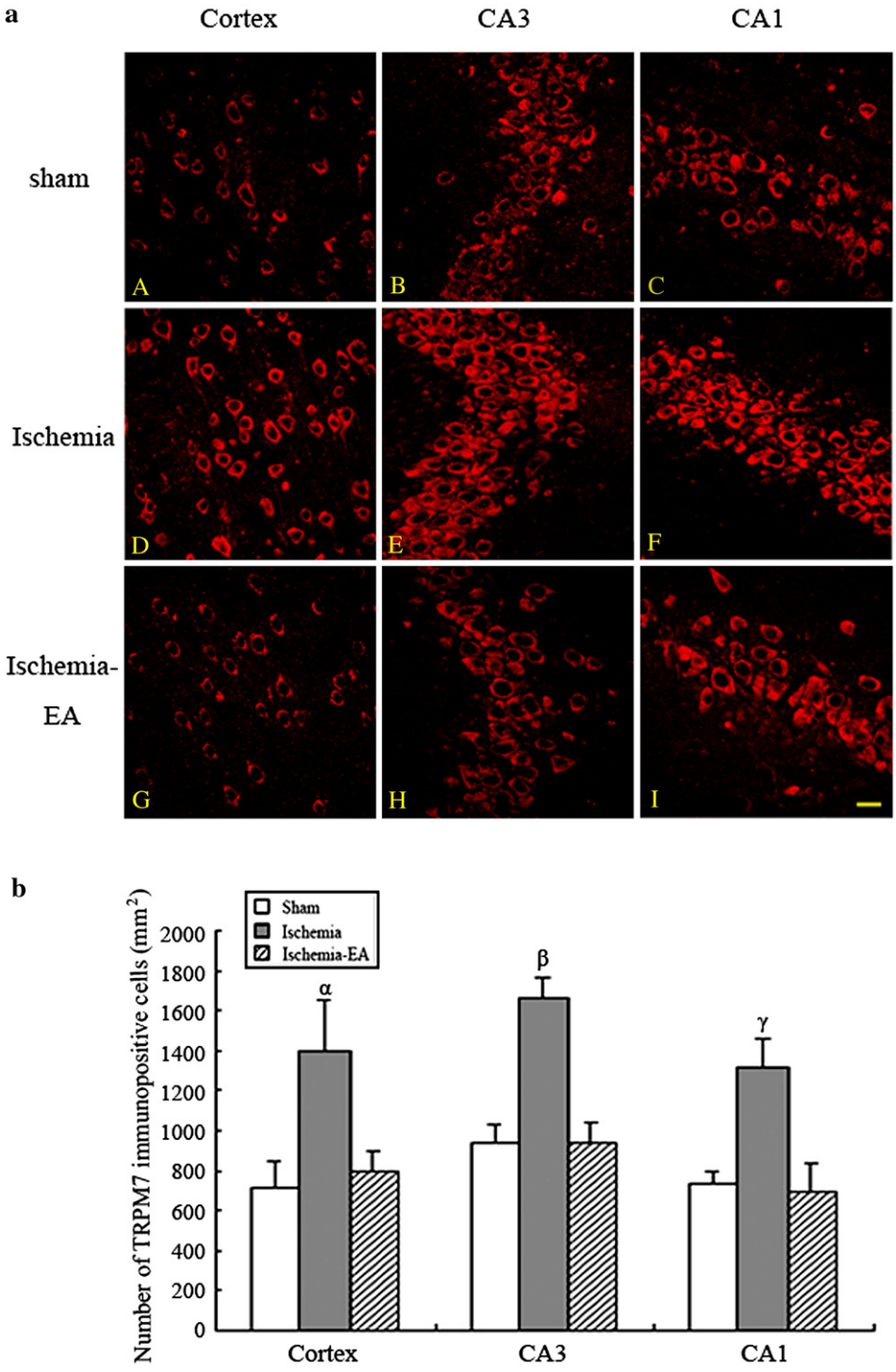

Fig. 1. Effect of EA on TRPM7 expression in the ischemic cortex and hippocampus. (a) Representative immunofluorescence staining for TRPM7 in the cortex (A, D, G) and the hippocampal CA3 (B, E, H) and CA1 regions (C, F, I). The red cells are TRPM7-positive neurons. Sham = no MCA occlusion and EA treatment; Ischemia = ischemic reperfusion; and Ischemia-EA = ischemic reperfusion with EA treatment. TRPM7-ir was moderately detectable in the sham group (A, B, C). A significant increase in both the number of stained cells and the staining strength was observed in the ischemia group (D, E, F). EA treatment diminished the high expression of TRPM7 induced by ischemia (G, H, I). Scale bar = 20 μm. (b) Quantitative representation of TRPM7 immunopositive cells in the cortex and hippocampal CA3 and CA1 regions in each group. Values are mean ± SD. $n=5$ in each group. $^{\alpha}$Significant difference at $P<0.05$ compared to the sham and ischemia-EA groups in the cortex, respectively; $^{\beta}$significant difference at $P<0.05$ compared to the sham and ischemia-EA groups in the hippocampal CA3 region, respectively; $^{\gamma}$Significant difference at $P<0.05$ compared to the sham and ischemia-EA groups in the hippocampal CA1 region, respectively. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

coupled to Texas red (1:200, Proteintech, Chicago, IL, USA) was applied to sections for 2 h at room temperature. Control staining experiments were performed by omission of the primary antibody with the same staining protocol.

