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Baifuzi reduces transient ischemic brain damage through an interaction with the STREX domain of BK$_{Ca}$ channels

S Chi[1,3,7], W Cai[2,7], P Liu[3,6,7], Z Zhang[2], X Chen[4], L Gao[5], J Qi[5], L Bi[4], L Chen*,[2] and Z Qi*,[1,3]

Stroke is a long-term disability and one of the leading causes of death. However, no successful therapeutic intervention is available for the majority of stroke patients. In this study, we explored a traditional Chinese medicine *Baifuzi* (*Typhonium giganteum* Engl.). We show, at first, that the ethanol extract of *Baifuzi* exerts neuroprotective effects against brain damage induced by transient global or focal cerebral ischemia in rats and mice. Second, the extract activated large-conductance Ca^{2+}-activated K$^+$ channel (BK$_{Ca}$) channels, and BK$_{Ca}$ channel blockade suppressed the neuroprotection of the extract, suggesting that the BK$_{Ca}$ is the molecular target of *Baifuzi*. Third, *Baifuzi* cerebroside (*Baifuzi*-CB), purified from its ethanol extract, activated BK$_{Ca}$ channels in a manner similar to that of the extract. Fourth, the stress axis hormone-regulated exon (STREX) domain of the BK$_{Ca}$ channel directly interacted with *Baifuzi*-CB, and its deletion suppressed channel activation by *Baifuzi*-CB. These results indicate that *Baifuzi*-CB activated the BK$_{Ca}$ channel through its direct interaction with the STREX domain of the channel and suggests that *Baifuzi*-CB merits exploration as a potential therapeutic agent for treating brain ischemia.

Stroke is a devastating condition that annually affects 15 million people worldwide, and is the leading cause of adult disability in industrialized countries.[1] Although there has been progress in our understanding of the pathophysiology of stroke during the past few decades, stroke continues to pose major therapeutic challenges, both to neuroscientists and to clinicians.[2] For example, a large number of neuroprotective agents have been shown to successfully reduce infarct size in animal models of stroke, but they have failed to demonstrate efficacy in phase III clinical trials in humans.[3] To date, no successful therapeutic interventions are available for the majority of stroke patients.[1,3] The intravenous recombinant tissue plasminogen activator (rt-PA) is the only drug therapy available for thrombolytic treatment of stroke in the United States, Canada and Europe. However, the requirement to exclude hemorrhagic stroke and the restriction to use within 3 h of the onset of symptoms limit rt-PA therapy to only ∼2%

of all stroke patients.[1] Therefore, there is an urgent need for new therapeutic options for the treatment of stroke.

One of the options that might offer fertile ground for modern drug development is traditional medicines, because traditional medicines, such as traditional Chinese medicine (TCM), have long time since documented the effects of many natural products on humans, and extensive human experience can provide some degree of comfort with regard to their safety.[4] Clinical application of artemisinin, triptolide, celastrol, capsaicin and curcumin has demonstrated the power and promise of turning traditional medicines into modern drugs. One of the most recent examples is veregen, an extract of green tea leaves, which was approved in 2006 by the US Food and Drug Administration as a new drug to treat perianal and genital condyloma.[5] As a matter of fact, more than half of the pharmaceutical drugs are either natural products or their derivatives.[6]

[1]Department of Physiology, Medical College of Xiamen University, 168 Daxue Road, Xiamen, China; [2]Department of Physiology, Nanjing Medical University, 140 Hanzhong Road, Nanjing, China; [3]State Key Laboratory of Brain and Cognitive Science, Institute of Biophysics, 15 Datun Road, Beijing, China; [4]National Laboratory of Biomacromolecules, Institute of Biophysics, 15 Datun Road, Beijing, China; [5]College of Pharmaceutical Sciences, Zhejiang University, 388 Yuhangtang Road, Hangzhou, China and [6]Graduate University of Chinese Academy of Sciences, 19A Yuquan Road, Beijing, China
*Corresponding authors: Z Qi, Department of Physiology, Medical College of Xiamen University, 168 Daxue Road, Xiamen 361005, China. Tel: +86 0592 218 1330; Fax: +86 0592 218 2736; E-mail: qizhi@xmu.edu.cn or L Chen, Department of Physiology, Nanjing Medical University, 140 Hanzhong Road, Nanjing 210029, China. E-mail: lingchen@njmu.edu.cn
[7]These authors contributed equally to this work.
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Abbreviations: 4VO, 4-vessel occlusion; BK$_{Ca}$ channel, large-conductance Ca^{2+}-activated K$^+$ channel; *Baifuzi*-CB, *Baifuzi* cerebroside; [Ca^{2+}]i, intracellular free Ca^{2+} concentration; e-*Baifuzi*, ethanol extract of *Baifuzi*; CTX, charybdotoxin; IPI, interpulse interval; MCAO, middle cerebral artery occlusion; P_o, single channel open probability; PLO, protein lipid overlay; PPF, paired-pulse facilitation; PPR, paired-pulse ratio; SIGD, synaptically induced glial depolarization; STREX, stress axis hormone-regulated exon; TCM, traditional Chinese medicine; TLC, thin layer chromatography
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Dried tubers of the herb *Typhonium giganteum* Engl. (Chinese name: *Baifuzi*), as a TCM, has been recorded in Chinese pharmacopeia and used for the treatment of brain stroke for a long time.[7] However, unlike industrially manufactured pharmacological drugs used in Western medicine, its active components and molecular targets have not been specified. Obviously, identification of its effects on well-defined molecular targets and the subsequent identification of its active component(s) underlying these effects are important for the evaluation of its therapeutic efficacy. Brain ischemia begins with energy depletion, followed by the disruption of ion homeostasis, membrane depolarization, excessive neurotransmitter (glutamate) release and elevation of intracellular calcium, all of which initiate a neurotoxic cascade that results in neurodegeneration and death of ischemic neurons.[8,9] The large-conductance Ca^{2+}-activated K^+ (BK_{Ca}) channels are widely expressed in the brain[10] and are preferentially located at glutamatergic synaptic terminals.[11,12] They react to either increases in intracellular Ca^{2+} or membrane depolarization by increasing K^+ efflux. Thus, they function as an 'emergency break' to limit Ca^{2+} influx for regulating synaptic transmission under conditions of enhanced neuronal excitability.[13] It has been shown that activation of BK_{Ca} channels in ischemic cells by BK_{Ca} channel openers could minimize neuronal depolarization, limit accumulation of potentially pathological levels of Ca^{2+} from a number of potential sources, reduce neurotransmitter release and significantly attenuate infarct growth during ischemic stroke in animal models.[14,15] In addition, blockade of BK_{Ca} channels enhanced cell damage in organotypical slice cultures after oxygen and glucose deprivation.[16] In this study, we show that the ethanol extract of *Baifuzi* (e-*Baifuzi*) shows significant neuronal protective activity in the rat and mice model of ischemic stroke. Second, we show that the BK_{Ca} channel is the molecular target of the ethanol extract. Third, we identified that *Baifuzi* cerebroside (*Baifuzi*-CB) is the active component of *Baifuzi* in activating the BK_{Ca} channel. Finally, we show that the stress axis hormone-regulated exon (STREX) domain of the BK_{Ca} channel, a 59-amino-acid splice insertion located in the cytoplasmic side of the channel, is required for the activation of the BK_{Ca} channel by *Baifuzi*-CB, probably due to direct interaction between *Baifuzi*-CB and the STREX domain.

