# Electroacupuncture inhibits hippocampal neuronal apoptosis and improves cognitive dysfunction in mice with vascular dementia via modulating JNK signaling pathway

**Yaru Liu** ( liuyaru@bucm.edu.cn )
Beijing University of Chinese Medicine https://orcid.org/0000-0002-8530-2547
**Chunyan Li**
Shizhong District People's Hospital of Jinan
**Zhenyang Yan**
Weifang Traditional Chinese Hospital
**Yafei Ren**
Beijing University of Chinese Medicine
**Woyu Wang**
Beijing University of Chinese Medicine
**Yinze Ke**
Beijing University of Chinese Medicine
**Yifan Wang**
Beijing University of Chinese Medicine
**Rongming Qi**
Beijing University of Chinese Medicine

**Research Article**

**Keywords:** Electroacupuncture, Vascular dementia, hippocampal, Apoptosis, JNK signaling pathway

**Posted Date:** June 11th, 2021

**DOI:** https://doi.org/10.21203/rs.3.rs-595105/v1

# Abstract

Background: Nerve cell apoptosis is an important pathological mechanism of vascular dementia (VaD). There are evidences that Electroacupuncture (EA) can reduce cognitive impairment in VaD patients and protect nerve cells. However, the mechanism remains unknown. JNK signaling pathway play an important role in apoptosis. And JNK induces cell apoptosis in two main ways, namely JNK transcription-dependent pathway and JNK transcription-independent route. We focus on whether EA can inhibit apoptosis and alleviate cognitive impairment by regulating JNK signaling pathway.

Method: In this study, Bilateral common carotid artery clipping (BCCAo) was used as a method to establish VaD mouse model. Longa scoring method and Morris water maze were used to evaluate whether EA could improve the behavioral score of VaD mice. The neuroprotective effects of EA were evaluated by the Hematoxylin and Eosin (HE) staining, TUNEL and Flow Cytometry (FCM). Western blot and real-time PCR were used to detect the expression of JNK-3, AP-1, P53, Bcl -2, Bax, and Caspase-3.

Results: Our findings indicated that EA could improve the behavioral scores of VaD mice. VaD mice treated with EA reduced hippocampal neuronal apoptosis. In addition, EA could reduce the expression of JNK-3, AP-1, P53, Bax and Caspase-3 proteins and mRNA in the hippocampus of VaD mice, and increase the expression of Bcl-2.

Conclusion: Our findings suggested that the mechanism of action of EA to treat VaD may be related to its regulation of JNK transcription-dependent pathway and JNK transcription-independent route.


# 1. Introduction

Although the main cause of Alzheimer's disease (AD) in Western countries, cognitive impairment due to vascular disease is the second most common cause of clinically diagnosed dementia, and may be the main cause in East Asia (Kalaria RN 2017). Vascular dementia (VaD) has a more serious natural course than AD, with a shorter survival rate and a worse long-term prognosis (Vinters HV et al. 2018), which seriously affects the quality of life and health of patients. VaD is a state of impairment in the memory functioning secondary to problems related to blood supply to the brain.

Cerebral ischemia can activate various programs of cell death, such as necrosis, apoptosis or autophagy-associated cell death (Zille M et al. 2012; Qin AP et al. 2010; Bredesen DE et al. 2006). Among these, apoptosis has been regarded as the key event for brain damage in cerebral ischemia (Bredesen DE et al. 2006). Nerve cell apoptosis is one of the important pathological mechanisms leading to cognitive impairment.

JNK is the main signal pathway that mediates apoptosis of nerve cells. Current research reveals that JNK induces cell apoptosis in two main ways, namely JNK transcription-dependent pathway and JNK transcription-independent route. Among them, the specific process of the JNK transcription-dependent pathway is that after the cell receives some extracellular stimulus to activate the JNK located in the

cytoplasm, the partially activated JNK will translocate into the nucleus, thereby activating AP-1 (Dunn C et al. 2002), P53, and other nuclear transcription factors (Davis RJ 2000; Chang L et al. 2001; Lin A 2003; Shaulian E et al. 2002), which in turn regulate the transcription of downstream apoptosis-related target genes and the expression of apoptotic proteins. In addition, the JNK transcription-independent route is to rely on the substrates in the cytoplasm, such as the Bcl-2 family (Bcl-2, Bcl-xL, Bim, BAD) etc (Yamamoto K et al. 1999; Maundrell K et al. 1997; Yu C et al. 2004).

In East Asian medicine, acupuncture is one of the most widely used treatments and has been applied in clinics for thousands of years (Kaptchuk TJ 2002). Electroacupuncture (EA) combines traditional acupuncture and modern electrotherapy and is effective in the treatment of various diseases of the brain (Zhou H et al. 2013). Previous studies indicate that acupuncture exerts its therapeutic effects via promoting cerebral blood flow (Ding N et al. 2019; Moon SK et al. 2019; Kim YI et al. 2018), preventing apoptosis of neuronal cells (Jang JH et al. 2020; Sun X et al. 2020), improving neurobehavioral performance (Lin L et al. 2019), and regulating the expression of key genes and proteins involved in neural development and functions (Sha R et al. 2019; Wang H et al. 2019). Due to its beneficial effects on neurological diseases, EA has been used as an improvement over traditional acupuncture (Li X et al. 2012). Our previous research found that EA at Baihui (DU20), Geshu (BL17) and Shenshu (BL23) can reduce the apoptosis of hippocampal neurons in VaD mice (Zhao JX et al. 2011; Zhao J et al. 2013).

However, it is unclear whether the effect of EA on VaD is through the JNK signaling pathway and how to participate. Therefore, we studied the role of EA, focusing on the JNK pathway. The purpose of this study is to determine whether EA reduces neuronal apoptosis in VaD mice by regulating the JNK signaling pathway.

## 2. Materials And Methods

# 2.1 Animals

62 Adult healthy male C57BL/6 mice weighing 22–28 g, aged 8 weeks, were from Beijing Vital River Laboratory Animal Technology Co., Ltd. (Beijing, China). Animals were acclimated to the animal quarters at least for 7 days before any experiment procedure with free access to standard laboratory diet and water. Room temperature (RT): 20–22℃. Relative humidity of the air: 65–70%. Also, the mice were deprived of water for 12 hours before surgery.

# 2.2 Modified Bilateral common carotid artery occlusion (BCCAo)

The surgical procedure was based on previous studies (Zhao JX et al. 2011; Zhao J et al. 2013). In brief, mice were anaesthetized by intraperitoneally injected with pentobarbital (0.35ml/100g). Bilateral carotid arteries were separated from the adjacent vagus nerves. The blood flow of bilateral carotid arteries was blocked for 20 minutes by using an arterial clip, then restored for 10 minutes, then blocked again for 20 minutes. While blocking the brain blood flow, 3 ml of blood was exsanguinated from the tail to aggravate

ischemia. The body temperature of each mouse was monitored at 37°C throughout the surgical procession. Surgical on the Sham group only exposes the common carotid artery, did not have the procedure of blocking the blood flow or exsanguinating blood from the tail.

# 2.3 EA treatment

On the third day after operation, perform EA at Baihui (DU20), Geshu (BL17) and Shenshu (BL23) or non-acupoints. Sterilized disposable needles (0.25×25 mm, Hwato, China) were selected to stimulate acupoints or non-acupoints. In addition, the acupuncture needles were connected to an electro-acupuncture device (KWD-808, Yingdi, Changzhou, China), and a continuous wave stimulation of 3 Hz was given for the duration of 10 minutes. The BL17 and BL23 on the same side connected a pair of electrodes to form a current loop. The other electrode that forms a current loop with DU20 is wrapped in wet gauze and fixed on the animal's forelimb. The intensity was adjusted to the muscle twitch threshold. The location and stimulation parameters of the acupoints are shown in Table 1. The mice in the EA was operated by experienced acupuncturists. EA treatment was performed once a day, 7 times in total. Sham and BCCAo groups did not receive EA, but were fixed in the same position for 10 minutes.