To double-label TRPM7 and trkA, sections from ischemia-EA rats were first incubated in a blocking solution of 3% BSA in 0.01 M PBS for 1 h. They were then incubated for 1 h at room temperature and over 48 h at 4 °C with a mixture of the commercially available polyclonal antibody of rabbit anti-trkA (1:100, Chemicon, Temecula, CA, USA) and goat anti-TRPM7 (1:50, Santa Cruz, CA, USA). Sections were washed in 0.01 M PBS and incubated in fluorescent secondary antibodies for 3 h at room temperature. The previously mentioned secondary antibody for TRPM7 and donkey anti-rabbit IgG conjugated with fluorescein isothiocyanate (FITC, Southern Biotechnology Associates, Birmingham, AL, USA) for trkA were used. The control experiments omitted both primary antibodies.

Fluorescent-stained sections in the ischemic penumbral cortex and hippocampus were examined using an Olympus FV500 confocal laser scanning microscope (Olympus, Japan), and the green and red channels were simultaneously acquired with a ×60 (oil) lens.

Immunohistochemistry

At 24 h following reperfusion, rat brains were treated as described above for immunofluorescent labeling, and the sections from animals receiving different treatment were collected and stained free-floating under identical conditions. After blocking with 3% BSA for 1 h, the slides were incubated for 48 h at 4 °C with a first antibody: rabbit anti-trkA (1:1000, Chemicon, Temecula, CA, USA) or goat anti-TRPM7 (1:200, Santa Cruz, CA, USA). The brain tissue sections were transferred to biotinylated secondary antibodies, rinsed, and exposed to a solution of avidin–biotin complex. The secondary antibody for trkA was goat anti-rabbit IgG and rabbit anti-goat IgG for TRPM7. Finally, the sections were reacted for 3 min in 0.05% 3, 3-diaminobenzidine tetrahydrochloride and 0.01% H_2O_2 in 0.05 TBS (0.05 M Tris, 0.9% NaCl, pH 7.6). The optical density of TRPM7 immunoreactivity in the ischemic penumbral cortex and hippocampus was detected by an Image Analyzer (Kontron, Karl Zeiss, Germany).

Western blot analysis

Western blot analysis was performed as described (Bennecib et al., 2000). In brief, extracts of ischemic cortex and hippocampus were separated by 10% sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE), and the protein bands were electrophoretically transferred to polyvinylidene difluoride (PVDF) membranes and immunoblotted with anti-TRPM7 antibody (1:50, Santa Cruz, CA, USA). Immunoreactive materials were detected using the enhanced chemical luminescence (ECL) method, according to the

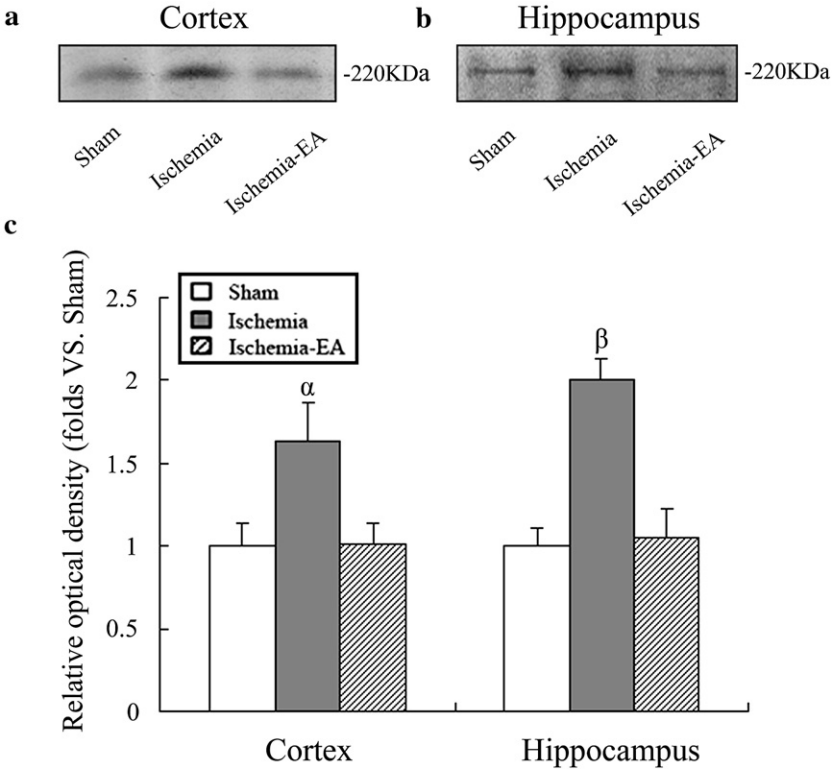

Fig. 2. Western blot analysis showing the effects of EA on TRPM7 level in the cortex (a) and hippocampus (b). The level of TRPM7 was increased in the ischemia group compared to the sham group. However, EA treatment inhibited the increase of TRPM7. TRPM7 proteins are detected at a molecular weight of 220 kilodaltons (kDa). (c) The relative optical density was represented as fold vs. sham control. Values are mean ± SD. $n=3$ in each group. $^{\alpha}$Significant difference at $P<0.05$ compared to the sham and ischemia-EA groups in the cortex, respectively; $^{\beta}$significant difference at $P<0.05$ compared to the sham and ischemia-EA groups in the hippocampus, respectively.

manufacturer's recommendation. The blots were scanned, and the sum optical density was quantitatively analyzed by Kodak Digital Science 1D software (Eastman Kodak Company, Bridgeport, CT, USA).

