

Sigma 1 receptor activation regulates brain-derived neurotrophic factor through NR2A-CaMKIV-TORC1 pathway to rescue the impairment of learning and memory induced by brain ischaemia/reperfusion

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Abstract

Rationale Sigma-1 receptor (Sig-1R) agonists showed anti-amnesic properties in Alzheimer's disease models and anti-inflammatory properties in cerebrum ischaemia models. The agonist of Sig-1R was reported to up-regulate brain-derived neurotrophic factor (BDNF) levels in the hippocampus of mice. Here, we investigate whether the activation of Sig-1R attenuates the learning and memory impairment induced by ischaemia/reperfusion and how it affects the expression of BDNF.

Objectives Bilateral common carotid artery occlusion (BCCAO) was induced for 20 min in C57BL/6 mice.

Materials and methods Sig-1R agonist, PRE084, sigma 1/2 non-selective agonist, DTG, Sig-1R antagonist and BD1047 were injected once daily throughout the experiment. Behavioural tests were performed from day 8. On day 22 after BCCAO, mice were sacrificed for biochemical analysis.

Results PRE084 and DTG ameliorated learning and memory impairments in the Y maze, novel object recognition, and water maze tasks and prevented the decline of synaptic proteins and BDNF expression in the hippocampus of BCCAO mice. Furthermore, PRE084 and DTG up-regulated the level of NMDA receptor 2A (NR2A), calcium/calmodulin-dependent protein kinase type IV (CaMKIV) and CREB-specific co-activator transducer of regulated CREB activity 1 (TORC1). Additionally, the effects

of PRE084 and DTG were antagonised by the co-administration of BD1047.

Conclusions Sig-1R activation showed an attenuation in the ischaemia/reperfusion model and the activation of Sig-1R increased the expression of BDNF, possibly through the NR2A-CaMKIV-TORC1 pathway, and Sig-1R agonists might function as neuroprotectant agents in vascular dementia.

Keywords Sigma-1 receptor · PRE084 · DTG · Brain ischaemia/reperfusion model · Learning and memory · Brain-derived neurotrophic factor · NR2A-CaMKIV-TORC1

Introduction

Sigma-1 receptor (Sig-1R) is recognised as a chaperone protein of the endoplasmic reticulum (ER) and is putatively located on membranes forming focal contacts between the ER and mitochondria (Hayashi and Su 2007). Sig-1R is highly expressed in the brain (Alonso et al. 2000; Langa et al. 2003). In the nervous system, Sig-1R regulates neurogenesis; K⁺ channels; inositol 1,4,5-triphosphate receptor (IP3R)-mediated Ca²⁺ signalling; and drug addiction (Maurice and Lockhart 1997; Hayashi and Su 2001, 2004; Aydar et al. 2002; Matsumoto et al. 2003). Sig-1Rs are well known to exert anti-depressant-like effects (Yagasaki et al. 2006; Takebayashi et al. 2004). Agonists of Sig-1R have been shown to have anti-amnesic properties in amnesia models, including amyloid-induced AD models (Villard et al. 2009), cholinergic deficit models (Zou et al. 2000) and aging-related memory loss models (Maurice 2001; Phan et al. 2003). Recently, the effects of Sig-1R on brain ischaemia have gained attention from researchers. The endogenous ligand of Sig-1R,

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DHEA, exerted neuroprotective effects by reducing the ischaemia-induced neuronal death and improving spatial learning deficits 3 to 48 h after ischaemia (Li et al. 2009). However, the detailed role of Sig-1R in ischaemia-induced learning and memory impairments is still unclear.