Table 1
The location and stimulation parameters of the acupoints

| PIONTS | ANOTOMIND POSITIONS | HERTZ(Hz) | TIME |
|---|---|---|---|
| Baihui (DU20) | Midline of the head, approximately midway on the line connecting the apices of the auricles | 3 | 20 min |
| Geshu (BL17) | The two sides below the spinous of the seventh thoracic vertebra, in the intercosta | 3 | 20 min |
| Shenshu (BL23) | The two sides below the spinous of the second lumbar vertebra, in the intercosta | 3 | 20 min |
| Non-acupoints | On the bilateral hypochondrium, 5 mm above iliac crest | 3 | 20 min |

# 2.4 Experimental design and grouping

# 2.4.1 Experiment A: Longa scoring method and Morris water maze (MWM)

In order to clarify whether there is a difference in effect between EA (model with EA at DU20, BL17 and BL23) and placebo acupuncture (the same stimulation was applied to a non-acupoint), we set up 4 groups. They were Sham group, BCCAo group, EA group and Non-EA group. There were 8 mice in each group, a total of 32 mice. At the same time on the second day after 7 times EA treatment, mice in different groups were scored for neurological function based on the Longa scoring method. (Table 2) (Longa EZ et al. 1989).

Table 2
Longa scoring method

| Score | Neurological behavior |
|---|---|
| Score 0 | No nerve function defect |
| Score 1 | The paw on the paralytic side cannot be fully extended |
| Score 2 | When walking, the rat circles to the paralytic side |
| Score 3 | When walking, the rat body topples to the paralytic side |
| Score 4 | Unable to walk spontaneously, loss of consciousness |
| Judging criteria | Scores of 1 or above indicate successful modeling |

Before modeling, all mice were trained for directional cruise. In short, each mouse trains 3 times a day for 5 consecutive days. After the mice in the EA group received 7 treatments, the MWM test was performed on the mice in each group. The purpose is to evaluate their spatial learning and memory. Mice swim freely until they found the platform. If the animal did not reach the platform within 60 seconds, it was guided into position. After 24 hours of the last treatment, the mice were allowed to swim freely in the pool without a platform for 60 seconds. We used a tracking system (TECHMAN WMT-100S Water Maze Video Analysis System, China) to record the mouse's movement trajectory and stay time in each quadrant, and used a computer for data analysis.

## 2.4.2 Experiment B: TUNEL and HE

In order to observe the apoptosis and tissue morphology of the CA1 region of the hippocampus of each group of mice, 18 mice were randomly divided into 3 groups: Sham group, BCCao group, EA group (n = 6 each group).

TUNEL: The whole-brain tissues of fresh mice were fixed in 4% paraformaldehyde for more than 1 week, and the target tissues were trimmed. It is then dehydrated and embedded in paraffin. The wax block was coronally sectioned with a thickness of 4 μm. DeadEnd™ Fluorometric TUNEL System (Promega G3250) was used for TUNEL. Three consecutive sections of hippocampus CA1 region of each tissue were taken to observe under 400x light microscope. Leica Quin analysis software (Soft Imaging System GmbH, Münster, Germany) was used to analyze positive cells. The ratio of positive cells was calculated.

HE: Slices were incubated with xylene and 100% ethanol for 5 min twice, and then with 95%, 90%, and 80% ethanol for 3 min each. Afterward, the slices were stained with hematoxylin for 10–15 min, followed by a rinse with water for 10 min. Following this, 1% hydrochloric acid alcohol was added for 1 second to visualize blue. After washing with water for 10 min, the slices were stained with eosin for 5 min and then dehydrated with 80%, 95%, and 100% ethanol for 1 min twice. The resulted samples were cleared using xylene for 5 min twice, mounted with neutral gum. Three consecutive sections of the hippocampus CA1 region of each tissue were taken to observe under the light microscope.

## 2.4.3 Experiment C: Flow cytometry (FCM)

To analyze the effect of EA on inhibiting hippocampal nerve apoptosis in I/R mice, further 18 mice were randomly divided into 3 groups: Sham group, BCCAo group, EA group (n = 6 each group).

The hippocampus tissues were placed in a 300 mesh nylon bag and gently squeezed. The squeezed cells were collected in a flow tube and washed once with PBS at 500 g for 3 mins. A total of 1´106 cells were harvested and then washed twice with PBS. Cells were re-suspended with a 500 µl 1´Annexin V binding buffer at a concentration of 1´106/ml, followed by the addition of 5 µl of Annexin V FITC dye and 5 µl of PI dye. After vortex, the samples were incubated at RT for 15 mins and subjected to flow cytometry (FCM) detection thereafter. Annexin V-/PI- cells represented living cells; while Annexin V+/PI- and Annexin V+/ PI + cells represented apoptotic and dead cells, respectively.

## 2.4.4 Experiment D: Western blot

To determine the effect of EA in the hippocampus of VaD mice at protein level, we divided a further 18 rats (n = 6 each group) into 3 groups: Sham, BCCAo, and EA.

RIPA lysis buffer containing a mixture of complete protease inhibitors (Roche Diagnostics) was used for sample homogenizing. BCA method was used to determine protein concentration. The primary antibodies include anti-JNK-3 (Santa Cruz, sc-507), anti-AP-1 (Proteintech, 50599-2-Ig), anti-P53 (Proteintech, 12789-1-AP), anti-Bcl-2 (Proteintech, 12789-1-AP), anti-Bax (Proteintech, 50599-2-Ig), anti-Caspase-3 (Proteintech, 19677-1-AP-150),and GAPDH (sigma, P7769-5MG). Secondary antibodies were used to incubated membranes for 2 hours at room temperature. Membranes were scanned and analyed by Odyssey 9120 imaging system (LICOR, Inc).

## 2.4.5 Experiment E: Real-time polymerase chain reaction (real-time PCR)

Total RNA was extracted from hippocampus tissues using TRIzol Reagent (Invitrogen, 15596-026, USA). The primers were designed for mice as follows: JNK-3, CTGATGGACGCCAACCTGTGT (forward primer), GCGGAGTGGAGGTGCTTGAT (reverse primer), AP-1, AAGGAAGAGCCGCAGACCGT (forward primer), GCCTTGATCCGCTCCTGAGACT (reverse primer), P53, AACCGCCGACCTATCCTTACCA (forward primer), CAGGGCAGGCACAAACACGAA (reverse primer), Bcl-2, 5'-TTCTTGTGGCCTTCGTGAGTTGTG-3' (forward primer), 5'-GGTTCAGGTATGCCGGCTCAGTCA-3' (reverse primer), Bax, 5'-CCACTGTGCACAGGTGCCGGAACT-3'(forward primer), 5'-CTCTGCCTGGCACATCCACCTGGC-3' (reverse primer), Caspase-3, 5'-TACCCAGGGAGGAGCAATAC-3' (forward primer), 5'-GAGGCAGCTTGAACAACAAC-3'(reverse primer),and GAPDH, AAGGTCGGTGTGAACGGATT (forward primer), TGAGTGGAGTCATACTGGAACAT (reverse primer). The cDNA was obtained from 2 µg of total RNA using cDNA First Strand Synthesis Kit (TaKaRa, RR036B, Japan). Quantitative PCR was used to analyze corresponding mRNA levels by Stepone Real-Time PCR System (Applied Biosystems, 4376357, USA).

## 2.5 Sample collection

After the experiment, the mice were euthanized by pentobarbital overdose. The brains were taken out of, and the hippocampi were dissociated for protein extraction. The remaining mice were perfused with 4% paraformaldehyde via the heart. The brain was taken out and immersed in 4% paraformaldehyde for histopathological evaluation.

## 2.6 Data and statistical analysis

We used Statistical Package for the Social Sciences (SPSS) 20.0 software for data analysis. Student's $t$-test or ANOVA was used to test data that meet the hypothesis of homogeneity of variance. The Rank-sum test was used to detect data that did not meet the assumption of homogeneity of variance All data were presented as mean ± SEM. Statistically significant was considered if $P < 0.05$.