Statistical analysis

All morphological data were analyzed by a double blind method. Data were expressed as mean±standard deviation.

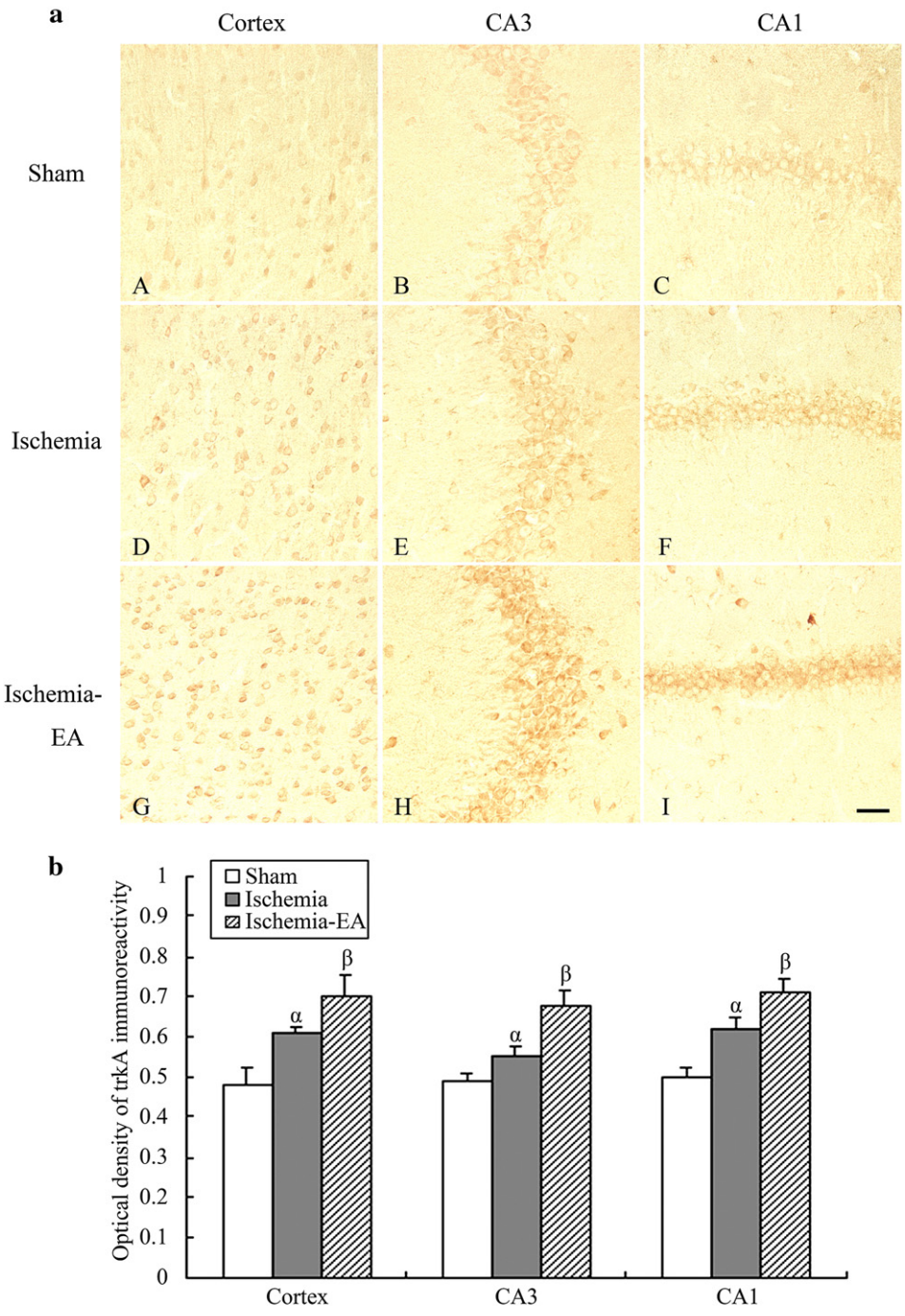

Fig. 3. Effect of EA on trkA expression in the ischemic cortex and hippocampus. (a) Representative immunohistochemical staining for trkA in the cortex (A, D, G) and hippocampal CA3 (B, E, H) and CA1 regions (C, F, I). Very weak trkA-ir existed in the neurons of the sham group (A, B, C). The level of trkA-ir was enhanced in the ischemia group compared to the sham group (D, E, F). TrkA-ir was further increased after EA treatment (G, H, I). Scale bar = 50 μm. (b) Graphic representation of the optical density of trkA immunoreactivity in the ischemic cortex and hippocampal CA3 and CA1 regions in each group. Values are mean±SD. $n=5$ in each group. $^{\alpha}$Significant difference at $P<0.05$ compared to the sham group in the cortex and hippocampal CA3 and CA1 regions, respectively; $^{\beta}$significant difference at $P<0.05$ compared to the ischemia group in the cortex and hippocampal CA3 and CA1 regions, respectively.