Brain-derived neurotrophic factor (BDNF) plays key roles in the maintenance and repair of the nervous system (Lewin and Barde 1996) and has emerged as an important regulator of synaptic plasticity and learning and memory (Cunha et al. 2010; Korte et al. 1995). A recent report suggested that chronic anti-depressant treatment modulated the BDNF signalling pathway through the Sig-1R chaperone (Yagasaki et al. 2006). Kikuchi-Utsumi and Nakaki (2008) found that chronic administration of Sig-1R agonist SA4503 regulated the region-specific BDNF functions in the rat brains. Recently, Fujimoto et al. (2012) reported that SA4503 increased Sig-1R protein and concomitantly increased the expression of endogenous BDNF. Overexpression of Sig-1R potentiated the conversion of the precursor pro-BDNF to mature BDNF and enhanced the secretion of mature BDNF into extracellular spaces (Fujimoto et al. 2012). These studies suggest that Sig-1R is a potential modulator for the BDNF signal pathway. Therefore, in this study, we hypothesise that Sig-1R stimulation will modulate BDNF in the hippocampus, thereby enhancing synaptic plasticity and improving deficits of learning and memory in brain ischaemia/reperfusion.

Methods

Animals

Equal numbers of male and female C57BL/6 mice (11 weeks old, $n=96$ in total) were used in the experiments. C57BL/6 mice were chosen because this strain is one of the most susceptible mouse strains to neuronal damage after cerebral ischaemia due to their poorly developed anastomosis between the carotid artery (posterior communicating artery) (Fujii et al. 1997; Yang et al. 1997). The animals were purchased from Beijing HFK Bioscience Co., Ltd. The animals were kept in a polycarbonate cage (15.0 cm×30.0 cm×10.0 cm) with six mice per cage and maintained under standard housing conditions (room temperature 25 ± 2 °C and humidity 50 ± 5 %) with a 12-h light and 12-h dark cycle. Food and water were available ad libitum. An acclimation period of at least 1 week was provided before initiating the experiment. All animal studies were performed in strict accordance with the P.R. China legislation on the use and the care of laboratory animals and with the guidelines established by the Institute for Experimental Animals at Shenyang Pharmaceutical University.

Surgical procedure

Bilateral common carotid artery occlusion (BCCAO) animal models were widely used to study the effects of brain ischaemia. BCCAO results in a systemic decrease of blood flow in the whole brain, which clinically resembles transient global cerebral ischaemia followed by a number of pathological changes, such as excitotoxicity, loss of ATP in the neurons and neurological deficits (Huang and McNamara 2004; Kim et al. 2007). In this study, we used the BCCAO model to investigate the effects of Sig-1R on extensive brain injury by ischaemia/reperfusion. The mice were anaesthetised with chloral hydrate (300 mg/kg i.p.). After a lateral neck incision, brain ischaemia was induced by BCCAO for 20 min as described (Wu et al. 2001; Zhao et al. 2005; Yamamoto et al. 2009). Body temperature was kept at 37 ± 0.5 °C using a heating pad during and for 1 h after the surgery. Sham-operated animals underwent all of the procedures, except that their arteries were not occluded. After surgery, the mice were housed individually and were allowed to recover for 7 days before the start of the behavioural assays.

Y maze task

The spontaneous alteration behaviour in a Y maze is assessed as a spatial working memory task (Yamamoto et al. 2009). The apparatus consists of three identical arms made of wood: Each arm was 40 cm long, 12 cm high, 5 cm wide at the bottom and 10 cm wide at the top. This behavioural test was performed 8 days after the BCCAO, according to a previous study (Lu et al. 2009), with minor modifications. Each mouse was placed at the end of one fixed arm and was allowed to move freely through the maze during a 5-min session. The total number of arm entries (N) and the sequence of entries were recorded. A successful alternation was defined as entries into all three arms on consecutive choices. The floor was cleaned with ethanol (10 %) after each individual trial to eliminate olfactory cues. The number of successive alternations was recorded. Alternation behaviour was calculated as number of alternations/($N-2$)×100 %.