# 3. Result

## 3.1 EA improved neurological function and the learning and memory deficits in VaD mice

Longa scoring method was used to evaluate the neurological function of 4 groups of mice. EA at DU20, BL17, and BL23 can lower the score. As shown in Fig. 1A, the score of the BCCAo group was significantly higher than that of the Sham group. On the contrary, EA can greatly reduce the Longa score. Moreover, there is a significant difference between the Non-EA group and the EA group.

The 4 groups of mice showed different spatial learning and memory abilities in the MWM experiment. EA at DU20, BL17 and BL23 can improve the cognitive ability of mice. As shown in Fig. 1B, the escape latency of the BCCAo group was significantly longer than that of the Sham group. On the contrary, EA could greatly shorten the escape latency. In the space exploration experiment, the mice in the BCCAo group stayed in the target quadrant for significantly shorter time than the Sham group. EA significantly increased the time of VaD mice in the target quadrant. Moreover, there is a significant difference between the Non-EA group and the EA group (Fig. 1C). Figure 1D is a representative diagram of the trajectory of each group of mice in the MWM.

These results indicate that EA at appropriate acupoints can improve the neurological function and the spatial learning and memory abilities of VaD mice. Since the effect of acupuncture points has been confirmed, the Non-EA group will no longer be used in subsequent experiments.

## 3.2 EA attenuated apoptosis of hippocampal neurons in VaD mice

By using HE staining method, we observed pathological changes in the CA1 hippocampus in different groups and the results awere shown in Fig. 2 observed as follows: In the Sham group (Fig. 2A), the arrangement of pyramidal cells was tight and orderly in the hippocampus. Cells showed normal morphology, with framework integrity by light staining and clear nucleolus. In the BCCAo group (Fig. 2B),

the arrangement of cells is disordered and sparse. In the picture, we can see neural cell loss and deformation, along with nuclear pyknosis and unclear nucleolus by dark staining. Compared with the BCCAo group, the cells in the EA group (Fig. 2C) were arranged more regularly and the nucleoli were clearer. Besides, the loss and deformation of nerve cells in the EA group were relieved.

TUNEL was used to detect the apoptosis of neurons in the CA1 area of the hippocampus. Sham group hippocampal CA1 pyramidal cells were arranged in a multilayer, circular, blue staining, clear nucleoli, fibers arranged in neat rows. Increased positive cells, swollen cells, and incomplete cells were observed in BCCAo group, while alleviation of swollen neurons and descresed positive staining were shown in EA group (Fig. 3A). The ratio of apoptosis cells in Sham group, BCCAo group, and EA group were 0.61 ± 0.55%, 6.09 ± 2.03%, and 2.82 ± 1.12% respectively. The ratio of apoptosis cells in BCCAo group was significantly higher than that in Sham group ($P < 0.05$). The ratio of apoptosis cells were decreased compared with the BCCAo group after EA treatment ($P < 0.05$) (Fig. 3B).

FCM was used to detect the apoptosis rate of neurons in the hippocampus. The ratio of apoptosis in the Sham group is 10.44 ± 2.0%. BCCAo group is 16.00 ± 1.27%. And EA group is 14.09 ± 1.02%. Cell apoptosis rate in the BCCAo group was higher than that in the Sham group and EA group ($P < 0.05$). After EA treatment, the ratio of apoptosis cells decreased compared with the BCCAo group (Fig. 3D).

We investigated protein and mRNA levels of Caspase-3 in the hippocampus using Western blot and real-time PCR. Compared with Sham group, the expression of Caspase-3 in protein level were increased in the BCCAo group. EA treatment decreased the expression of Caspase-3 in protein level, compared with the BCCAo group (Fig. 4A, B). The expression trend of Caspase-3 mRNA was similar to their protein expression trend (Fig. 4C). These results indicated that EA inhibited the expression of Caspase-3 protein and mRNA after BCCAo surgery.

## 3.3 EA inhibited activation of JNK transcription-dependent pathway

We investigated protein and mRNA levels of JNK-3, AP-1 and P53 in the hippocampus using Western blot and real-time PCR. Compared with Sham group, the expressions of JNK-3, AP-1 and P53 in protein level were increased in the BCCAo group. EA treatment decreased the expressions of JNK-3, AP-1 and P53 in protein level, compared with the BCCAo group (Fig. 5A-D). The expressions of JNK-3, AP-1 and P53 mRNA and protein expression showed similar trends (Fig. 5E-G). These results indicate that EA treatment can inhibit the expression of JNK-3, AP-1 and P53 protein and mRNA after BCCAO. EA inhibited the activation of the JNK transcription-dependent pathway.

## 3.4 EA up-regulated the level of hippocampal Bcl-2 and down-regulated the level of Bax

We investigated protein and mRNA levels of Bcl-2 and Bax in the hippocampus using Western blot and real-time PCR. Compared with Sham group, the expression of Bax in protein level were increased in the BCCAo group. While, the expression of Bcl-2 in protein level were decreased. EA treatment decreased the expression of Bax in protein level and increased the expression of Bcl-2 in protein level, compared with the BCCAo group (Fig. 6A-C). The expression trends of Bcl-2, Bax mRNA were similar to their protein expression trends (Fig. 6D, E). These results indicate that EA treatment can promote the expression of Bcl-2 protein and mRNA, and inhibit the expression of Bax protein and mRNA after BCCAo surgery. EA inhibited the activation of JNK transcription-independent route.

# 4. Discussion

In these experiments, we proved that the neuroprotective effect of EA is related to the JNK signaling pathway that mediates neuronal apoptosis. EA could prevent cognitive dysfunction mediated by cerebral ischemia-reperfusion injury by regulating both JNK transcription-dependent pathway and JNK transcription-independent route. EA inhibited the expression of JNK-3, AP-1, and P53 to regulate the neuronal apoptosis program mediated by JNK transcription-dependent pathway. In addition, EA decreased the expression of Bax and increased the expression of Bcl-2 to regulate the neuronal apoptosis program mediated by JNK transcription-independent route.

VaD refers to severe cognitive dysfunction syndrome caused by ischemic stroke, hemorrhagic stroke, and cerebrovascular disease that causes hypoperfusion of brain areas such as memory, cognition, and behavior. Many studies have proved that the death of nerve cells caused by whole brain or local cerebral ischemia is mainly apoptosis (Endo H et al. 2006; Wang HQ et al. 2007). Ischemia–reperfusion induced cerebral injury is one of more commonly employed models to induce vascular dementia in animals (Wu L et al. 2015; Wan L et al. 2015). In the present study, a state of cerebral ischemia reperfusion was induced by occlusion of both carotid arteries.

The BCCAo model has been developed and been widely used in global ischemia studies in mice (Spray S et al. 2016; Soares LM et al. 2013; Wahul AB et al. 2018). C57BL/6 mice have been widely used in the production of global cerebral ischemia models. They are easily affected by neurological deficits and brain damage, and the carotid artery of the carotid artery is simply and briefly occluded (Berry K et al. 1975), even as short as 5 minutes. Compared with other strains of mice, C57BL/6 mice undergoing BCCAo surgery showed reliable ischemic neurological symptoms, and had highly reproducible histological damage in the hippocampus and papillae (Yang G et al. 1997).

The JNK signaling pathway is one of the key pathways that mediate cell apoptosis, which plays a role in the cell apoptosis of various organs. In recent years, studies have found that the activation of the JNK signaling pathway can mediate ovarian granulosa cell apoptosis, and the mitochondrial apoptosis of pancreatic cancer is also closely related to the activation of JNK (Yang H et al. 2017; Zhang X et al. 2019). What's more, the vascular dementia models typically exhibit downregulated anti-apoptotic Bcl-2 and upregulated pro-apoptotic Bax, cleaved caspase-3, and cleaved-PARP levels in the brain, for which

modulated p38 MAPK and JNK phosphorylation pathways play a vital role (Wang XX et al. 2020). Our previous studies have found that EA treatment can enhance the expression of endogenous BDNF, thereby improving the living environment of neurons in the brain and inhibiting hippocampal cell apoptosis (Zhao J et al. 2013). The present study showed EA could alleviate cognitive dysfunction, which may be achieved by regulating the JNK signaling pathway and inhibiting hippocampal nerve cell apoptosis.