Results

e-*Baifuzi* protects ischemia-induced brain injury
e-Baifuzi against ischemia-induced neuronal cell death. Transient global ischemia leads to a dramatic loss of neurons in the CA1 region of the hippocampus.[17,18] Therefore, we examined whether e-*Baifuzi* has neuroprotective roles in the CA1 region in the rat model of transient global ischemia, which was made by 20 min 4-vessel occlusion (4VO). In the CA1 region of the ischemic hemisphere, the number of pyramidal neurons on the eighth day post-4VO was <50% of the control (2VO rats, $n=8$, $P<0.01$; Figures 1a and b). Surprisingly, a single injection of e-*Baifuzi* (5 or 50 mg/kg) at 1 h post-4VO significantly attenuated neuronal loss in a dose-dependent manner (*versus* vehicle-treated 4VO rats, $n=8$, $P<0.01$), although

no neuroprotective effect was observed at a dosage of 1 mg/kg ($P>0.05$). In contrast, the administration of e-*Baifuzi* at a dosage of 5 mg/kg did not affect the number of pyramidal neurons in 2VO rats ($n=8$, $P>0.05$). Furthermore, the administration of e-*Baifuzi* (5 mg/kg) even at 4–8 h post-4VO also significantly reduced ischemia-induced neuronal loss (*versus* vehicle-treated 4VO rats, $n=8$, $P<0.01$; Figure 1c), whereas it could not protect neuronal cells when administered at 24 h post-4VO ($n=8$, $P>0.05$). These results suggested that e-*Baifuzi* could protect the brain from transient global cerebral ischemia and that its therapeutic time window may last up to 8 h after the onset of ischemia.

e-Baifuzi improves ischemia-induced deficits in spatial memory. Neuronal damage induced by transient global ischemia is accompanied by severe impairments in performance on hippocampally dependent spatial learning tasks.[19] To investigate whether e-*Baifuzi* could improve the behavioral disorder induced by transient global cerebral ischemia, we evaluated its effects on the performance of rats in a Morris water maze test. Results showed that rats subjected to 20 min global cerebral ischemia (4VO) significantly extended the escape latency to the hidden platform compared with 2VO rats ($n=8$, $P<0.01$; Figure 1d), whereas the swimming speed was not detectably different between them (data not shown). In agreement with its neuroprotection, e-*Baifuzi* (5 mg/kg) reduced ischemia-induced prolongation of the escape latency when administered between 1 and 8 h post-4VO (*versus* vehicle-treated 4VO rats, $n=8$, $P<0.01$). In contrast, it did not affect the escape latency of 2VO rats at a dosage of 5 mg/kg ($n=8$, $P>0.05$). Thus, e-*Baifuzi* could improve spatial memory impairment induced by global cerebral ischemia.

e-Baifuzi reduces MCAO-produced cerebral infarction. To determine the effect of e-*Baifuzi* against focal cerebral ischemia-induced injury, we used a mouse model of middle cerebral artery occlusion (MCAO) by the intraluminal filament insertion technique. Infarct size was measured at 24 h of reperfusion as described previously.[20] As shown in Figure 2a and b, a single injection of e-*Baifuzi* (5 mg/kg) at 1 h post-MCAO remarkably reduced the hemispheric infarct, from $42\pm2\%$ in vehicle (saline)-injected MCAO mice ($n=10$) to $19\pm5\%$ in e-*Baifuzi*-injected MCAO mice ($n=11$, $P<0.01$), indicating that e-*Baifuzi* could reduce cerebral infarct induced by focal ischemia-induced damage.

e-*Baifuzi* protects the brain from ischemic injury through activation of BK_{Ca} channels
e-Baifuzi activates BK_{Ca} channels. The above study confirmed the protective role of e-*Baifuzi* in ischemic injury. As BK_{Ca} channels have been shown to have an important role in ischemic stroke,[14,15] we explored whether the BK_{Ca} channel is a potential target for the effect of e-*Baifuzi*. Whole-cell configuration was used to investigate the effect of e-*Baifuzi* on the whole-cell currents of BK_{Ca} channels expressed in CHO cells. All test experiments ($n=28$) showed an increase in current amplitude with the application of e-*Baifuzi*. Figure 3a clearly shows increases in whole-cell BK_{Ca} channel currents from the same cell in
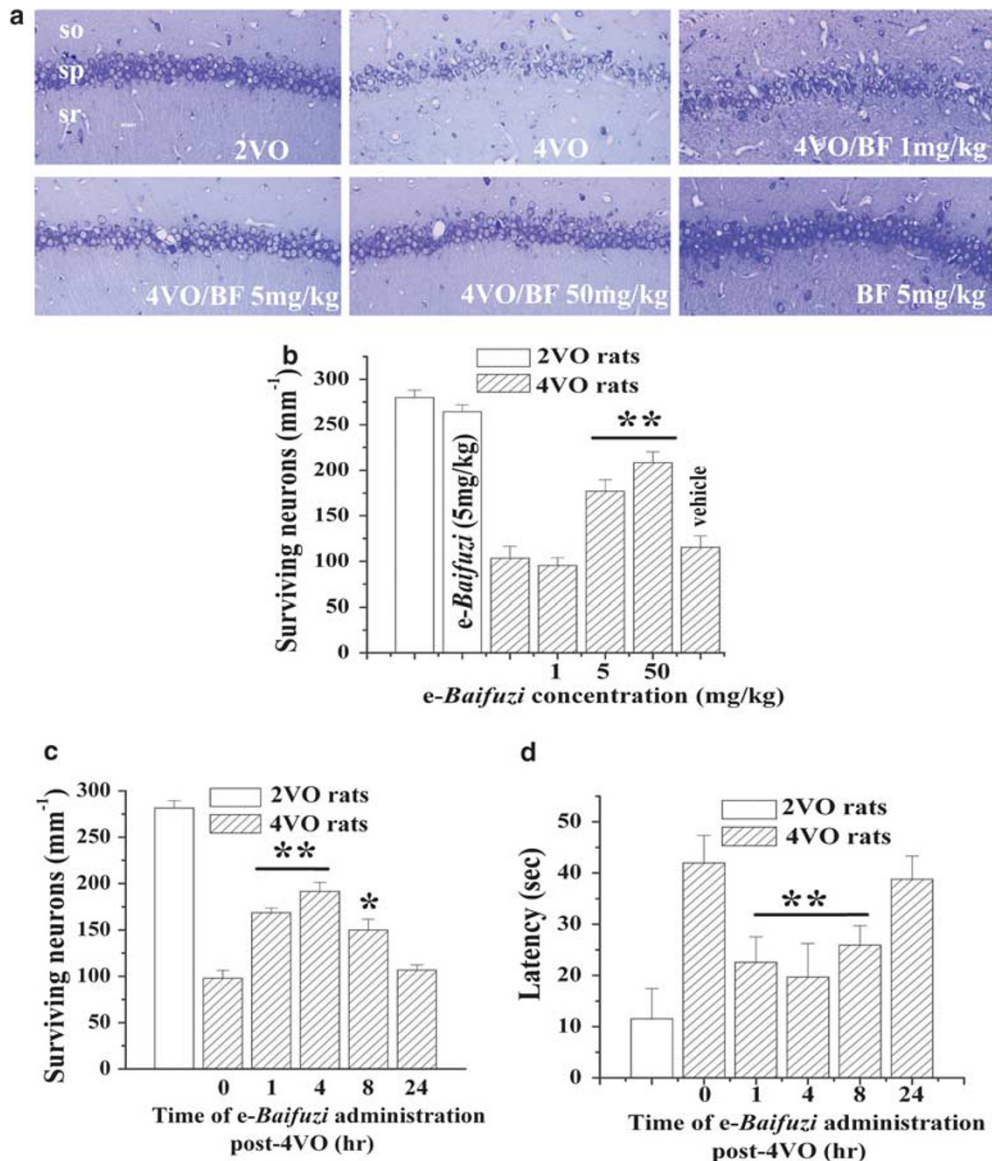
Figure 1 Effects of e-*Baifuzi* on global cerebral ischemia. (**a**) Representative sections in 2VO and 4VO rats treated with vehicle or e-*Baifuzi* (0, 1, 5 and 50 mg/kg) at 1 h post-4VO. (**b**) Bar graphs represent the number of surviving pyramidal neurons of hippocampal CA1 in 2VO (open bars) and 4VO rats (hatched bars). Histological changes in ischemic hippocampal CA1 were assessed on the eighth day post-4VO. (**c**) Time window of e-*Baifuzi* neuroprotection post-4VO. A single injection (i.p.) of e-*Baifuzi* (5 mg/kg) is administered at 1, 4, 8 or 24 h post-4VO. *$P < 0.05$, **$P < 0.01$. (**d**) Effects of e-*Baifuzi* on 4VO-impaired special memory. The Morris water maze test was carried out at 3–7 days post-4VO. Bar graphs represent the results of escape latency to reach the hidden platform on the fifth day of Morris water maze training. It must be noted that a single administration of e-*Baifuzi* within 1–8 h post-4VO decreases the ischemia-induced death of neuronal cells and prolongation of latency. so, stratum oriens; sp, stratum pyramidal; sr, stratum radiatum; BF, e-*Baifuzi*.

response to e-*Baifuzi* at a concentration of 0.45 μg/μl. At a voltage of $+100$ mV, 0.45 μg/μl e-*Baifuzi* increased the current two and threefold at an intracellular Ca^{2+} concentration of 0.1 and 1 μM, respectively (Figure 3b). At the single channel level, e-*Baifuzi* increased the single channel open probability (P_o) from $2.1 \pm 0.9\%$ of control ($n = 11$) to $18.4 \pm 6.3\%$ in the presence of 0.45 μg/μl e-*Baifuzi* ($n = 8$) without affecting its amplitude (Figure 3c and d). These results indicated that e-*Baifuzi* could activate the BK$_{Ca}$ channel and implied that the BK$_{Ca}$ channel is the molecular target of *Baifuzi*.