Novel object recognition test

The novel object recognition test was performed on the 10th day after the BCCAO, as a non-aversive learning paradigm that relies on the spontaneous exploratory behaviour of animals. The experimental arena consisted of a square box (L 45 × W 45 × H 15 cm) with wood walls. Two days prior to the experiments, the mice received a habituation of 5 min in the empty open-field arena, twice per day (Damgaard et al. 2010; McClean et al. 2011). On the day of the experiment, each mouse received a 3-min habituation. Two identical objects, A1 and A2, were placed at the centre of the box in a

symmetrical position approximately 15 cm away from the black wall. The animals were singly placed in the box and allowed to explore for 5 min (training session), and the total time spent exploring each of the two objects (when the animal's snout was directly pointed toward the object at a distance ≤ 2 cm) was recorded. One hour after the training session, the mice were placed back into the arena and exposed to the familiar object A1 and a novel object B for an additional 5 min. After a 24-h retention interval, the mice were placed again in the same box with the novel object B replaced with another novel object C and were allowed to explore freely for 5 min. The placement of the novel and familiar objects alternated between the left and right to reduce potential bias for a particular location. Throughout the experiments, the objects were used in a counterbalanced manner in terms of their physical complexity and emotional neutrality. Any faeces and urine were removed with paper towels, and the floor was cleaned with 10 % ethanol after each individual trial to eliminate olfactory cues. The exploration time(s) for each object in the training session and the retention sessions were recorded. The preferential index (PI) was calculated as time spent exploring novel object/total exploration time. The discrimination index (DI) was calculated as [(time spent exploring novel object – time spent exploring familiar object)/total exploration time].

Morris water maze task

The maze was a circular pool (100 cm in diameter and 40 in height) filled with water at 25 ± 1 °C to avoid hypothermia. A small escape platform (10 cm in diameter) was placed at a fixed position in the centre of one quadrant and hidden 1 cm beneath the water surface. The room contained a number of fixed visual cues on the walls.

Acquisition phase

In the acquisition trial phase, mice were trained twice a day for five consecutive days (days 1–5) with an intertrial interval of 3 h. Four points equally spaced along the circumference of the pool (north, south, east and west) served as the starting position, which was randomised during the two trials each day. If an animal did not reach the platform within 60 s, it was guided to the platform where it had to remain for 10 s before being returned to its home cage. The mice were kept dry between trials in a plastic holding cage filled with paper towels. Escape latency (time to find the hidden platform), distance swum and swimming speed were recorded ($n=10$ –12 per group). All of the trials were completed between 09:00 and 18:00 (McClellan et al. 2011).

Probe trial

One day after finishing the acquisition task (day 6), a probe trial was performed to assess the spatial memory (after a 24-h delay). The platform was removed from the maze, and each mouse was allowed to explore freely for 60 s. Spatial acuity was expressed as the amount of time spent in the exact area where the escape platform was located (McClellan et al. 2011).

Immunohistochemical staining

Immunohistochemical staining was performed 22 days after the BCCAO as described previously (Andersberg et al. 2002). Briefly, sections were incubated with rabbit anti-BDNF antibody (1:100, Santa Cruz) and mouse anti-NeuN antibody (1:100, Millipore) at 4 °C overnight. After washings (three times) with PBS, the sections were incubated with biotin-labelled goat anti-rabbit/mouse antibody (1:200, Santa Cruz) at 37 °C for 30 min. The sections were treated with avidin-biotin enzyme reagent (Santa Cruz), and DAB was used to visualise the positive signal. The intensity of each section was quantified using Image Pro-Plus 6.0 software by Olympus IX 71.

Western blotting

The mice were sacrificed 22 days after the BCCAO, and the hippocampi were dissected for Western blotting analysis. The protein (40 μ g) was run on a 12 % gradient SDS-PAGE gel and electrophoretically transferred to polyvinylidene difluoride (PVDF) membranes (Millipore, Billerica, MA, USA). After blocking with 5 % skim milk for 2 h at room temperature, the membranes were incubated with primary antibodies at 4 °C overnight. The membranes were incubated with horseradish peroxidase-labelled anti-mouse IgG or anti-rabbit IgG antibodies (Santa Cruz) for 2 h at room temperature. Protein bands were visualised with an ECL Western blotting kit (Kangwei Biotechnology, China). The intensity was quantified by densitometry using Quantity One 4.6.2 software (Bio-Rad, Hercules, CA, USA) and corrected with the corresponding β -actin level. The results were expressed as a percentage of the control. The following antibodies were used: anti-synaptophysin (SYP, 1:1000; sc-17750, Santa Cruz), anti-GAP-43 (1:400; sc-17790, Santa Cruz), anti-PSD95 (1:1000; ab18258, Abcam), anti-Nogo-A (1:400; sc-25660, Santa Cruz), anti-CaMKIV (1:400; sc-55501, Santa Cruz), anti-TORC1 (1:300; sc-67146, Santa Cruz), anti-NeuN (1:300; MAB377, Millipore), anti-TrkB (1:800; BS6683, Bioworld Technology), anti-TrkB (phospho-Y706, 1:200; BS4201, Bioworld Technology), anti-ERK1/2 (1:500; sc-292838, Santa Cruz), anti-p-ERK1/2 (Thr 202, 1:400; sc-101760, Santa Cruz), anti-CREB-1 (1:400; sc-25785, Santa Cruz), anti-p-CREB-1 (Ser 133, 1:400; sc-101663, Santa Cruz) and β -actin (1:400; sc-47778, Santa Cruz), anti-