The JNK of resting cells is mainly located in the cytoplasm. When activated by stimulators, the partially activated JNK is translocated to the nucleus and activated by phosphorylation to activate a variety of nuclear transcription factors, such as activated protein-1 (AP-1), P53 and so on (Davis RJ 2000; Pei H et al. 2016). After AP-1 activity is enhanced, it promotes P53 protein expression (Shaulian E et al. 2001). In recent years, studies have found that inhibiting the activity of JNK can reduce neuronal apoptosis in cerebral ischemic diseases (Wen XR et al. 2016). In present study, we found that BCCAo could increase the activity of JNK-3, AP-1 and P53. In addition, the results also showed that EA could inhibit the expression of JNK-3, AP-1 and P53. It is speculated that the therapeutic effect of EA may be through the regulation of JNK transcription-dependent pathway.

JNK signaling pathway can not only regulate the transcription of target genes in the nucleus, but also rapidly exert corresponding biological effects by directly regulating the structure and function of cytoplasmic substrates. A part of activated JNK stays in the cytoplasm and directly regulates the activity of Bcl-2 family members (Bim, Bax, Bcl-2, etc.) through phosphorylation, thereby mediating apoptosis in the mitochondrial pathway (Bogoyevitch MA et al. 2006; Carboni S et al. 2005; Tournier C et al. 2000; Perier C et al. 2007). This process does not rely on the expression of new genes. The Bcl-2 family is the main regulator of the JNK transcription-independent route. It is divided into three categories: pro-apoptotic proteins, such as Bak and Bax; anti-apoptotic proteins, such as Bcl-2 and Bcl-xL, and BH3- such as Bim and Bid. only protein. Among them, Bax is the main mediator of the mitochondrial pathway (Bogoyevitch MA et al. 2006; Perier C et al. 2007). Activated Bax translocates to the outer mitochondrial membrane to increase the permeability of the mitochondrial membrane and release pro-apoptotic mitochondrial proteins (Perier C et al. 2007). Mitochondrial proteins released into the cytoplasm initiates the caspase-dependent mitochondrial pathway of apoptosis by forming apoptotic bodies. Caspase-3 is a key factor in the process of cell apoptosis and is called the executor of cell apoptosis (Fan TJ et al. 2005). Our current study found that the expression of Bcl-2 decreased in the hippocampus of BCCAo model mice, while the expression of Bax and Caspase-3 increased. The research results also showed that EA could increase the expression of Bcl-2 and reduce the expression of Bax and Caspase-3. It is speculated that EA may play a neuroprotective effect by regulating the JNK mitochondrial pathway.

In summary, this study showed possible mechanisms by which EA at DU20, BL17 and BL23 promotes functional recovery in VaD mice. EA inhibited apoptosis of hippocampal neurons, improved cognitive impairment, and down-regulated the expression of JNK-3, AP-1, P53, Bax, Caspase-3 and up-regulated the expression of Bcl-2 in VaD mice. The mechanism of action of EA to treat VaD may be related to its regulation of JNK transcription-dependent pathway and JNK transcription-independent route. These investigations provided new insights into the mechanism of EA-induced anti-apoptotic effects in VaD.

# Declarations

## Consent for publication:

Written informed consent for publication was obtained from all participants.

## Availability of data and materials:

All data generated or analyzed during this study are included in this published article.

## Competing interests:

The authors have no conflicts of interest to declare that are relevant to the content of this article.


## Funding:

This study was supported by the Beijing University of Chinese Medicine New Teacher Startup Fund (No. 2019-JYB-XJSJJ014).


## Authors' contributions:

Yaru Liu, Chunyan Li, and Zhenyang Yan contributed to the study conception and design. Material preparation, data collection and analysis were performed by Yaru Liu, Yafei Ren, Woyu Wang, Yinze Ke, Yifan Wang, and Rongming Qi. Acupuncture treatment and data processing were performed by Yaru Liu. The first draft of the manuscript was written by Yaru Liu and, Chunyan Li and Zhenyang commented on previous versions of the manuscript. All authors read and approved the final manuscript.

## Authors' information:

## Affiliations


**Shizhong District People’s Hospital of Jinan, Jinan 250000, China**
Chunyan Li
**Weifang Traditional Chinese Hospital, Weifang 261041, China**
Zhenyang Yan
**School of Acupuncture-Moxibustion and Tuina, Beijing University of Chinese Medicine, Beijing 100029, China**
Yafei Ren
**School of Acupuncture-Moxibustion and Tuina, Beijing University of Chinese Medicine, Beijing 100029, China**
Woyu Wang
**School of Acupuncture-Moxibustion and Tuina, Beijing University of Chinese Medicine, Beijing 100029, China**
Yinze Ke
**School of Acupuncture-Moxibustion and Tuina, Beijing University of Chinese Medicine, Beijing 100029, China**
Yifan Wang
**School of Acupuncture-Moxibustion and Tuina, Beijing University of Chinese Medicine, Beijing 100029, China**
Rongming Qi


## Corresponding author


Correspondence to Yaru Liu, School of Acupuncture-Moxibustion and Tuina, Beijing University of Chinese Medicine, Beijing 100029, China. liuyaru@bucm.edu.cn, ORCID: 0000-0002-8530-2547


## Acknowledgements:

Not Applicable

## Ethics Approval:

All procedures performed in studies were in accordance with the ethical standards of the guide for the care and use of laboratory animals. The study was approved by the Ethics Committee for Animal Experimentation and Use Committee of Beijing University of Chinese Medicine (Permit No. Kj-dw-32-20191012).

# References


1. Berry K, Wiśniewski HM, Svarzbein L, Baez S (1975 May;25(1):75–92) On the relationship of brain vasculature to production of neurological deficit and morphological changes following acute unilateral common carotid artery ligation in gerbils. J Neurol Sci. doi: 10.1016/0022-510x(75)90188-4. PMID: 1141958
2. Bogoyevitch MA, Kobe B (2006 Dec) Uses for JNK: the many and varied substrates of the c-Jun N-terminal kinases. Microbiol Mol Biol Rev 70(4):1061–1095. doi:10.1128/MMBR.00025-06. PMID: 17158707; PMCID: PMC1698509
3. Bredesen DE, Rao RV, Mehlen P. Cell death in the nervous system. Nature. 2006 Oct 19;443(7113):796–802. doi: 10.1038/nature05293. PMID: 17051206; PMCID: PMC3970704
4. Carboni S, Antonsson B, Gaillard P, Gotteland JP, Gillon JY, Vitte PA (2005 Mar;92(5):1054-60) Control of death receptor and mitochondrial-dependent apoptosis by c-Jun N-terminal kinase in hippocampal CA1 neurones following global transient ischaemia. J Neurochem. doi: 10.1111/j.1471-4159.2004.02925.x. PMID: 15715656
5. Chang L, Karin M (2001) Mammalian MAP kinase signalling cascades. Nature. Mar 1;410(6824):37–40. doi: 10.1038/35065000. PMID: 11242034
6. Davis RJ (2000) Signal transduction by the JNK group of MAP kinases. Cell. Oct 13;103(2):239 – 52. doi: 10.1016/s0092-8674(00)00116-1. PMID: 11057897
7. Ding N, Jiang J, Xu A, Tang Y, Li Z. Manual Acupuncture Regulates Behavior and Cerebral Blood Flow in the SAMP8 Mouse Model of Alzheimer's Disease. Front Neurosci. 2019 Jan 31;13:37. doi: 10.3389/fnins.2019.00037. PMID: 30766475; PMCID: PMC6365452
8. Dunn C, Wiltshire C, MacLaren A, Gillespie DA (2002 Jul;14(7):585 – 93) Molecular mechanism and biological functions of c-Jun N-terminal kinase signalling via the c-Jun transcription factor. Cell Signal. doi: 10.1016/s0898-6568(01)00275-3. PMID: 11955951
9. Davis RJ (2000) Signal transduction by the JNK group of MAP kinases. Cell. Oct 13;103(2):239 – 52. doi: 10.1016/s0092-8674(00)00116-1. PMID: 11057897
10. Endo H, Saito A, Chan PH (2006) Mitochondrial translocation of p53 underlies the selective death of hippocampal CA1 neurons after global cerebral ischaemia. Biochem Soc Trans. Dec;34(Pt 6):1283-6. doi: 10.1042/BST0341283. Erratum in: Biochem Soc Trans. 2007 Dec;35(Pt 6):1649. PMID: 17073802