e-*Baifuzi* reduces presynaptic glutamate release. Excessive glutamate release and subsequent excitotoxicity are major pathological factors leading to neurodegeneration and death of ischemic neurons.[8,21] Previous studies have reported that BK$_{Ca}$ channels located at glutamatergic presynaptic terminals[12] regulate presynaptic Ca^{2+} entry,[10] which in turn regulates presynaptic glutamate release.[11] Therefore, we investigated the effect of e-*Baifuzi* on presynaptic glutamate release. First, the excitatory postsynaptic potential (EPSP) slopes in the presence of e-*Baifuzi* (0.45 μg/μl) were consistently less than those in its absence
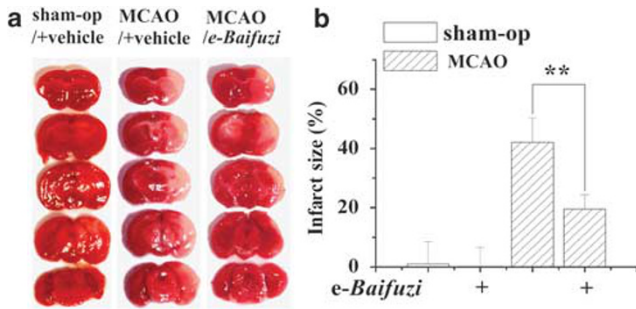
Figure 2 Effects of e-*Baifuzi* on brain infarct after MCAO. (**a**) Representative section of vehicle-treated sham, vehicle-treated MCAO and e-*Baifuzi* (5 mg/kg)-treated MCAO rats. (**b**) Bar graphs represent the mean infarct volume percentage in sham-op (open bars) and MCAO-mice (hatched bars) treated with vehicel or e-*Baifuzi* (5 mg/kg) at 1 h post-MCAO. **P < 0.01
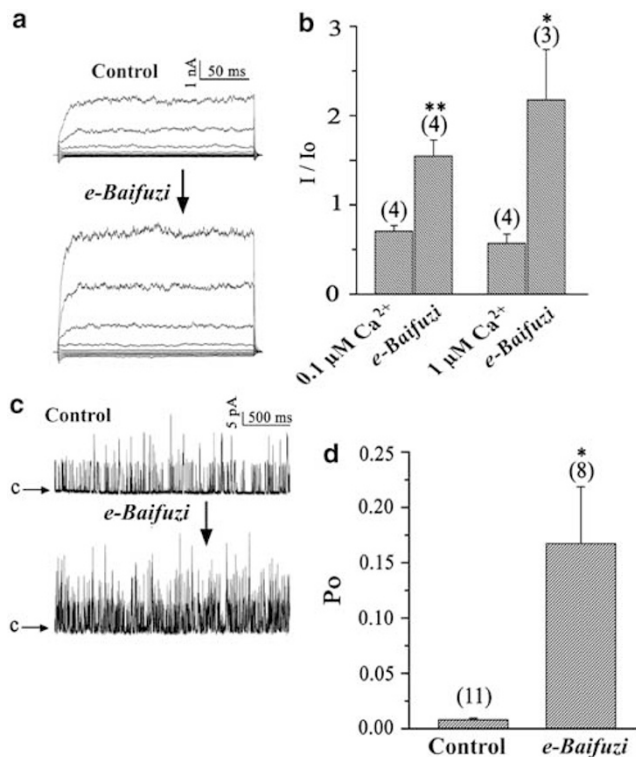

Figure 3 e-*Baifuzi* activates BK$_{Ca}$ channels. (**a**) Representative current trace of whole-cell BK$_{Ca}$ channel current before (control) and after 10 min of extracellular application of 0.45 μg/μl e-*Baifuzi*. The current was activated by rectangular voltage steps from -80 to $+100$ mV for 200 ms with 10 mV increment. (**b**) Statistics on ratio values (I/I_o) of the whole-cell BK$_{Ca}$ current before (I_o) and after (I) application of 0.45 μg/μl e-*Baifuzi* at $+100$ mV in the presence of 0.1 and 1 μM intracellular Ca^{2+}, respectively.(**c**) Representative single channel recordings of the BK$_{Ca}$ channels in excised inside-out configuration at $+50$ mV. Channel openings are shown as an upward deflection. Arrows indicate the level that all the channels are at their closed state. (**d**) Statistical data for open probability (P_o) of the single BK$_{Ca}$ channels in the presence of 0.45 μg/μl e-*Baifuzi*

(Figure 4a, P < 0.05; $n = 10$ slices per 5 rats). In addition, the reduction of the EPSP slope completely recovered within 10 min after e-*Baifuzi* washout ($n = 12$ slices per 6 rats; Figure 4b), indicating that e-*Baifuzi* reversibly suppresses synaptic transmission. Second, paired-pulse facilitation (PPF), a sensitive indicator of presynaptic glutamate

release, was measured by delivering two successive stimuli with 25–150 interpulse intervals (IPIs). Compared with that before the addition of e-*Baifuzi*, the paired-pulse ratio (PPR) at 50–75 IPI was markedly increased after addition of e-*Baifuzi* (0.45 μg/μl) (P < 0.05, $n = 15$ slices per 7 rats; Figure 4c). Furthermore, we measured the synaptically induced glial depolarization (SIGD), an indicator of the amount of presynaptic glutamate release,[22] in the presence of CNQX (10 μM)/AP5 (50 μM) (see the 'Materials and Methods' section). Consistent with the result of PPF, the area of SIGD was significantly reduced by perfusion of e-*Baifuzi* (0.45 μg/μl) (P < 0.05, $n = 12$ slices per 6 mice), and could be recovered after the washout of e-*Baifuzi* (Figure 4d). Thus, e-*Baifuzi* could reversibly reduce presynaptic glutamate release, which in turn might protect the brain from the excitotoxicity of excessive glutamate release.

Blockade of BK$_{Ca}$ channels abolishes the neuroprotection and glutamate release induced by e-Baifuzi. If the BK$_{Ca}$ channel is the molecular target, then its blocker should suppress the neuroprotective effect of e-*Baifuzi*. To determine whether the neuroprotection of e-*Baifuzi* is associated with BK$_{Ca}$ channel activation, charybdotoxin (CTX, 400 nM) was administered (i.v.c) for 30 min before the injection of e-*Baifuzi* (5 mg/kg) at 1 h post-4VO. Blockade of the BK$_{Ca}$ channel by CTX pretreatment significantly prevented the neuroprotection of e-*Baifuzi* (Figure 5a, P < 0.01, $n = 8$), whereas CTX alone had no significant effect on either 2VO or 4VO rats in comparison with the corresponding vehicle-treated control groups. As CTX is a specific blocker of the BK$_{Ca}$ channel, the result strongly suggests that the BK$_{Ca}$ channel is the molecular target of e-*Baifuzi* for its neuroprotective effect. Furthermore, we found that administration of 100 nM CTX could prevent e-*Baifuzi* from reducing the area of SIGD (P < 0.05, $n = 12$ slices per 6 rats; Figure 5b). These results strongly suggest that e-*Baifuzi* protects ischemia-induced brain injury by opening BK$_{Ca}$ channels to decrease presynaptic glutamate release, and gives further evidence that the BK$_{Ca}$ channel is the molecular target of *Baifuzi*.