BDNF (1:500; sc-546, Santa Cruz), anti-NR2A (1:300; Cell Signaling) and anti-VEGF (1:400; sc-7269, Santa Cruz), to detect protein levels in the hippocampus of mice.

Statistical analysis

The data were expressed as the mean±SEM. Statistical analysis was conducted using SPSS 16.0. Differences between more than two groups were determined by one-way ANOVA followed by post hoc tests. The analysis of acquisition phase in the water maze test was determined by two-way ANOVA. $p < 0.05$ was considered significant.

Results

The effects of Sig-1R activation on the impairment of spontaneous alteration behaviour induced by brain ischaemia/reperfusion in the Y maze test

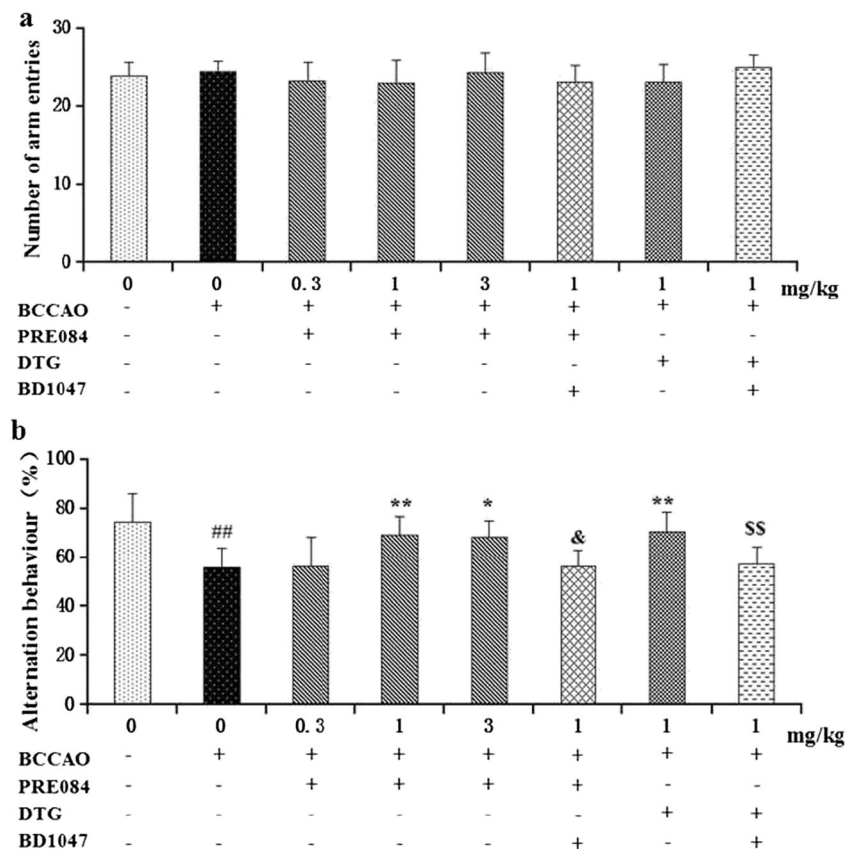
The effect of Sig-1R on the impairment of spontaneous alteration behaviour was evaluated on the 8th day after the BCCAO using the Y maze test. No significant differences in the total number of arm entries were found among the groups ($F_{7, 88} = 1.426$, $p = 0.205$; Fig. 1a). As shown in Fig. 1b, the