11. Fan TJ, Han LH, Cong RS, Liang J (2005 Nov;37(11):719 – 27) Caspase family proteases and apoptosis. Acta Biochim Biophys Sin (Shanghai). doi: 10.1111/j.1745-7270.2005.00108.x. PMID: 16270150
12. Jang JH, Yeom MJ, Ahn S, Oh JY, Ji S, Kim TH, Park HJ (2020 Oct) Acupuncture inhibits neuroinflammation and gut microbial dysbiosis in a mouse model of Parkinson's disease. Brain Behav Immun 89:641–655 doi: 10.1016/j.bbi.2020.08.015. Epub 2020 Aug 19. PMID: 32827699
13. Kalaria RN (2018) The pathology and pathophysiology of vascular dementia. Neuropharmacology. May 15;134(Pt B):226–239. doi: 10.1016/j.neuropharm.2017.12.030. Epub 2017 Dec 19. PMID: 29273521
14. Kaptchuk TJ (2002) Acupuncture: theory, efficacy, and practice. Ann Intern Med. Mar 5;136(5):374 – 83. doi: 10.7326/0003-4819-136-5-200203050-00010. PMID: 11874310
15. Kim YI, Kim SS, Sin RS, Pu YJ, Ri G, Rim KS. Study on the Cerebral Blood Flow Regulatory Features of Acupuncture at Acupoints of the Governor Vessel. Med Acupunct. 2018 Aug 1;30(4):192–197. doi: 10.1089/acu.2018.1285. PMID: 30147820; PMCID: PMC6106755
16. Lin A (2003 Jan;25(1):17–24) Activation of the JNK signaling pathway: breaking the brake on apoptosis. Bioessays. doi: 10.1002/bies.10204. PMID: 12508278
17. Lin L, Yu L, Xiang H, Hu X, Yuan X, Zhu H, Li H, Zhang H, Hou T, Cao J, Wu S, Su W, Li M (2019 Oct) Effects of Acupuncture on Behavioral Stereotypies and Brain Dopamine System in Mice as a Model of Tourette Syndrome. Front Behav Neurosci 15:13:239. doi:10.3389/fnbeh.2019.00239. PMID: 31680895; PMCID: PMC6803462
18. Li X, Luo P, Wang Q, Xiong L (2012) Electroacupuncture Pretreatment as a Novel Avenue to Protect Brain against Ischemia and Reperfusion Injury. Evid Based Complement Alternat Med 2012:195397. doi:10.1155/2012/195397. Epub 2012 Apr 26. PMID: 22611425; PMCID: PMC3350954
19. Longa EZ, Weinstein PR, Carlson S, Cummins R. Reversible middle cerebral artery occlusion without craniectomy in rats. Stroke. 1989 Jan;20(1):84–91. doi: 10.1161/01.str.20.1.84. PMID: 2643202
20. Maundrell K, Antonsson B, Magnenat E, Camps M, Muda M, Chabert C, Gillieron C, Boschert U, Vial-Knecht E, Martinou JC, Arkinstall S. Bcl-2 undergoes phosphorylation by c-Jun N-terminal kinase/stress-activated protein kinases in the presence of the constitutively active GTP-binding protein Rac1. J Biol Chem. 1997 Oct 3;272(40):25238-42. doi: 10.1074/jbc.272.40.25238. PMID: 9312139
21. Moon SK, Kwon S, Cho SY, Park SU, Jung WS, Park JM, Ko CN, Cho KH, Son JH (2019 Oct) Effects of GB34 acupuncture on hyperventilation-induced carbon dioxide reactivity and cerebral blood flow velocity in the anterior and middle cerebral arteries of normal subjects. Acupunct Med 37(5):277–282. doi:10.1136/acupmed-2017-011474. Epub 2019 Aug 15. PMID: 31412706
22. Pei H, Jiang T, Liu G, Li Z, Luo K, An J, Li G, Guo Y. The Effect of Minimally Invasive Hematoma Aspiration on the JNK Signal Transduction Pathway after Experimental Intracerebral Hemorrhage in Rats. Int J Mol Sci. 2016 May 13;17(5):710. doi: 10.3390/ijms17050710. PMID: 27187368; PMCID: PMC4881532

23. Perier C, Bové J, Wu DC, Dehay B, Choi DK, Jackson-Lewis V, Rathke-Hartlieb S, Bouillet P, Strasser A, Schulz JB, Przedborski S, Vila M. Two molecular pathways initiate mitochondria-dependent dopaminergic neurodegeneration in experimental Parkinson's disease. Proc Natl Acad Sci U S A. 2007 May 8;104(19):8161-6. doi: 10.1073/pnas.0609874104. Epub 2007 May 2. PMID: 17483459; PMCID: PMC1876588
24. Qin AP, Liu CF, Qin YY, Hong LZ, Xu M, Yang L, Liu J, Qin ZH, Zhang HL (2010 Aug;6(6):738 – 53) Autophagy was activated in injured astrocytes and mildly decreased cell survival following glucose and oxygen deprivation and focal cerebral ischemia. Autophagy. doi: 10.4161/auto.6.6.12573. Epub 2010 Aug 3. PMID: 20574158
25. Sha R, Han X, Zheng C, Peng J, Wang L, Chen L, Huang X. The Effects of Electroacupuncture in a Rat Model of Cerebral Ischemia-Reperfusion Injury Following Middle Cerebral Artery Occlusion Involves MicroRNA-223 and the PTEN Signaling Pathway. Med Sci Monit. 2019 Dec 28;25:10077–10088. doi: 10.12659/MSM.919611. PMID: 31883264; PMCID: PMC6946047
26. Shaulian E, Karin M. AP-1 in cell proliferation and survival. Oncogene. 2001 Apr 30;20(19):2390 – 400. doi: 10.1038/sj.onc.1204383. PMID: 11402335
27. Shaulian E, Karin M (2002 May;4(5):E131-6) AP-1 as a regulator of cell life and death. Nat Cell Biol. doi: 10.1038/ncb0502-e131. PMID: 11988758
28. Soares LM, Schiavon AP, Milani H, de Oliveira RM. Cognitive impairment and persistent anxiety-related responses following bilateral common carotid artery occlusion in mice. Behav Brain Res. 2013 Jul 15;249:28–37. doi: 10.1016/j.bbr.2013.04.010. Epub 2013 Apr 17. PMID: 23602921
29. Spray S, Edvinsson L (2016 Jul;234(7):1925–1934) Improved assessment of outcomes following transient global cerebral ischemia in mice. Exp Brain Res. doi: 10.1007/s00221-016-4597-5. Epub 2016 Feb 25. PMID: 26914479
30. Sun X, Liu H, Sun Z, Zhang B, Wang X, Liu T, Pan T, Gao Y, Jiang X, Li H. Acupuncture protects against cerebral ischemia-reperfusion injury via suppressing endoplasmic reticulum stress-mediated autophagy and apoptosis. Mol Med. 2020 Nov 10;26(1):105. doi: 10.1186/s10020-020-00236-5. PMID: 33167857; PMCID: PMC7653860
31. Tournier C, Hess P, Yang DD, Xu J, Turner TK, Nimnual A, Bar-Sagi D, Jones SN, Flavell RA, Davis RJ. Requirement of JNK for stress-induced activation of the cytochrome c-mediated death pathway. Science. 2000 May 5;288(5467):870-4. doi: 10.1126/science.288.5467.870. PMID: 10797012
32. Vinters HV, Zarow C, Borys E, Whitman JD, Tung S, Ellis WG, Zheng L, Chui HC. Review: Vascular dementia: clinicopathologic and genetic considerations. Neuropathol Appl Neurobiol (2018 Apr;44(3):247–266) doi: 10.1111/nan.12472. Epub 2018 Mar 1. PMID: 29380913
33. Wahul AB, Joshi PC, Kumar A, Chakravarty S. Transient global cerebral ischemia differentially affects cortex, striatum and hippocampus in Bilateral Common Carotid Arterial occlusion (BCCAo) mouse model. J Chem Neuroanat. 2018 Oct;92:1–15. doi: 10.1016/j.jchemneu.2018.04.006. Epub 2018 Apr 24. PMID: 29702163