Statistical comparisons were made using analysis of variance (ANOVA) followed by Student–Newman–Keuls post hoc analysis. In all cases, $P<0.05$ was considered statistically significant.

Results

Effect of EA on TRPM7 expression in the ischemic cortex and hippocampus

To determine whether TRPM7 expression was altered after MCA occlusion and EA treatment, TRPM7 expression in the ischemic cortex and hippocampus was observed in sham, ischemia and ischemia-EA groups by immunofluorescence and Western blot.

Moderate TRPM7 staining was detected in the cortex and hippocampal CA3 and CA1 regions in the sham group ($n=5$ per group, Fig. 1a). TRPM7 immunoreactivity (TRPM7-ir) noticeably increased both in terms of the number of cells stained and staining strength after ischemic insult ($n=5$ per group, Fig. 1a and b). EA treatment after ischemia–reperfusion reversed the ischemia-induced increase of TRPM7-ir ($n=5$ per group, Fig. 1a and b).

The Western blot study showed a pattern of TRPM7 expression similar to the immunofluorescence changes in the three groups. Extracts from the cortex and hippocampus on the ischemic side were subjected to Western blot analysis with anti-TRPM7 antibody. Compared with the sham group, the TRPM7 level was increased significantly in the ischemia group ($n=3$ per group, Fig. 2). After EA treatment, the level of TRPM7 protein decreased noticeably and was nearly restored to normal ($n=3$ per group, Fig. 2). Taken together, these data indicated that EA treatment reversed TRPM7 over-expression induced by ischemia.

Effect of EA on trkA expression in the ischemic cortex and hippocampus

To determine whether trkA expression was altered after MCA occlusion and EA treatment, trkA expression in the ischemic cortex and hippocampus was observed by immunohistochemistry in the three groups.

In the sham group, trkA immunoreactivity (trkA-ir) was very weakly detectable in cortex and hippocampal CA3 and CA1 regions ($n=5$ per group, Fig. 3a). The level of trkA expression was enhanced in the ischemia group compared to that of the

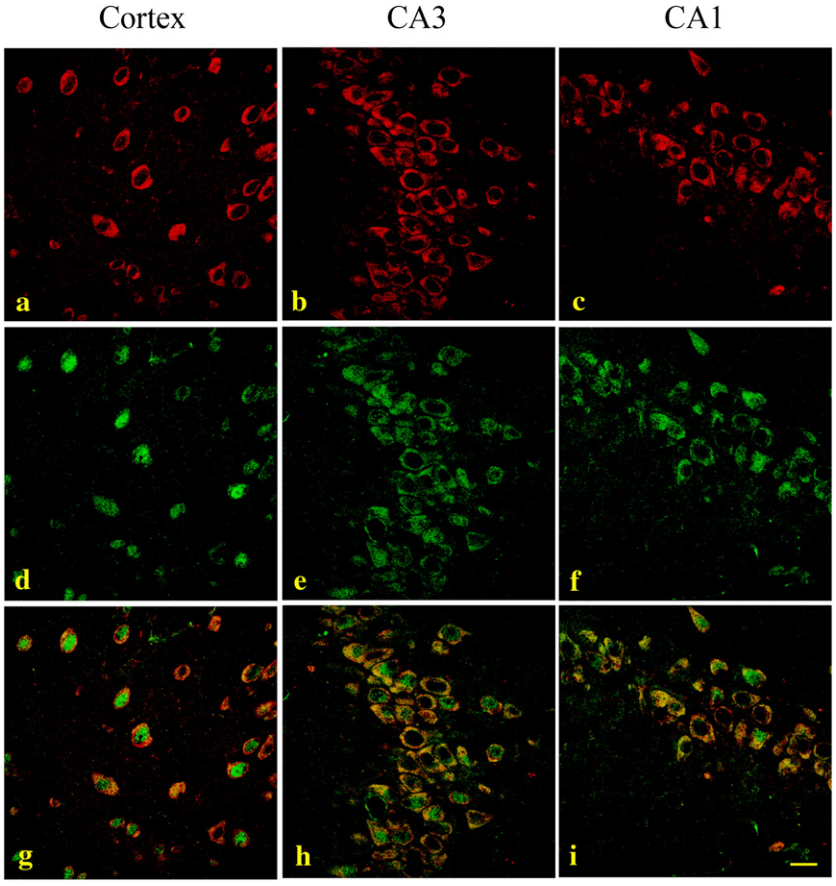

Fig. 4. Double immunofluorescence staining for TRPM7 and trkA in the ischemic cortex (a, d, g) and hippocampal CA3 (b, e, h) and CA1 regions (c, f, i) after EA treatment, as examined by confocal laser scanning microscopy. TRPM7-(a, b, c, red) or trkA-(d, e, f, green) positive neurons, as well as double-labeled neurons (g, h, i, yellow), can be seen in the ischemic cortex and hippocampal CA3 and CA1 regions, indicating colocalization of TRPM7 and trkA in the same neurons. Scale bar=20 μm. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

sham group ($n=5$ per group, Fig. 3a and b). Moreover, trkA-ir was further strengthened after EA treatment in the ischemic area ($n=5$ per group, Fig. 3a and b). The results suggest that the neuroprotective effects of EA treatment may be mediated through trkA up-regulation.