BCCAO group exhibited a significantly reduced spontaneous alteration behaviour compared with the sham group ($F_{7, 88} = 9.820$, $p < 0.001$; post hoc, $p < 0.01$). PRE084 (3 and 1 mg/kg) dose-dependently attenuated the BCCAO-induced impairment of spontaneous alteration behaviour ($F_{7, 88} = 9.820$, $p < 0.001$; PRE084 at 3 mg/kg, $p < 0.05$; PRE084 at 1 mg/kg, $p < 0.01$; Fig. 1b). The post hoc analysis showed that after co-administration with the antagonist BD1047 (1 mg/kg), the effect of PRE084 (1 mg/kg) was completely blocked ($p < 0.05$; Fig. 1b). DTG also improved the impairment of spontaneous alteration behaviour ($p < 0.01$), whereas BD1047 (1 mg/kg) completely antagonised this improvement ($p < 0.01$; Fig. 1b).

The effects of Sig-1R activation on visual recognition impairment induced by brain ischaemia/reperfusion in the novel object recognition test

We evaluated the effect of Sig-1R activation on the impairment of visual recognition through a novel object recognition test. The level of exploratory preferences for the novel object B (both the PI and DI were calculated, DI not shown) in the BCCAO mice group was significantly decreased compared with that in the sham mice group ($F_{7, 87} = 9.398$, $p < 0.001$; post hoc, $p < 0.01$; Fig. 2a). A strong increase in the PI was observed in PRE084 (3 and 1 mg/kg)-treated mice in the 1-h test

Fig. 1 Effects of Sig-1R activation on the impairment of spontaneous alteration behaviour induced by brain ischaemia/reperfusion in Y maze test. **a** Total number of arm entries; **b** spontaneous alteration behaviour (%). All results are presented as mean±SEM. $n = 12$; $^{##} p < 0.01$ vs sham; $^{*} p < 0.05$, $^{**} p < 0.01$ vs BCCAO; $^{\&} p < 0.05$ vs PRE084 (1 mg/kg), $^{SS} p < 0.01$ vs DTG (1 mg/kg)