Baifuzi-CB is the major active ingredient

The above studies indicated that e-*Baifuzi* had a significant neuroprotective effect on the ischemic brain through activation of BK$_{Ca}$ channels. This result prompted us to seek the active ingredient(s) of *Baifuzi*. We isolated *Baifuzi*-CB from e-*Baifuzi*,[23] which was verified by ^{1}H and ^{13}C NMR spectra (Supplementary Figures 1b and c). They appeared as one point on a normal silica gel thin layer chromatography (TLC) plate (Supplementary Figure 1a). Although phytochemical studies have shown that *Baifuzi* contains several other chemicals, including amino acids, fatty acids, choline, uracil, daucosterol and dipalmin,[23] we began our studies by focusing on *Baifuzi*-CB, because our previous study showed that sulfatide, a kind of cerebroside, can potently activate BK$_{Ca}$ channels.[24] In whole-cell patch-clamp recordings, *Baifuzi*-CB dose-dependently increased BK$_{Ca}$ channel currents. Intracellular application of 10 μM *Baifuzi*-CB increased the relative BK$_{Ca}$ channel current (I/I_o) from 0.80 ± 0.06 of control to
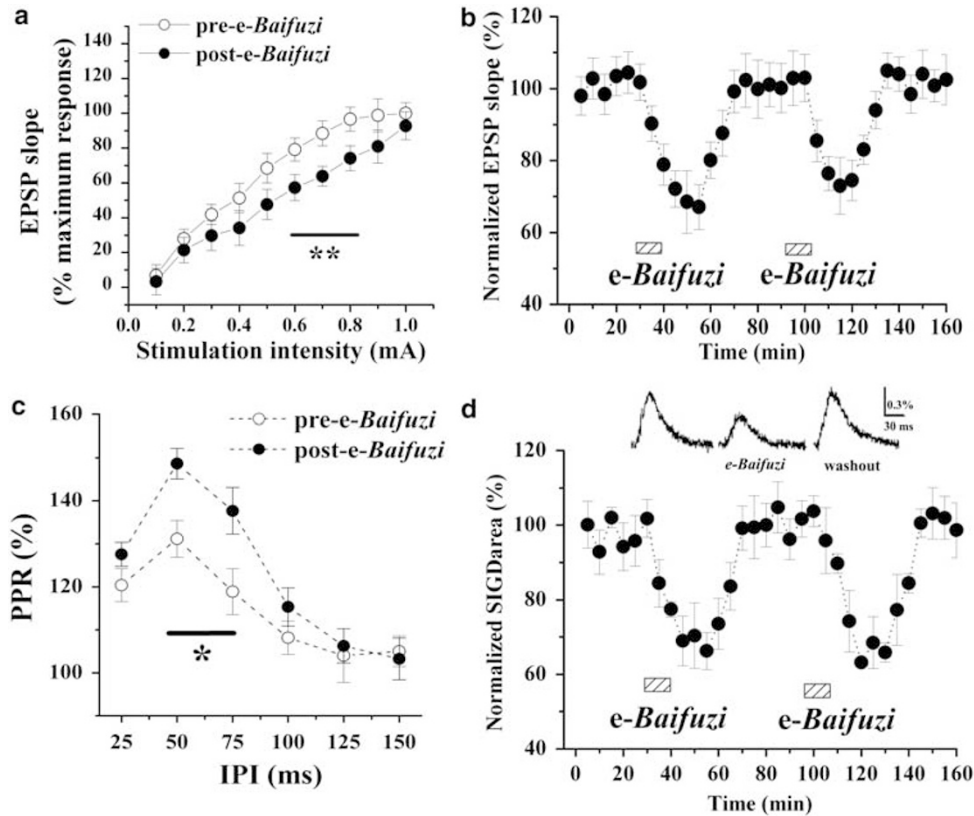
Figure 4 e-*Baifuzi* reduces presynaptic glutamate release. (**a**) Stimulus–response curve, in which the EPSP slope were evoked by stimulating the Schaffer collaterale-CA1 path with a current from 0.1 to 1.0 mA in the absence and presence of e-*Baifuzi* (0.45 μg/μl), was constructed by plotting the EPSP slope against stimulus intensity (0.1–1.0 mA). (**b**) Reversible inhibition of the EPSP slope by a transient application of e-*Baifuzi* (0.45 μg/μl). Horizontal hatched bar indicates the duration of e-*Baifuzi* application. (**c**) Effect of e-*Baifuzi* on presynaptic glutamate release. Paired-pulses facilitation (PPF) was evoked before and after the addition of e-*Baifuzi* (0.45 μg/μl). Paired-pulses ratios (PPR) are plotted against various IPIs. (**d**) Sample traces of SIGD were obtained just before, during and after the application of e-*Baifuzi* (0.45 μg/μl). Hippocampal slices were stained with RH155, a voltage-sensitive dye. SIGD: synaptically induced glial depolarization. Horizontal hatched bar indicates the duration of e-*Baifuzi* application. **$P < 0.01$
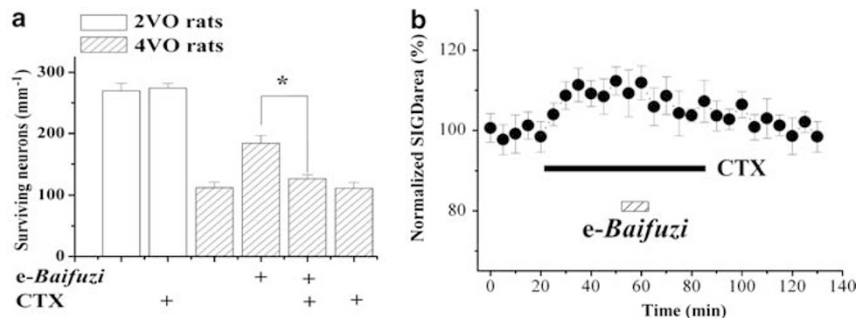

Figure 5 Effect of e-*Baifuzi* on neuroprotection and glutamate release in the presence of BK$_{Ca}$ channel blocker. (**a**) Role of CTX in e-*Baifuzi* neuroprotection. CTX at 400 nM was infused into the cereboventricle (i.c.v.) at 30 min before the injection of e-*Baifuzi* (5 mg/kg). Bar graphs represent the number of surviving pyramidal neurons of hippocampal CA1 in 2VO (open bars) and 4VO rats (hatched bars). **$P < 0.01$. (**b**) Effect of e-*Baifuzi* on the SIGD area in the presence of 100 nM CTX

3.32 ± 0.88 (Figure 6a). On the other hand, extracellular application of 10 μM *Baifuzi*-CB increased the relative BK$_{Ca}$ channel current from 0.70 ± 0.07 of control to 1.31 ± 0.15 (Figure 6b), suggesting that the action site of *Baifuzi*-CB is easier to access from the intracellular side. To examine whether the activation of BK$_{Ca}$ channels by *Baifuzi*-CB was due to some diffusible intracellular signal molecules, the effect of *Baifuzi*-CB on BK$_{Ca}$ channels was further examined at a single channel level in excised outside-out patches. As shown in Figure 6c, application of 10 μM *Baifuzi*-CB to the outside-out patch enhanced P_o from 3.1 ± 1.7% to 12.0 ± 3.2%. Activation of BK$_{Ca}$ channels by *Baifuzi*-CB in patches excised from the cell membrane strongly suggests that their actions are not mediated by diffusible intracellular signal molecules. On the basis of the amount of *Baifuzi*-CB purified from e-*Baifuzi*, it is calculated that e-*Baifuzi* contains 0.87% of *Baifuzi*-CB. Thus, 0.45 μg/μl of
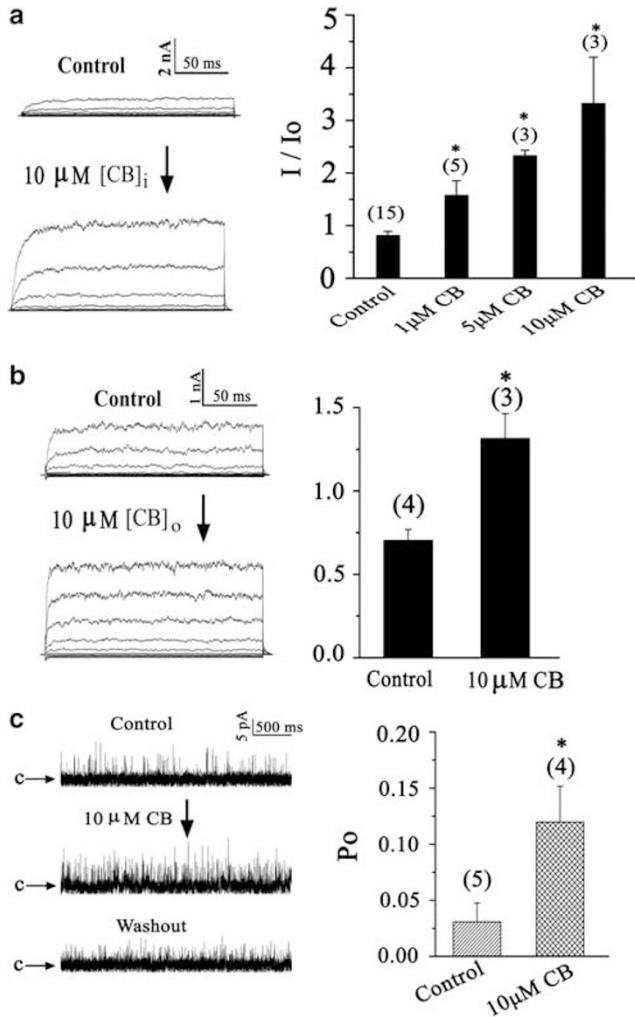
Figure 6 Effect of intracellularly and extracellularly applied *Baifuzi*-CB on BK_{Ca} channels. (**a**) Left: Representative current traces before (control) and after 10 min of intracellular application of 1 μM *Baifuzi*-CB ([CB]$_i$). Right: Statistics on I/I_o at different concentrations of *Baifuzi*-CB at $+100$ mV. (**b**) Representative current traces (left) and statistical summary on I/I_o (right) for whole-cell BK_{Ca} channel current before and after 10 min of extracellular application of 10 μM *Baifuzi*-CB ([CB]$_o$). (**c**) Representative single BK_{Ca} channel current traces at $+10$ mV (left) and statistical summary on its P_o (right) with and without *Baifuzi*-CB in the outside-out configuration. Asterisk indicates significant differences between control and *Baifuzi*-CB-treated BK_{Ca} current. *$P < 0.05$; CB, *Baifuzi*-CB