Coexpression of trkA and TRPM7 in the ischemic cortex and hippocampus after EA treatment

To explore the possible role of trkA activation in the regulation of TRPM7 expression, we used double immunofluorescence to examine the distribution of trkA and TRPM7 immunoreactivity in the ischemic cortex and hippocampal CA3 and CA1 regions of rats after EA treatment ($n=3$ per group, Fig. 4). TrkA and TRPM7 were coexpressed in the neurons of the ischemic cortex and hippocampal CA3 and CA1 regions (Fig. 4g,h, and i). We also found that trkA staining was located in both the neuronal cytoplasm and neuronal membrane (Fig. 4d,e, and f), while TRPM7 was only located in the neuronal membrane (Fig. 4a,b, and c).

Effect of K252a on TRPM7 expression in the ischemic cortex and hippocampus after EA treatment

To explore whether trkA was necessary for the effect of EA on the expression of TRPM7, we used K252a, a trkA inhibitor, in the present study.

Ischemia-EA group rats ($n=5$ per group) were ICV injected with DMSO (vehicle) or K252a just before MCA occlusion. As shown in Fig. 5a,b, and c, a low level of TRPM7-ir was observed in ischemia-EA rats treated with DMSO in the ischemic cortex

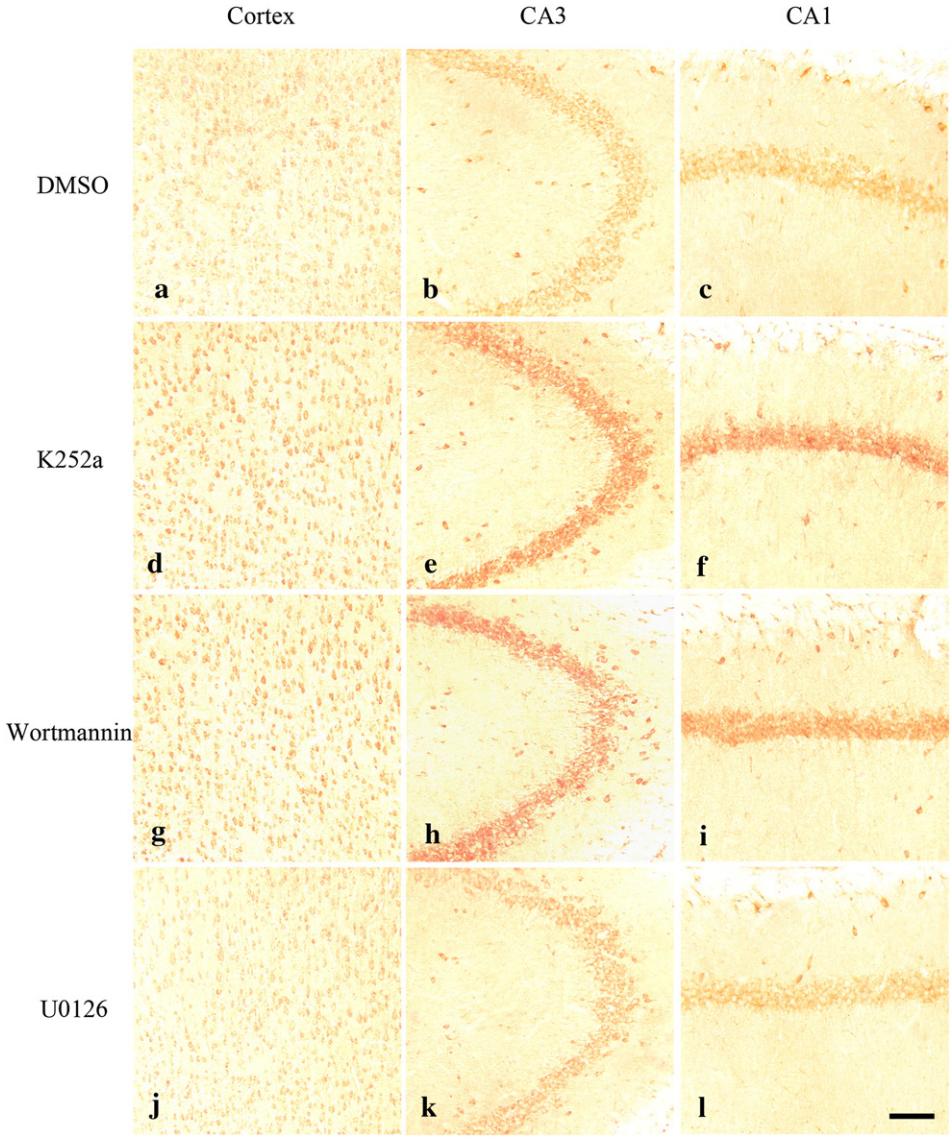

Fig. 5. Representative immunohistochemical staining for TRPM7 in the ischemic cortex (a, d, g, j) and hippocampal CA3 (b, e, h, k) and CA1 regions (c, f, i, l). Each animal was ICV injected with 5 μl solution containing DMSO (vehicle), K252a (200 nM/rat), Wortmannin (0.1 nmol/rat), or U0126 (1 nmol/rat) just before MCA occlusion. DMSO = ischemic reperfusion with EA and DMSO treatments; K252a = ischemic reperfusion with EA and K252a treatments; Wortmannin = ischemic reperfusion with EA and Wortmannin treatments; U0126 = ischemic reperfusion with EA and U0126 treatments. TRPM7-ir was significantly increased in the ischemia-EA group after administration of K252a or Wortmannin compared to DMSO and U0126 treatments. Scale bar=100 μm.