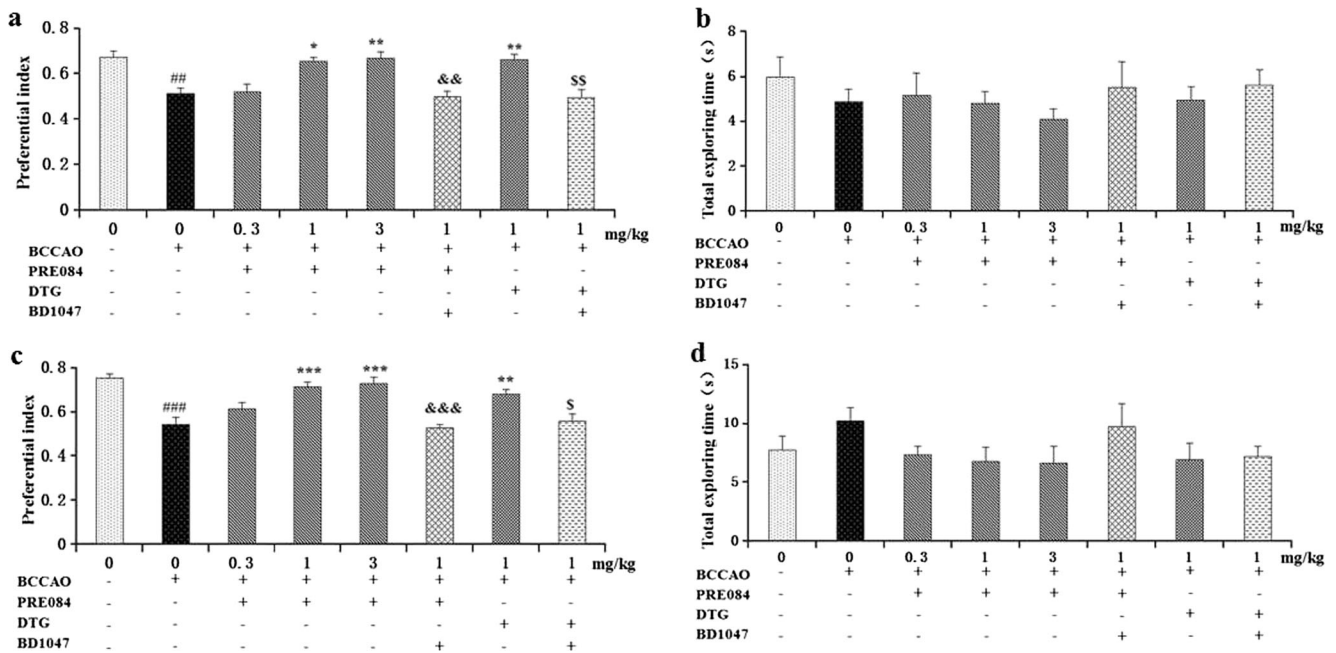


Fig. 2 Effects of Sig-1R activation on visual recognition impairment induced by brain ischaemia/reperfusion in the novel object recognition test. **a** Preferential index of 1 h; **b** total exploring time of 1 h; **c** preferential index of 24 h; **d** total exploring time of 24 h; all results

are presented as mean±SEM. $n=11-12$; ## $p<0.01$, ### $p<0.001$ vs sham; * $p<0.05$, ** $p<0.01$, *** $p<0.001$ vs BCCAO; && $p<0.01$, &&& $p<0.001$ vs PRE084 (1 mg/kg), \$ $p<0.05$, \$\$ $p<0.01$ vs DTG (1 mg/kg)

(PRE084 at 3 mg/kg, $p<0.01$; PRE084 at 1 mg/kg, $p<0.05$; Fig. 2a). However, restored recognition memory was not observed after co-administration with the antagonist BD1047 ($p<0.01$; Fig. 2a). BD1047 also reversed the improvement of the recognition memory of the DTG (1 mg/kg) group after BCCAO ($p<0.01$; Fig. 2a). Similar results were observed in the BCCAO mice when the test was performed 24 h after training ($F_{7, 87}=11.932$, $p<0.001$; post hoc, $p<0.001$; Fig. 2c). The data showed that PRE084 (3 and 1 mg/kg) significantly increased the PI ($p<0.001$ for both dose; Fig. 2c) in a dose-dependent manner. Compared with PRE084 (1 mg/kg), co-administration with BD1047 decreased the PI ($p<0.001$; Fig. 2c). The DTG group increased the PI after the BCCAO ($p<0.01$; Fig. 2c), but co-administration with BD1047 showed a significant inversion after DTG (1 mg/kg) treatment ($p<0.05$; Fig. 2c). No significant differences in the total exploring time after 1 or 24 h were found among the groups (1 h: $F_{7, 87}=0.555$, $p=0.791$; 24 h: $F_{7, 87}=1.152$, $p=0.339$; Fig. 2b, d). These results indicate that Sig-1R activation could restore the recognition memory deficit induced by brain ischaemia/reperfusion.

The effects of Sig-1R activation on learning and memory deficits induced by the BCCAO in the Morris water maze test

To determine whether Sig-1R activation improves spatial memory deficits induced by brain ischaemia/reperfusion in mice, we assessed their performance using the Morris water maze test. During the acquisition trial phase, there was a