e-*Baifuzi* corresponds to 5.1 μM of *Baifuzi*-CB (MW $\sim$720 Dalton). Comparing Figure 3a and 6b, the channel activated by 10 μM *Baifuzi*-CB is at the same level as that of 0.45 μg/μl e-*Baifuzi*, indicating that *Baifuzi*-CB is the major active component responsible for the activation of the BK_{Ca} channel.

Molecular mechanism of BK_{Ca} channel activation by *Baifuzi*-CB

STREX is required for BK_{Ca} channel activation by Baifuzi-CB. It has been shown in our previous studies that STREX is important for activation of BK_{Ca} channels by lipid-soluble chemicals.[24,25] Besides, it has been demonstrated that inclusion of the STREX exon facilitates voltage- and Ca^{2+}-dependent activation of BK_{Ca} channels.[26] To test whether STREX contributes to the activation of the BK_{Ca} channel by *Baifuzi*-CB, we studied its effect on the STREX-deleted mutant of the channel. Both intracellularly (1 μM, Figure 7) and extracellularly (10 μM, Figure 6b) applied *Baifuzi*-CB significantly increased whole-cell current for wild-type BK_{Ca} channels. The relative whole-cell current was increased from 0.80 ± 0.06 of control to 1.57 ± 0.28 in the presence of 1 μM *Baifuzi*-CB applied intracellularly (Figure 7b), whereas extracellularly applied *Baifuzi*-CB (10 μM) increased the current from 0.70 ± 0.06 of control to 1.31 ± 0.15 (Figure 6b). In contrast, the STREX-deleted mutant BK_{Ca} channel could not be activated by *Baifuzi*-CB, whether it was applied intracellularly (Figure 7b) or extracellularly (Supplementary Figure 2), indicating that STREX is required for BK_{Ca} channel activation by *Baifuzi*-CB.

Interaction between Baifuzi-CB and STREX. We investigated the manner in which *Baifuzi*-CB is sensed by STREX, which is located at the cytoplasmic side, to activate the BK_{Ca} channel. A simple explanation may be that STREX interacts directly with *Baifuzi*-CB and consequently conveys the signal to gate the channel. To test whether the STREX domain is sufficient for *Baifuzi*-CB binding, we performed a protein lipid overlay (PLO) assay. No interaction was detected between STREX and POPE (1-Palmitoyl-2-Oleoyl-sn-Glycero-3-Phosphoethanolamine), 1-Palmitoyl-2-Oleoyl-sn-Glycero-3-[Phospho-rac-(1-glycerol (POPG) or DL-α-Phosphatidylcholine Dipalmitoyl (DPPC). In contrast, the STREX domain of the BK_{Ca} channel could interact with both
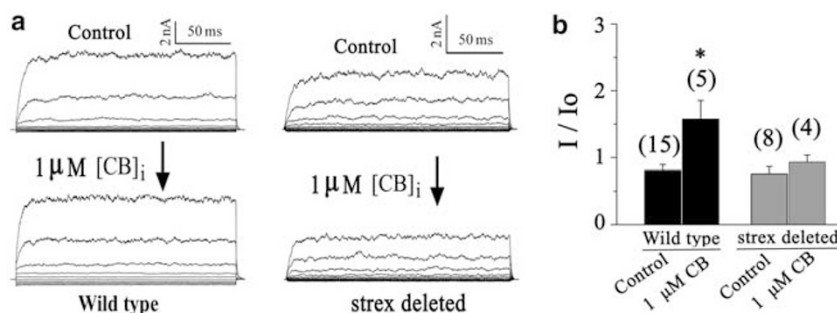

Figure 7 STREX is required for BK_{Ca} channel activation by *Baifuzi*-CB. (**a**) Representative current traces for wild-type (left) and STREX-deleted (right) BK_{Ca} channels in control and in the presence of 1 μM *Baifuzi*-CB applied intracellularly ([CB]$_i$). (**b**) Statistics on I/I_o for wild-type and STREX-deleted BK_{Ca} channels at $+100$ mV. CB, *Baifuzi*-CB
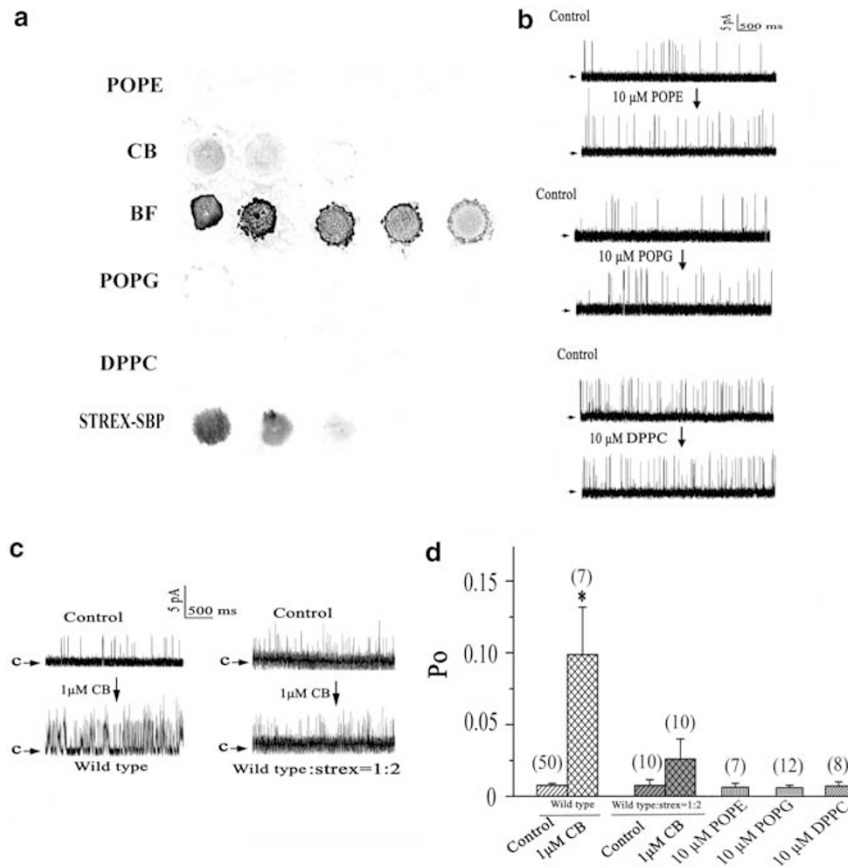
Figure 8 Direct interaction between *Baifuzi*-CB and the STREX domain of the BK$_{Ca}$ channel. (**a**) The ability of STREX to bind various lipid-soluble materials. The concentration of the lipid-soluble material was increased from right to left and spotted onto nitrocellulose membranes. (**b**) Effect of POPE, POPG and DPPC on BK$_{Ca}$ channels at $+20$ mV. (**c**) Intracellularly applied *Baifuzi*-CB (1 μM) on the single wild-type BK$_{Ca}$ channels (left) and on the channels with an overexpression of STREX fragments (BK$_{Ca}$/STREX $= 1:2$, q/q) (right) at $+10$ mV. (**d**) Statistical summary for the experiment in panels **b** and **c**

e-*Baifuzi* and *Baifuzi*-CB in a concentration-dependent manner (Figure 8a), indicating that *Baifuzi*-CB can bind to the BK$_{Ca}$ channel through its STREX domain. Interestingly, *Baifuzi*-CB and e-*Baifuzi*, which can bind to STREX, could activate the channel (Figure 3 and Figure 6), whereas POPE, POPG and DPPC, which cannot bind to STREX could not activate the channel (Figures 8b and d). These results strongly suggest that the interaction with the STREX domain is critical for BK$_{Ca}$ channel activation by *Baifuzi*-CB. If this is true, then the overexpression of STREX fragments will interrupt the interaction between *Baifuzi*-CB and the STREX domain of the channel, which might subsequently suppress the ability of *Baifuzi*-CB to activate the channel. To confirm this hypothesis, the STREX fragment was overexpressed with BK$_{Ca}$ channels (BK$_{Ca}$/STREX $= 1:2$, q/q). This overexpression significantly inhibited the ability of *Baifuzi*-CB to activate the channel (Figure 8c and d). This result further confirmed that the BK$_{Ca}$ channel activated by *Baifuzi*-CB is through its interaction with the STREX domain of the channel.