34. Wang H, Chen S, Zhang Y, Xu H, Sun H (2019) Electroacupuncture ameliorates neuronal injury by Pink1/Parkin-mediated mitophagy clearance in cerebral ischemia-reperfusion. Nitric Oxide. Oct 1;91:23–34. doi: 10.1016/j.niox.2019.07.004. Epub 2019 Jul 16. PMID: 31323277

35. Wang HQ, Imai Y, Kataoka A, Takahashi R (2007 May;9(5):533 – 42) Cell type-specific upregulation of Parkin in response to ER stress. Antioxid Redox Signal. doi: 10.1089/ars.2006.1522. PMID: 17465879

36. Wan L, Cheng Y, Luo Z, Guo H, Zhao W, Gu Q, Yang X, Xu J, Bei W, Guo J. Neuroprotection, learning and memory improvement of a standardized extract from Renshen Shouwu against neuronal injury and vascular dementia in rats with brain ischemia. J Ethnopharmacol. 2015 May 13;165:118–26. doi: 10.1016/j.jep.2015.02.027. Epub 2015 Feb 19. PMID: 25704930

37. Wang XX, Zhang B, Xia R, Jia QY (2020 Sep;24(18):9601–9614) Inflammation, apoptosis and autophagy as critical players in vascular dementia. Eur Rev Med Pharmacol Sci. doi: 10.26355/eurrev_202009_23048. PMID: 33015803

38. Wen XR, Tang M, Qi DS, Huang XJ, Liu HZ, Zhang F, Wu J, Wang YW, Zhang XB, Guo JQ, Wang SL, Liu Y, Wang YL, Song YJ. Butylphthalide Suppresses Neuronal Cells Apoptosis and Inhibits JNK-Caspase3 Signaling Pathway After Brain Ischemia /Reperfusion in Rats. Cell Mol Neurobiol. 2016 Oct;36(7):1087–95. doi: 10.1007/s10571-015-0302-7. Epub 2016 Mar 25. PMID: 27015680

39. Wu L, Feng XT, Hu YQ, Tang N, Zhao QS, Li TW, Li HY, Wang QB, Bi XY, Cai XK (2015 Sep;237(1):57–67) Global Gene Expression Profile of the Hippocampus in a Rat Model of Vascular Dementia. Tohoku J Exp Med. doi: 10.1620/tjem.237.57. PMID: 26353909

40. Yamamoto K, Ichijo H, Korsmeyer SJ (1999 Dec) BCL-2 is phosphorylated and inactivated by an ASK1/Jun N-terminal protein kinase pathway normally activated at G(2)/M. Mol Cell Biol 19(12):8469–8478. doi:10.1128/MCB.19.12.8469. PMID: 10567572; PMCID: PMC84954

41. Yang G, Kitagawa K, Matsushita K, Mabuchi T, Yagita Y, Yanagihara T, Matsumoto M. C57BL/6 strain is most susceptible to cerebral ischemia following bilateral common carotid occlusion among seven mouse strains: selective neuronal death in the murine transient forebrain ischemia. Brain Res. 1997 Mar 28;752(1–2):209 – 18. doi: 10.1016/s0006-8993(96)01453-9. PMID: 9106459

42. Yang H, Xie Y, Yang D, Ren D (2017) Oxidative stress-induced apoptosis in granulosa cells involves JNK, p53 and Puma. Oncotarget. Apr 11;8(15):25310–25322. doi: 10.18632/oncotarget.15813. PMID: 28445976; PMCID: PMC5421932.)

43. Yu C, Minemoto Y, Zhang J, Liu J, Tang F, Bui TN, Xiang J, Lin A. JNK suppresses apoptosis via phosphorylation of the proapoptotic Bcl-2 family protein BAD. Mol Cell. 2004 Feb 13;13(3):329 – 40. doi: 10.1016/s1097-2765(04)00028-0. PMID: 14967141

44. Zhao JX, Tian YX, Xiao HL, Hu MX, Chen WR (2011 Dec;31(4):349 – 55) Effects of electroacupuncture on hippocampal and cortical apoptosis in a mouse model of cerebral ischemia-reperfusion injury. J Tradit Chin Med. doi: 10.1016/s0254-6272(12)60017-x. PMID: 22462244

45. Zhao J, Xu H, Tian Y, Hu M, Xiao H (2013 Apr;33(2):253-7) Effect of electroacupuncture on brain-derived neurotrophic factor mRNA expression in mouse hippocampus following cerebral ischemia-

reperfusion injury. J Tradit Chin Med. doi: 10.1016/s0254-6272(13)60135-1. PMID: 23789227

46. Zhang X, Jiang J, Chen Z, Cao M (2019 Sep;215(9):152530) Silibinin inhibited autophagy and mitochondrial apoptosis in pancreatic carcinoma by activating JNK/SAPK signaling. Pathol Res Pract. doi: 10.1016/j.prp.2019.152530. Epub 2019 Jul 11. PMID: 31351801
47. Zhou H, Zhang Z, Wei H, Wang F, Guo F, Gao Z, Marsicano G, Wang Q, Xiong L. Activation of STAT3 is involved in neuroprotection by electroacupuncture pretreatment via cannabinoid CB1 receptors in rats. Brain Res. 2013 Sep 5;1529:154–64. doi: 10.1016/j.brainres.2013.07.006. Epub 2013 Jul 20. PMID: 23880371
48. Zille M, Farr TD, Przesdzing I, Müller J, Sommer C, Dirnagl U, Wunder A (2012 Feb) Visualizing cell death in experimental focal cerebral ischemia: promises, problems, and perspectives. J Cereb Blood Flow Metab 32(2):213–231. doi:10.1038/jcbfm.2011.150. Epub 2011 Nov 16. PMID: 22086195; PMCID: PMC3272608