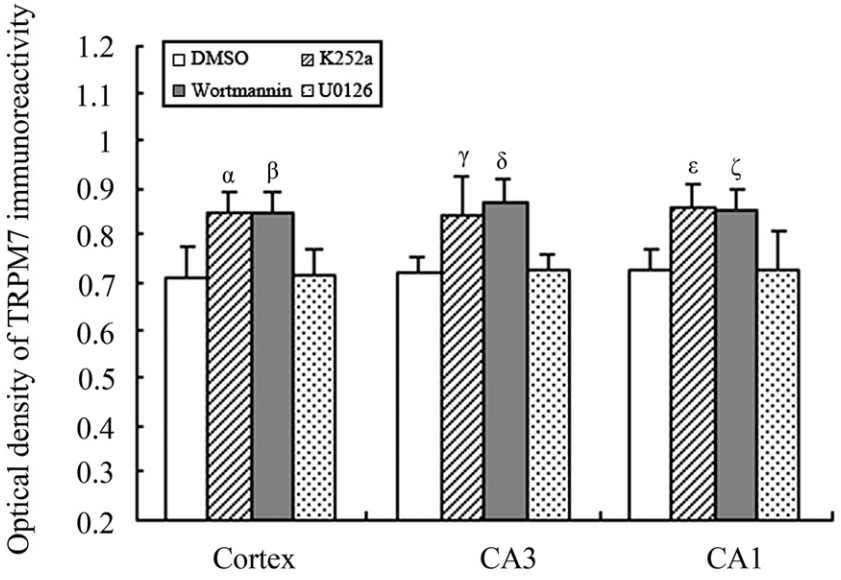

Fig. 6. Graphic representation of the optical density of TRPM7 immunoreactivity in the ischemic cortex and hippocampal CA3 and CA1 regions treated with DMSO, K252a (200 nM), Wortmannin (0.1 nmol), or U0126 (1 nmol). The injection volume was 5 μl for each animal. Note that treatment with K252a or Wortmannin in the ischemia-EA group significantly increased TRPM7-ir compared to DMSO and U0126 treatments. There was no difference in density between the DMSO group and U0126 group. Values are mean±SD. $n=5$ in each group. $^{\alpha,\beta}$Significant difference at $P<0.05$ compared to the DMSO and U0126 groups in the cortex, respectively; $^{\gamma,\delta}$significant difference at $P<0.05$ compared to the DMSO and U0126 groups in the hippocampal CA3 region, respectively; $^{\varepsilon,\zeta}$Significant difference at $P<0.05$ compared to the DMSO and U0126 groups in the hippocampal CA1 region, respectively.

and hippocampal CA3 and CA1 regions. However, TRPM7-ir was significantly increased in the ischemia-EA group after administration of K252a (Figs. 5d,e,f and 6).

In parallel with the immunohistochemical analysis, Western blot analysis of the hippocampal and cortical homogenates using anti-TRPM7 antibody also showed that K252a treatment