Discussion

In this study, we show that e-*Baifuzi* protects ischemia-induced brain injury through activation of BK$_{Ca}$ channels,

which in turn could reduce the excitotoxic glutamate release at the condition of brain ischemia. This conclusion is supported by the following four results: (1) e-*Baifuzi* protected ischemia-induced brain injury in a dose-dependent manner when administered after global or focal cerebral ischemia in rats and mice. (2) e-*Baifuzi* activated the BK$_{Ca}$ channel by increasing its P_o without affecting its amplitude. (3) The neuroprotective effect of e-*Baifuzi* could be suppressed by the specific blocker of BK$_{Ca}$ channels. (4) e-*Baifuzi* suppressed presynaptic glutamate release through the activation of BK$_{Ca}$ channels in hippocampal slices.

Furthermore, we propose that the BK$_{Ca}$ channel activated by *Baifuzi*-CB is through the interaction between *Baifuzi*-CB and the STREX domain of the BK$_{Ca}$ channel according to the following pieces of evidence: (1) PLO assay indicated the direct interaction between *Baifuzi*-CB and the STREX domain; (2) the STREX domain is required for activation of the BK$_{Ca}$ channel by *Baifuzi*-CB, indicating that the interaction between the STREX domain and *Baifuzi*-CB is critical for channel activation. This is in accordance with the result that intracellular application of *Baifuzi*-CB is more effective than that applied extracellularly. (3) Lipid molecules that did not interact with the STREX domain did not activate the BK$_{Ca}$ channel. (4) Overexpression of STREX fragments suppresses the ability of *Baifuzi*-CB to activate the BK$_{Ca}$ channel.

Many BK_{Ca} channel modulators have been reported to prevent neuronal degeneration after ischemia in preclinical studies. However, there is no approval of a single BK_{Ca} channel modulator for clinical use so far. For example, BMS-204352, one of the potent BK_{Ca} channel activators described to prevent neuronal degeneration after ischemia, has failed to improve the clinical outcome in patients affected by stroke.[27] In this study, we demonstrated that *Baifuzi* could effectively reduce brain damage in both focal and global ischemia. We are aware that there is no guarantee that *Baifuzi* will be effective in the clinical treatment of stroke. However, the following reasons may suggest its potential of being regarded as a therapeutic agent: (1) *Baifuzi* has been effectively used for the treatment of brain stroke for a long time in China[7]. (2) Cerebrosides are rich in the brain, implying that *Baifuzi*-CB may have a target-specific effect on neurons. (3) As an analog of membrane cerebroside, *Baifuzi*-CB may be transported by the endogenous transport machinery to target cells to fulfill its action.

Lipid–protein interactions are of fundamental importance, as they partly determine the structures of integral membrane proteins and their activities.[28] Lipid regulation of channels has been demonstrated for NMDA receptor channels,[29,30] TRAAK channels,[31] transient receptor potential channels,[32] inward rectifier K^+ channels,[33] Ca^{2+} channels,[34] voltage-dependent K^+ channels,[35] BK_{Ca} channels[36,37] and Twik-related K^+ channels.[38] The KcsA potassium channel only opens if the membrane contains a small amount of an anionic lipid, and lipid association has actually been resolved in the crystal structure of KcsA K^+ channels.[39] Therefore, it is not surprising that *Baifuzi*-CB can activate BK_{Ca} channels. In agreement with this result, we have previously found that sulfatide, a kind of cerebroside, can potently activate BK_{Ca} channels.[24] In this study, we found that the BK_{Ca} channel can be activated by *Baifuzi*-CB even at the excised membrane, implying that channel activation was not due to some intracellular diffusible signals. Besides, *Baifuzi*-CB could not increase at the intracellular Ca^{2+} level in hippocampal neurons (data not shown), suggesting that BK_{Ca} channel activation is not due to an increase in intracellular Ca^{2+} concentration. Then, how does *Baifuzi*-CB activate the channel? There are at least three possibilities, which are not exclusive with each other: (1) The STREX domain of the BK_{Ca} channel is attracted toward the plasma membrane because of the direct interaction with *Baifuzi*-CB, leading to a stabilization of the open state. This is similar to what has been proposed for the $PI(4,5)P_2$-dependent gating of inward-rectifier K^+ channels.[40] (2) *Baifuzi*-CB alters the thickness of the membrane, and thus makes the BK_{Ca} channel easy to open. This mechanism has been shown for BK_{Ca} channel activation by phosphatidylcholines (PCs) of varying chain lengths.[41] (3) *Baifuzi*-CB may create stress in the membrane because of its large head group, as we reported previously for the BK_{Ca} channel activated by membrane stress created with amphipaths.[25] Obviously, further studies are required to judge which one or their combinations is correct.

In both global and focal ischemia, restriction of blood flow to the brain limits the delivery of oxygen and glucose to neurons, causing energy depletion. This is followed by a cascade of neurochemical events, including disruption of ion homeostasis, excessive release of glutamate, overload of intracellular Ca^{2+}, release of free radicals, inflammatory changes and finally cell death of neurons, glia and endothelial cells.[9,42] These injury cascades are interconnected in complex ways, as one event in the cascade can cause or can be caused by multiple other events, and cells suffering from different severity levels of ischemia may undergo different chemical processes. On the other hand, AKT has been shown to have a prominent part in signaling networks that result in the modulation of cellular proliferation, apoptosis and survival. Recently, activation of AKT has been demonstrated to be important in neuronal survival after cerebral ischemia.[43–46] The neuroprotective effects of nerve growth factor, osteopontin, erythropoietin, as well as preconditioning and post-conditioning to reduce ischemia-induced brain injury, are mediated by AKT [47–50]. In our study, we demonstrated that *Baifuzi* could activate the BK_{Ca} channel and reduce the release of glutamate, which is responsible for the neuroprotective effect of *Baifuzi*. Therefore, it would be very interesting to study whether the AKT pathway is involved in the effect of *Baifuzi* on brain ischemia.

In summary, we demonstrated that e-*Baifuzi* provided significant neuroprotection, not only in protecting neurons against cell death but also in promoting behavioral recovery in the animal model of stroke. We identified the BK_{Ca} channel as a molecular target for the therapeutic action of *Baifuzi*, and that *Baifuzi*-CB is the active component that effectively activates BK_{Ca} channels. Through BK_{Ca} channel activation, e-*Baifuzi* limited glutamate release, which is involved in excitotoxicity during ischemic damage. These data provide evidence that e-*Baifuzi* attenuates ischemia-induced neuronal damage by enhancing the activation of BK_{Ca} channels. Its neuronal protective effect provides pharmacological rationale for the traditional use of *Baifuzi* in stroke. As the natural source of *Baifuzi*, the medicinal tubers of *T. giganteum* Engl., has a long history in Chinese traditional medicine, it is hoped that *Baifuzi*-CB will be a well tolerated, promising therapeutic candidate for the treatment of ischemic brain stroke and other forms of neuronal damage.

Materials and Methods

Extraction and isolation. Tubers of *Baifuzi* were collected from Yuxian County (Henan Province, China). The dried material was powdered and then extracted with 95% ethanol for several days with shaking. The extract was concentrated to yield e-*Baifuzi*. e-*Baifuzi* was chromatographed on silica gel, then on ODS to afford *Baifuzi*-CB, which was confirmed to be a mixture of cerebrosides on the basis of ^1H and ^{13}C NMR spectra (Supplementary Figure 1b and c). TLC experiment indicated that *Baifuzi*-CB is one of the major spots of e-*Baifuzi* (Supplementary Figure 1a). The detailed process was described in our previous paper.[23]

Experimental animals. Male Sprague–Dawley rats (weighing 200–250 g, Oriental Bio Service Inc., Nanjing, China) and male mice (weighing 25–30 g, Oriental Bio Service Inc.) were used throughout the study. Animals were housed in a light-controlled room under a 12 h light–dark cycle starting at 0700 hours and kept at 25°C. They were given unrestricted access to food and water. All procedures were in accordance with the guidelines of the Institute for Laboratory Animal Research of the Nanjing Medical University.