# Figures

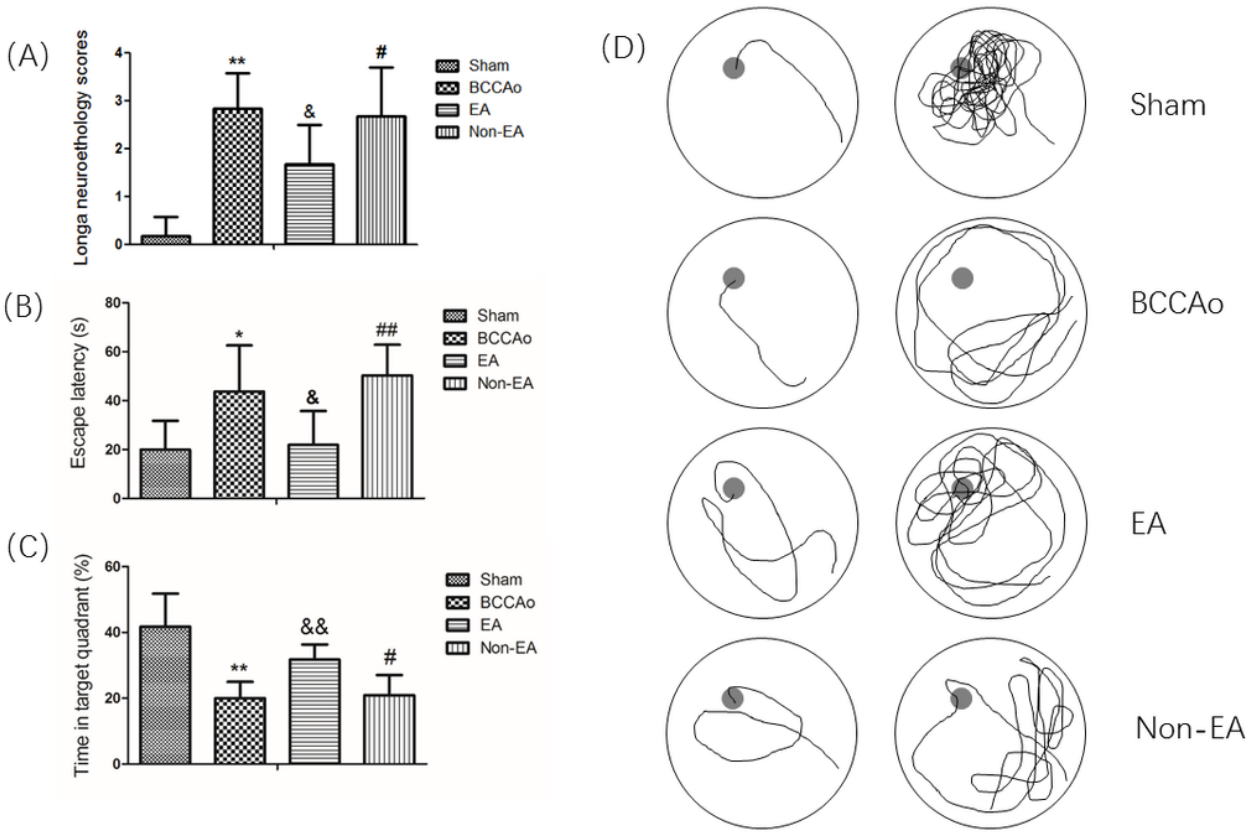
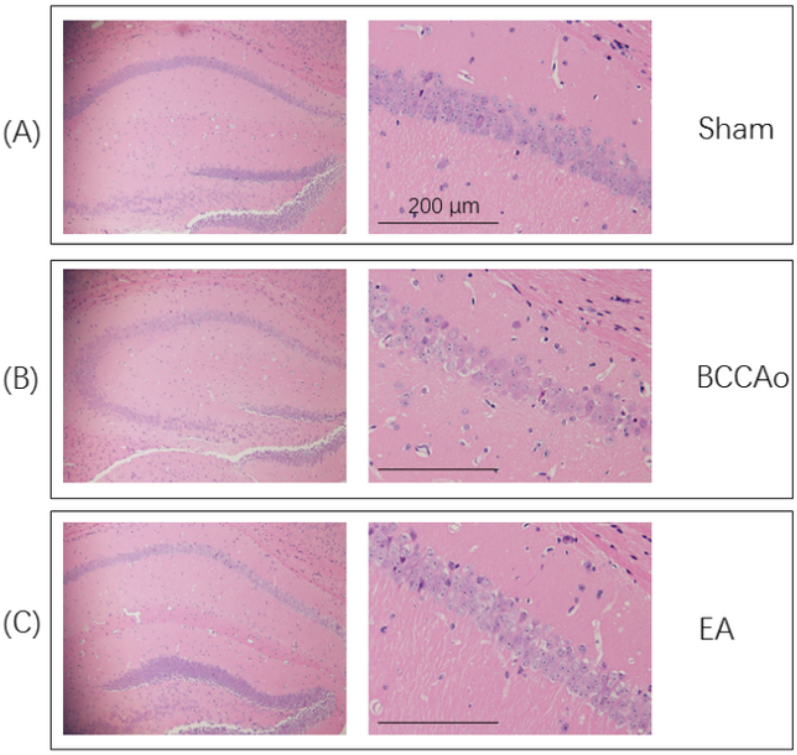



**Figure 1**

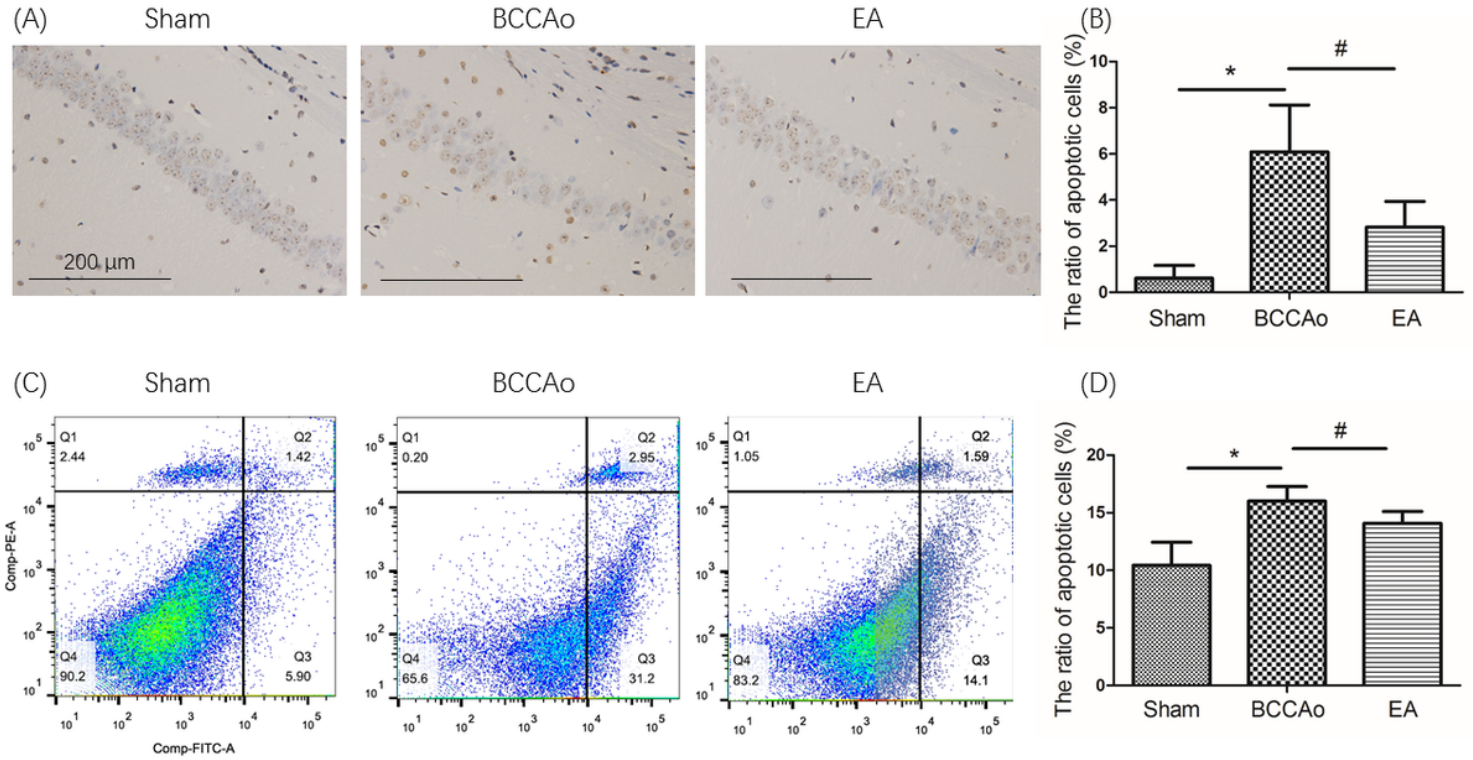
A improved the neurological function and learning and memory deficits in VaD mice. A, Longa score. B, escape latency. C, The time spent in target quadrant on the seventh day. D, the typical swimming trajectories of all groups before surgery and 24 hours after EA treatment. Values are expressed as means ± SEM (n = 8 in each group). *P <0.05, **P <0.01, BCCAo vs Sham. &P <0.05, &&P <0.01, EA vs BCCAo. #P