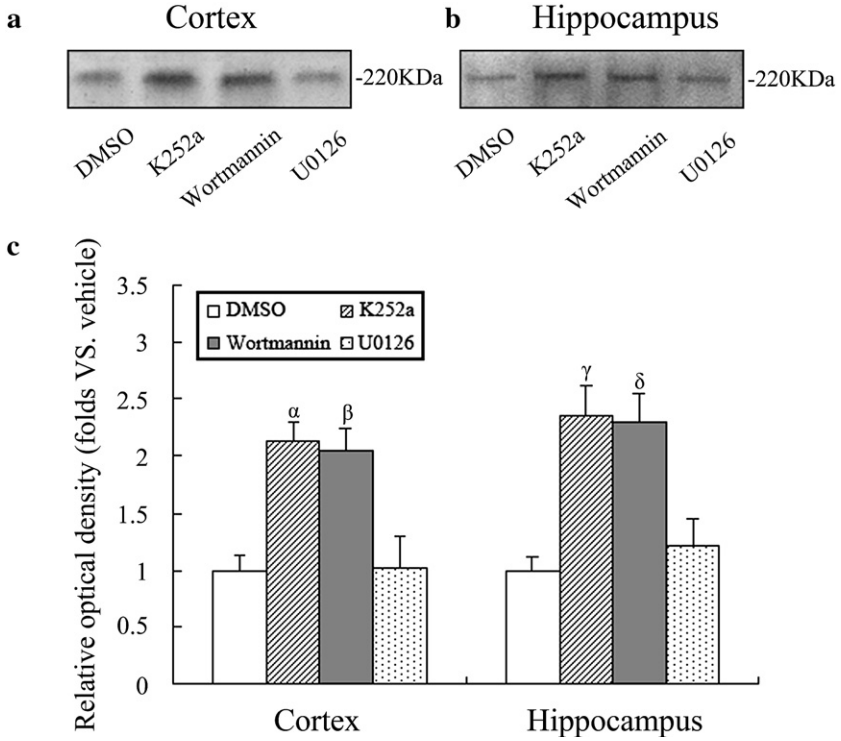

Fig. 7. Western blot analysis showing effects of protein kinase inhibitors on the TRPM7 level in the cortex (a) and hippocampus (b). The level of TRPM7 was enhanced in the ischemia-EA group after administration of K252a or Wortmannin compared to DMSO and U0126 treatments. TRPM7 proteins are detected at a molecular weight of 220 kDa. (c) The relative optical density was represented as folds vs. vehicle control. Values are mean±SD. $n=3$ in each group. $^{\alpha,\beta}$Significant difference at $P<0.05$ compared to the DMSO and U0126 groups in the cortex, respectively; $^{\gamma,\delta}$significant difference at $P<0.05$ compared to the DMSO and U0126 groups in the hippocampus, respectively.

in the ischemia-EA group increased the level of TRPM7 compared to DMSO treated controls ($n=3$ per group, Fig. 7). Our results indicated that the effect of EA on the expression of TRPM7 could be inhibited by the trkA inhibitor.

Effect of Wortmannin and U0126 on TRPM7 expression in the ischemic cortex and hippocampus after EA treatment

To explore which trkA-mediated signaling pathways were necessary for the effect of EA on TRPM7 expression, we used Wortmannin, a PI3K inhibitor, and U0126, an ERK inhibitor, in the present study.

Ischemia-EA group rats ($n=5$ per group) were ICV injected with DMSO (vehicle), Wortmannin, or U0126 just before MCA occlusion. No differences in density of TRPM7 expression were shown between U0126 and DMSO treatment (Figs. 5j,k,l and 6). However, TRPM7-ir was significantly increased in the ischemia-EA group after administration of Wortmannin compared with DMSO and U0126 treatments (Figs. 5g,h,i and 6).

In parallel with the immunohistochemical analysis, Western blot analysis of the hippocampal and cortical homogenates using anti-TRPM7 antibody also showed that U0126 treatment in the ischemia-EA group scarcely changed the level of TRPM7 compared to DMSO treatment. However, administration of Wortmannin significantly increased the TRPM7 level ($n=3$ per group, Fig. 7). Our results indicated that the effect of EA on TRPM7 could be suppressed by a PI3K inhibitor but not by an ERK inhibitor.

Discussion

Few effective therapies have been realized to treat stroke, and once promising avenues, such as anti-excitotoxic therapy with NMDA receptor antagonists, have not been proven to be useful in clinic. Thus, additional possible mechanisms may be involved in neuron loss. TRP channels are an exciting new family of cation channels that are expressed abundantly in the mammalian brain. Recently, several studies have indicated that members of the melastatin subfamily (TRPM) of TRP proteins, particularly TRPM7 and TRPM2, are implicated directly as central components of neuronal death pathways (Hara et al., 2002; Wehage et al., 2002; Monteilh-Zoller et al., 2003; Nadler et al., 2001; Schmitz et al., 2003). TRPM7, also called TRP-PLIK, ChaK1, and LTRPC7, functions as a calcium-permeable non-selective ion channel. Unique among ion channels, TRPM7 possesses an α-kinase at the C-terminus. TRPM7 appears to be regulated by Src-family kinases (Jiang et al., 2003), PIP_2 (Runnels et al., 2002), and its own α-kinase domain (Takezawa et al., 2004). The activity of TRPM7 channel has also been shown to be mediated by changes in $[Ca^{2+}]_e$ and blocked by Gd^{3+}. Over-expression of TRPM7 in HEK 293 cells results in cell swelling, detachment, and death within 48–72 h (Aarts et al., 2003; Nadler et al., 2001). TRP-like channels are activated by cellular stress and contribute to ischemia-induced membrane depolarization, intracellular calcium accumulation, and cell swelling (Lipski et al., 2006). TRPM7 activation during anoxia results in calcium overload. As is well-known, Ca^{2+} overload can trigger several downstream lethal reactions that culminate in cell death (Orrenius et al., 2003). Suppression of TRPM7 expression in primary cortical neurons with RNA interference blocks TRPM7 currents, anoxic Ca^{2+} influx, and reactive oxygen-species production, protecting cells from anoxia and demonstrating a role for endogenous TRPM7 in anoxic cell death (Aarts et al., 2003). On this basis, we investigated TRPM7 expression after focal cerebral ischemia in vivo. We found that TRPM7 was widely expressed in neurons of the cortex and hippocampus. The expression of TRPM7 was increased significantly 24 h after reperfusion. Our results support that the TRPM7 channel, which allows Ca^{2+} entry into the neuron, may contribute to ischemic brain damage.