Preparation of the global brain ischemia model. We used a 4VO method for transient global ischemia as described elsewhere.[51] Briefly, using sodium pentobarbital anesthesia (60 mg/kg, intraperitoneally (i.p.)), both common

carotid arteries (CCAs) of rats were dissected free, and the incision was closed. Immediately after this procedure, both vertebral arteries (VAs) between the first and second cervical vertebrae were exposed and electro-cauterized completely using a bipolar cauterizer under an operating microscope (SZH-ILLB, Olympus, Tokyo, Japan). After 24 h, the common arteries were occluded with aneurysm clips for 20 min. The clips were then removed, and blood flow though the arteries was confirmed before the wound was sutured. Rectal temperature was continually monitored and maintained at $\sim 37^{\circ}$C using a heating pad until the animal had fully recovered from anesthesia. Sham operation (control) groups had a permanent bilateral occlusion of VAs (2VO rats) that was carried out in the same manner as in 4VO rats, except that the CCAs were not occluded. All rats were allowed to survive for 8 days after the onset of cerebral ischemia.

Preparation of the focal cerebral ischemia model. Focal cerebral ischemia was induced by MCAO for 60 min using the intraluminal filament insertion technique, as described previously.[52] All mice were anesthetized with 2% halothane, and were then maintained with 1% halothane in 70% N_2O and 30% O_2 using a vaporizer. Briefly, a poly-L-lysine (0.1% per weight volume)-coated nylon monofilament thread (3/0 G with the tip heat blunted to a diameter of 0.104 mm) was inserted through the external carotid artery and advanced into the internal carotid artery to occlude the origin of the MCA (~ 12 mm). Adequacy of vascular occlusion and reperfusion was monitored in the front parietal cortex of the occluded side by Perimed PF5050 (Perimed, Jarfalla, Sweden) multichannel laser Doppler flowmetry. Body and head temperatures were monitored and controlled at $37 \pm 0.5^{\circ}$C using a homeothermic blanket and water pads. Arterial blood pressure and gases were monitored through a femoral catheter. After 60 min of occlusion, the filament was withdrawn to allow for reperfusion, and animals recovered from anesthesia and survived for 24 h. Sham-operated animals were treated identically, except that MCAs were not occluded after the neck incision.

Drug administration. Animals subjected to cerebral ischemia treatment or sham operation were blinded to drug-treated groups. e-*Baifuzi* was diluted in 0.9% saline to a final concentration of 1.0% ethanol that was used as vehicle control. CTX, a BK_{Ca} channel blocker, was dissolved in 0.9% saline and injected into the cerebroventricle (i.c.v.). e-*Baifuzi* was administered i.p. For i.c.v. implantation, mice were anesthetized with ketamine (80 mg/kg i.p.). A guide cannula (4 mm length, 23 G) aimed above the left lateral ventricle was implanted and anchored to the skull with four stainless steel screws and dental cement. The guide cannula was implanted above the rostral ventricle so as to not lesion the hippocampus. The drug was prepared freshly on the day of the experiment and injected using a stepper-motorized microsyringe (Stoelting, Wood Dale, IL, USA) at a rate of 0.5 μl/min (final volume $= 3\ \mu$l per mouse). Control mice were given an equal volume of vehicle.

Histological examination after 4VO. On the eighth day post-4VO, all animals were deeply anesthetized with pentobarbital (50 mg/kg) and were perfused transcardially with ice-cold phosphate-buffered saline (PBS), followed by 4% ice-cold phosphate-buffered paraformaldehyde. The brains were removed, postfixed in the same fixative for 24 h and then processed for paraffin embedding. Coronal sections (4 μm in thickness) including the dorsal hippocampus were cut and stained with toluidine blue. All slide preparations were randomly coded so that observers were blind to the experimental condition. Healthy CA1 pyramidal neurons, showing a round cell body with a plainly stained nucleus, were counted by eye using a conventional light microscope (PD70) with a 100 × objective. The number of surviving pyramidal neurons per 1 mm length along the extent of the CA1 pyramidal layer was counted as neuronal density (cells per mm) as described elsewhere.[53] We also conducted supplemental examinations on several slices stained with trypan blue that stains dead cells, and obtained results that were essentially the same as that determined by eye with the toluidine blue-stained slices. To exclude the possibility that alterations in cell density value might be a consequence of changes in the volume of reference, we used a subset of the sections for volume estimation of CA1 stratum radiatum using Scion Image software (Scion Corp., Frederick, MD, USA). Six slices were prepared from the right and left dorsal hippocampus each to count the number of surviving pyramidal neurons.

Lesion size measurement after MCAO. Using 2,3,5-triphenyltetrazolium chloride (TTC) staining, we measured the infarct size at 24 h of reperfusion, as described previously.[20] All animals were killed under deep halothane anesthesia, and the brains were quickly frozen and sliced into thick sections for infarct measurement. Tissue infarction was identified by TTC staining in thick (2 mm)

coronal sections. Slices were photographed, and images were studied using image-analysis software (MCID; Imaging Research, St. Catharines, ON, Canada). The infarct volume in all slices was expressed as a percentage of the contralateral hemisphere after correcting for edema.

Behavioral analysis. Animals were trained in the standard Morris water maze task[54]. For the Morris water maze task, a pool (90 cm diameter, 45 cm height) made of black-colored plastic was prepared, and was filled with water ($20 \pm 1^{\circ}$C) that was rendered opaque with nontoxic blue paint. Swimming paths were analyzed by a computer system using a video camera (AXIS-90 Target/2; Neuroscience, Inc., Tokyo, Japan). In the hidden-platform test, the platform (7 cm in diameter) was submerged 1 cm below the water surface. The first day of training began with a free swim, in which the swim speed was assessed in the absence of the platform, and then immediately after the free swim, mice were given four training trials per day over 5 days, in which they were given 90 s to swim to the hidden platform. If a mouse did not find the platform in the allotted time, it was placed on the platform for 15 s. The average swim speed (cm/s) and latency (s) to reach the platform were scored on all trials and analyzed. Data were obtained from groups of 10 mice throughout the experiments.

Electrophysiological analysis

Slice preparation. Rats were decapitated under deep anesthesia with ethyl ether. Brains were rapidly removed and coronal brain slices (400 μm) were cut using a vibrating microtome (Microslicer DTK 1500, Dousaka EM Co., Kyoto, Japan) in ice-cold cutting solution composed of (in mM) 94 sucrose, 30 NaCl, 4.5 KCl, 1.0 $MgCl_2$, 26 $NaHCO_3$, 1.2 NaH_2PO_4, and 10 D-glucose with pH 7.4. The hippocampal slices were continuously incubated in artificial cerebrospinal fluid (ACSF) at $36 \pm 1^{\circ}$C for more than 60 min to allow the slices to recover. ACSF was composed of (in mM) 124 NaCl, 2 $CaCl_2$, 4.5 KCl, 1 $MgCl_2$, 26 $NaHCO_3$, 1.2 NaH_2PO_4, and 10 D-glucose. pH was adjusted to 7.4. Both the cutting solution and ACSF were oxygenated with a gas mixture of 95% O_2 and 5% CO_2.

Field potential recording. For recordings, the slice was transferred into a recording chamber and perfused continuously with oxygenated ACSF at a flow rate of ~ 1 ml/min. The experiments were conducted at $30 \pm 1^{\circ}$C. Briefly, orthodromic stimuli were delivered using an electrically polished bipolar tungsten electrode that was placed in the outer third of the molecular stratum of hippocampal dentate gyrus to stimulate the lateral perforant path. Constant current pulses (0.05 Hz) were supplied by a stimulator (SEN-3301, Nihon Kohden, Tokyo, Japan). EPSP was recorded from the middle third of the molecular stratum with a 4–5 MΩ resistance glass microelectrode that was filled with 0.9% NaCl and connected to a neutralized, high input-impedance preamplifier with a high-pass filter at 5 kHz. The level of test stimulus was set to evoke ~ 50% of a minimum stimulus intensity that evoked a saturated EPSP in each slice to avoid possible deprivation of readily releasable transmitters. Signals were amplified using a differential AC amplifier (A-M Systems, model 1700, Seattle, WA, USA). EPSPs were digitized and saved using the pCLAMP system (Axon Instrument Inc., Foster City, CA, USA). Stability of baseline recordings was established by delivering single pulses (1 per min, 0.1 ms pulse width at an intensity yielding half-maximal EPSP amplitude for a given slice) for 15–30 min before collection of input/output functions. Baseline synaptic transmission was assessed by averaging the response to five pulses (from 0.1 to 1.0 mA) delivered at a rate of 0.05 Hz. PPF was examined at a range of IPIs (25–150 ms) and a stimulus intensity of half-maximal for elicitation of EPSP.