<0.05, ##P <0.01, Non-EA vs EA. (For Longa score, escape latency, two-way repeated measure analysis of ANOVE; For the time spent in target quadrant on the seventh day, one-way ANOVA)



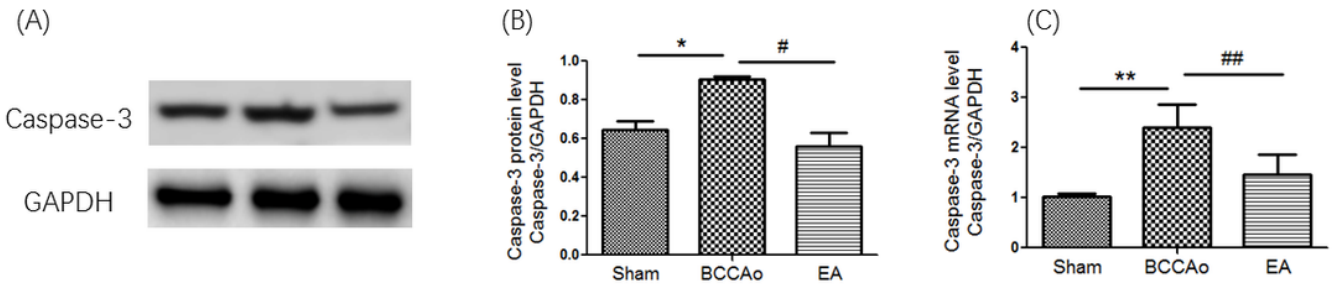

## Figure 2

EA improved the tissue morphology of hippocampal CA1 area in VaD mice. A, the sham group. B, the BCCAo group. C, the EA group (bar indicates 200 μm).



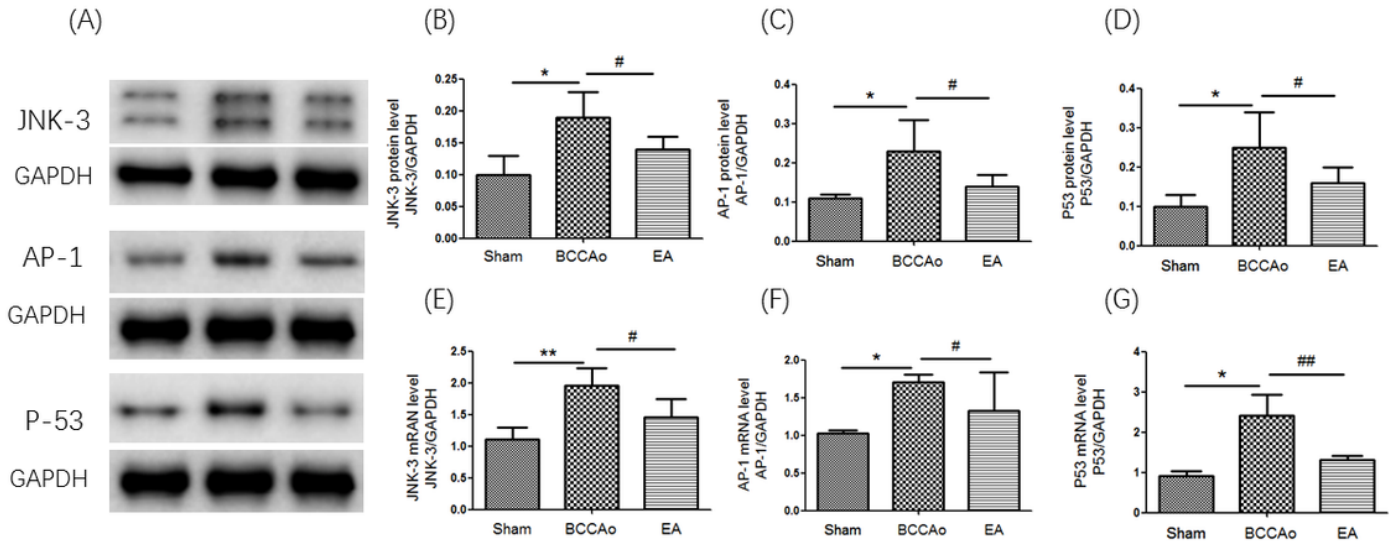

## Figure 3

EA inhibited neuronal apoptosis in the hippocampal CA1 region in VaD mice. A and B, TUNEL staining. C and D, FCM. Values are expressed as mean ± SEM (n = 6 in each group). Values are expressed as mean ± SEM. *P <0.05, BCCAo vs Sham, #P <0.05, EA vs BCCAo (one-way ANOVA).



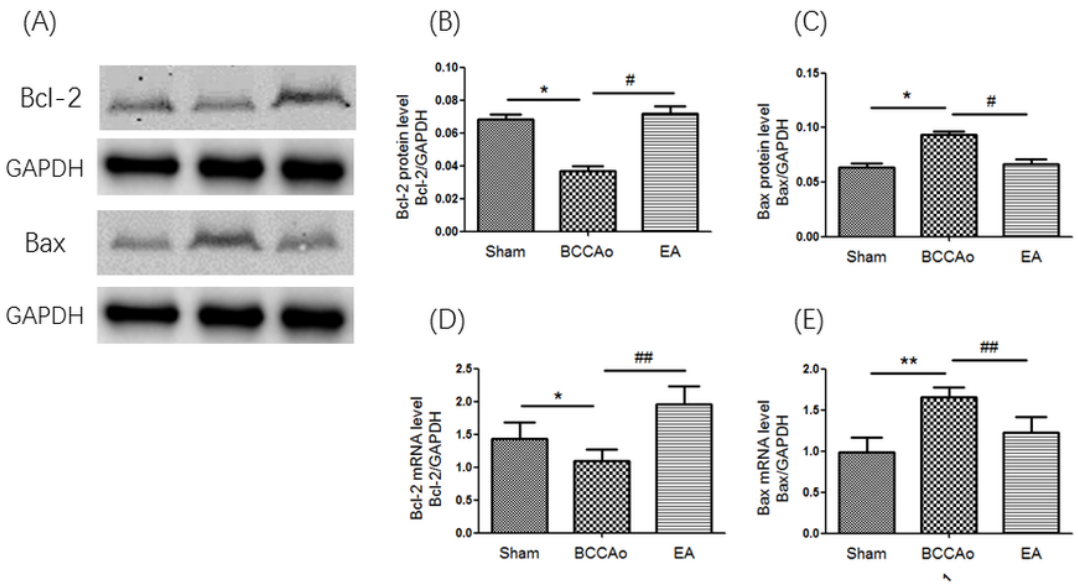

## Figure 4

EA down-regulated the levels of Caspase-3 in the hippocampus. A and B, the protein expression of Caspase-3 in the hippocampus. C, the mRNA level of Caspase-3. Data are presented as mean ± SEM (n = 6 in each group). *P <0.05, **P <0.01, BCCAo vs Sham. #P <0.05, ##P <0.01, EA vs BCCAo (one-way ANOVA).




## Figure 5

EA down-regulated the levels of JNK-3, AP-1, P53 in the hippocampus. A-D: the protein expressions of JNK-3, AP-1, P53 in the hippocampus. E-G, the mRNA levels of JNK-3, AP-1, P53. Data are presented as mean ± SEM (n = 6 in each group). *P <0.05, **P <0.01, BCCAo vs Sham. #P <0.05, ##P <0.01, EA vs BCCAo (one-way ANOVA).




## Figure 6

EA up-regulated the level of Bcl-2 in the hippocampus and down-regulated the level of Bax. A-C: the protein expressions of Bcl-2 and Bax in the hippocampus. D and E, the mRNA levels of Bcl-2 and Bax. Data are presented as mean ± SEM (n = 6 in each group). *P <0.05, **P <0.01, BCCAo vs Sham. #P <0.05, ##P <0.01, EA vs BCCAo (one-way ANOVA).