Cell survival is regulated by a balance between death and survival signals. Growth factors or neurotrophic factors may play a protective role in brain ischemic injury (Kitagawa et al., 1998; Schabitz et al., 1997). NGF is one of the most extensively studied neurotrophic factors. NGF can protect cultured cortical and hippocampal neurons against hypoglycemic damage or iron-induced degeneration (Cheng and Mattson, 1991; Zhang et al., 1993). NGF infusion into the lateral ventricle can delay the progress of cholinergic terminal damage and neuronal death after ischemia (Ishimaru et al., 1998; Shigeno et al., 1991). NGF expression is increased soon (within 4 h) after MCA occlusion (Lee et al., 1998). NGF interacts with the tyrosine kinase receptor trkA on the cell surface, triggers multiple intracellular phosphorylation cascades, and alters cellular functions of the nervous system (Kaplan and Miller, 1997; Klesse and Parada, 1999; Segal and Greenberg, 1996). The low level of trkA expression in the normal condition is compatible with our present study (Lee et al., 1998). The level of trkA at 24 h after cerebral reperfusion was found to be higher than that of the sham group in this study. TrkA-ir reportedly increases from 30 min after ischemia and peaks at 12 h (Hwang et al., 2005). A lack of trkA signal in hippocampal CA1 pyramidal cells is associated with delayed neuronal death after transient ischemia (Hu et al., 2000). TrkA over-expression after transient forebrain ischemia may be a compensatory mechanism that prevents ischemic damage after ischemic insult.

EA has been widely used for treatment of cerebral ischemia because it has been shown to reduce infarct areas induced by cerebral ischemia in clinical practice (Chen and Fang, 1990; Zhao et al., 1998). EA stimulation of low and high frequencies may exert different acceleratory effects on the release and synthesis of different peptides in the central nervous system (Guo et al., 1997). Recently, it was reported that low frequency EA can play a more important role in the EA-induced cerebral ischemic tolerance than high frequency EA (Yang et al., 2004). In this study we found that EA stimulation at 4/16 Hz frequencies reversed the high expression of TRPM7 induced by ischemic injury and up-regulated the level of trkA in a rat model of MCA occlusion. Meanwhile, we found that nearly all of the TRPM7-ir neurons in the ischemic cortex and hippocampus contained trkA. It is possible that trkA can regulate TRPM7 expression or function through multiple signal cascades in the same neuron. In order to explore whether trkA was necessary for the effect of EA on TRPM7 expression, we used K252a, a trkA inhibitor. We found that the effect of the EA-mediated down-regulation of TRPM7 was inhibited by K252a. In a previous study, we also found that

antisense trkA oligonucleotides, which inhibit trkA expression, can increase the level of TRPM7 mRNA in the ischemia-EA group (Zhao et al., 2005). Our results indicate that EA treatment may reverse the high expression of TRPM7 induced by ischemia through the trkA pathway.

The regulation of gene expression through trkA activity is mainly transduced by the Ras/ERK and PI3K cascades, although the Ras/ERK cascade may be involved more in differentiation and PI3K more in cell survival (Klesse and Parada, 1999). The PI3K pathway can inhibit glycogen synthase kinase-3 (GSK-3) by promoting its phosphorylation. GSK3 regulates many important metabolic and signaling proteins (Aplin et al., 1996; Diehl et al., 1998), structural proteins (Goold et al., 1999; Hanger et al., 1998), and transcription factors (Fiol et al., 1994; Hoeflich et al., 2000). The PI3K pathway also activates pp70 ribosomal S6 kinase (p70S6K) which mediates glycogen, DNA and protein synthesis (Dixon et al., 1999; Vinals et al., 1999; Sanchez-Margalet, 2000). The PI3K/Akt pathway plays a key anti-apoptotic role in the NGF signaling pathway (Dudek et al., 1997; Khreiss et al., 2004; York et al., 2000). The inhibition of PI3K activity induces apoptosis in PC12 cells in the presence of NGF (Yao and Cooper, 1995). The ERK cascade plays a major role in transducing NGF signals from the membrane to nuclear targets (Groot et al., 2000). Terminal effectors of this cascade, activated ERK1 and ERK2, are members of the mitogen-activated protein kinase (MAPK) family. They translocate to the nucleus (Groot et al., 2000; Khokhlatchev et al., 1998) or activate the pp90 ribosomal S6 kinase, which then translocates to the nucleus (Xing et al., 1998) to modify proteins involved in gene transcription. ERK is important for cell activation, including mitogenesis and cell proliferation (Boulton et al., 1991). ERK expression is induced in the MCA territory soon after permanent MCA occlusion (Kitagawa et al., 1999). Inhibition of the ERK pathway decreases cerebral ischemia/reperfusion injury secondary to focal cerebral hypoxia (Namura et al., 2001). In our study, we administrated Wortmannin (PI3K inhibitor) or U0126 (ERK inhibitor) in the ischemia-EA group before MCA occlusion. We found that the effect of EA treatment on TRPM7 was inhibited by the PI3K inhibitor, while the ERK inhibitor had no effect. Our study demonstrates that EA stimulation at both Renzhong (DU26) and Chengjiang (RN24) can reverse the ischemia-induced increase of TRPM7 by enhancing trkA activity, which triggers the downstream PI3K pathway. These findings may be beneficial for the clinical application of EA in stroke patients. Further study to detect how the PI3K signaling regulates TRPM7 gene expression will be required.

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