Optical recordings. All slices used in optical recording were stained with a voltage-sensitive dye (RH155 0.2 mg/ml, Nippon Kanko Shikiso Kenkyujo, Okayama, Japan) for 15 min, and were kept in a chamber containing oxygenated ACSF for at least 30 min for recovery as described previously.[55] The slices were then transferred into a recording chamber and placed on the stage of an Olympus inverted microscope (IMT-2, Nikon, Tokyo, Japan). Test stimulus was set at 50% maximal stimulus intensity to evoke optical EPSP (op-EPSP) in each slice. Light from a tungsten-halogen lamp (type JC-24v/200 W, Kondo Philips, Tokyo, Japan) was collimated, and an interference filter with a transmission maximum at 700 ± 10 nm (Olympus Optical, Tokyo, Japan) was used. Changes in light absorption associated with membrane potential changes were measured using an optical recording system equipped with a high-speed diode camera with metal oxide (MOS) image sensors (128 × 128 pixels, each of which receives light from a 25 μm × 25 μm sample area) and a data processing unit (HR Deltaron-1700 Fujix; Fuji Photo Film, Tokyo, Japan). To minimize bleaching of the dye molecules and

photodynamic damages to slices, a heat-absorption filter was placed on the slices, and the light path to the slice was opened only long enough to measure optical responses (1–2 s). In each trial, a background image recorded for 16 ms before electrical stimulation was stored as a reference image. To analyze the change in neuronal activities over time at a given region, data from each pixel were stored and retrieved, and the amplitude of the optical signal (indicated as a percentage change in optical absorbency) was plotted as a function of time. Signals obtained from six pixels in str. molecular were averaged. RH155 dye is reported to stain glial cells preferentially over neuronal cells.[56] Optical recording with the RH155 dye is used to monitor glial depolarization by the electrogenic glutamate transporter (GLT-1) uptaking the released glutamate.[57] When AMPA and NMDA receptors are blocked, a delayed depolarizing response can be recorded, which is completely abolished by the specific GLT-1 blocker. Thus, the size of SIGD is considered to reflect the probability of presynaptic glutamate release.

Patch-clamp recordings. Whole-cell currents were measured using a conventional tight seal whole-cell recording technique at room temperature. Pipettes had resistances at a range of 2–5 MΩ in recording solution containing (in mM) 145 KCl, 10 EGTA, 10 HEPES (pH 7.3 with KOH) and CaCl$_2$ adjusted to the desired [Ca^{2+}]$_{free}$. The intracellular free Ca^{2+} concentration ([Ca^{2+}]i) was 0.1 μM, if not stated otherwise. The extracellular solution was Hanks' balanced salt solution (Sigma, St Louis, MO, USA): 1.3 CaCl$_2$, 0.8 MgSO$_4$, 5.4 KCl, 0.4 KH$_2$PO$_4$, 136.9 NaCl, 0.3 Na$_2$PO$_4$, 10 D-glucose and 4.2 NaHCO$_3$. Compensation for cell capacitance and series resistance was made automatically using EPC-9 or EPC-10 amplifiers; only recordings with stable series resistances $\leqslant$ 25 MΩ were included in the study. Currents were amplified using EPC-9 or EPC-10 patch-clamp amplifiers (HEKA, Lambrecht, Germany), sampled at 2–5 kHz and filtered at 1.5–2.9 kHz using a 4-pole low-pass Bessel filter. Program packages Patchmaster 2.1 and TAC 4.1 (HEKA) were used for data acquisition and analysis. The probability of a single channel being open (P_o) was simply calculated from the total time spent in the open state divided by the total time of recording for the patches containing a single channel. Continuous recordings of 2000–4000 ms were used to estimate P_o. When multiple channels were present in a patch, P_o was calculated from the amplitude histogram as $P_o = (1 - P_C^{1/N})$, where P_C is the fraction of area under the closed state, and N is the number of channels. To avoid underestimating the number of channels in a patch as less as possible, the number of active single channels in the patch was counted at the condition in which the maximum number of channels was observed. Both e-*Baifuzi* and *Baifuzi*-CB activate chick BK$_{Ca}$ expressed in CHO cells, as well as rat BK$_{Ca}$ expressed in HEK293 cells, at both the single channel and whole-cell level. However, for convenience, we present data at the single channel level for chick BK$_{Ca}$ and at the whole-cell level for rat BK$_{Ca}$ channels. Both e-*Baifuzi* and *Baifuzi*-CB have low aqueous solubility and were dissolved in 100% dimethyl sulfoxide (DMSO) as a stock solution and diluted with intracellular or extracellular solutions to obtain different concentrations as required. The final concentration of DMSO was < 0.5% per assay, which had no effect on the BK$_{Ca}$ channel.

Plasmid construction and STREX–SBP fusion protein purification. The *STREX* gene of the chick BK$_{Ca}$ channel was amplified from pcDNA 3.1-*BK$_{Ca}$* and subcloned into PQE30(+) (Novagen, Madison, WI, USA). The STREX–SBP (streptavidin-binding peptide) fusion protein was constructed by insertion of a peptide linker (Ser-Gly)$_5$ between the STREX and SBP tag. After culturing in LB medium containing 0.1 mg/ml ampicillin at 37°C and 200 r.p.m./min, cells were induced to express the STREX–SBP fusion protein by adding 0.25 mM IPTG until OD$_{600}$ = 0.4. Cells were harvested by centrifugation at 8000 r.p.m./min for 10 min, ultrasonicated and centrifuged at 12 000 r.p.m./min for 30 min at 4°C to remove the cell fragments. The supernatant was applied to a Ni2+-NTA column (Amersham Life Sciences, Buckinghamshire, UK). Gradient imidazole was then used to elute the protein. Its purity was analyzed by SDS-PAGE. The purified protein was stored in buffer containing 50 mM Tris-HCl, pH 8.2, 200 mM NaCl, 1 mM 1,4-dithiothreitol (DTT) and 20% glycerol at −70°C until use.

PLO assay. Five types of lipid-soluble chemicals, including e-*Baifuzi*, *Baifuzi*-CB, POPE (Avanti, Alabama, USA), POPG (sodium salt, Avanti) and DPPC (Sigma) were diluted and spotted on a Hybond-C extra nitrocellulose membrane (Amersham Life Sciences) with concentration increased from right to left and air-dried for 1 h at room temperature as described previously.[58] Lipid spots were blocked in buffer 1 (fatty acid-free BSA buffer 40 mg/ml, 25 mM Tris-HCl pH 7.9, 150 mM NaCl) with gentle shaking for 2 h at room temperature and incubated overnight with 9.2 μM STREX–SBP fusion protein in buffer 2 (fatty acid-free BSA 10 mg/ml, 25 mM Tris-

HCl pH 7.9, 150 mM NaCl) at 4°C. Taken as a positive control, the purified STREX–SBP fusion protein was also incubated with lipid spots. The membrane was washed in PBS buffer (137 mM NaCl, 2.7 mM KCl, 10 mM Na$_2$HPO$_4$, 2 mM NaH$_2$PO$_4$, PH7.4) with 0.05% Tween20, and then incubated in a 1 : 3000 dilution of streptavidin-alkaline phosphatase (Promega, Co., Madison, WI, USA) for 1 h. The enzyme was bound to the membrane through interaction between streptavidin and the SBP tag. The protein bound to the lipids was detected with a mixture of 5-bromo-4-chloro-3-indolyl phosphate and nitroblue tetrazolium (BCIP/NBT, Ameresco, Solon, OH, USA) as a color development substrate.

Data analysis/statistics. Data were retrieved and processed with the software Micro cal Origin 6.1 (OriginLab, Northhampton, MA, USA). Group data were expressed as means ± S.E. Experimental results were compared among groups by ANOVA, followed by Bonferroni's *post hoc* test. Statistical analysis was performed using State7 software (STATA Corporation, TX, USA). Differences at $P < 0.05$ were considered to be statistically significant.

Conflict of interest

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