significant increase in escape latency in the BCCAO mice (two-way ANOVA, $group$, $F_{7,429}=10.136$, $p<0.001$; Fig. 3a). Mice in the PRE084 (3 and 1 mg/kg) groups required significantly less time to find the platform than the BCCAO group ($p<0.01$ for 3 mg/kg, $p<0.05$ for 1 mg/kg; Fig. 3a). In the probe test, as no significant differences in swimming speeds were shown among the groups ($F_{7,85}=0.760$, $p=0.622$; Fig. 3b), the exploration time and the exploration distance spent in the target quadrant were significantly reduced in the BCCAO mice compared with the sham group (exploration time $F_{7,85}=2.228$, $p<0.05$, post hoc, $p<0.05$; exploration distance $F_{7,85}=2.230$, $p<0.05$, post hoc, $p<0.05$; Fig. 3c, d). Mice in the PRE084 (3 and 1 mg/kg) groups spent more time ($p<0.01$ for 3 mg/kg, $p<0.05$ for 1 mg/kg; Fig. 3c) and swam longer distances ($p<0.01$ for 3 mg/kg, $p<0.05$ for 1 mg/kg; Fig. 3d) in the target quadrant than the BCCAO group. DTG (1 mg/kg) also increased the exploration time ($p<0.05$; Fig. 3c) as well as the exploration distance ($p<0.05$; Fig. 3c) in the target quadrant. Taken together, these data suggest that Sig-1R activation could ameliorate learning and memory deficits in brain ischaemia/reperfusion in mice.

The effects of Sig-1R activation on neuronal cell damage in the hippocampus induced by BCCAO

In BCCAO mice, NeuN-positive cells decreased compared with the sham group ($F_{7,32}=2.827$, $p<0.05$, post hoc, $p<0.01$; Fig. 4a, b, i). Treatment with PRE084 (3 and 1 mg/kg) significantly reduced neuronal cell loss ($p<0.05$ both; Fig. 4d, e, i)

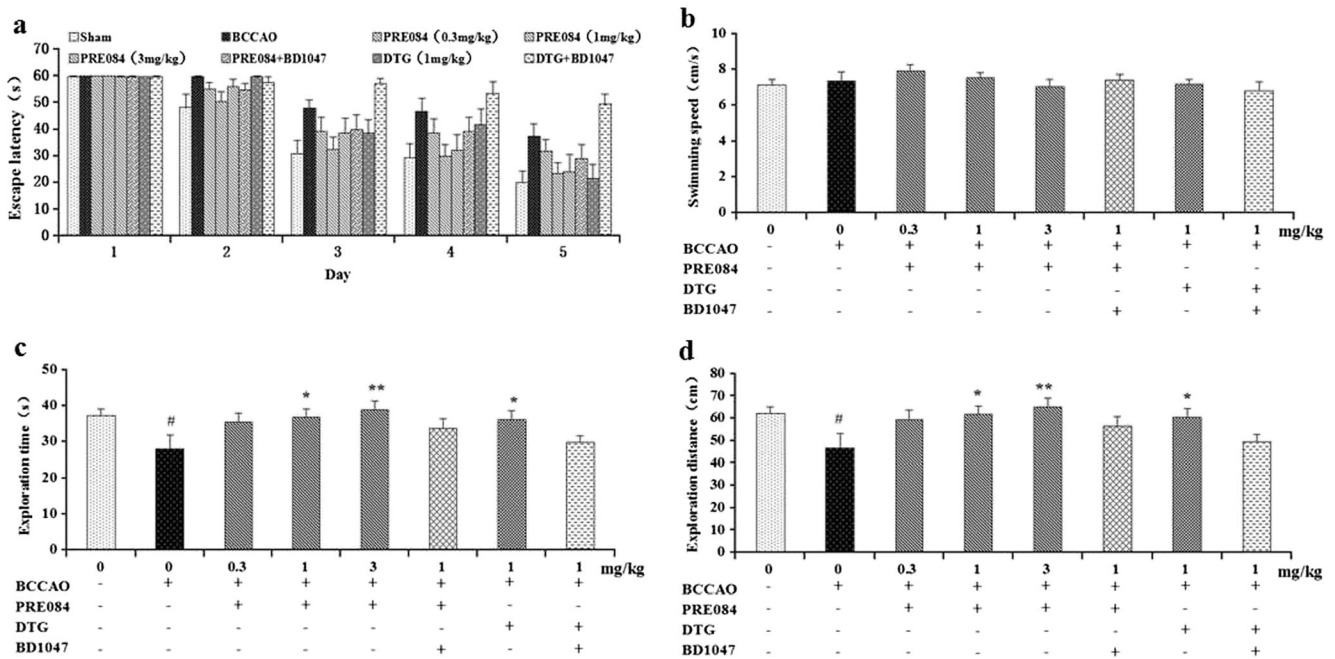


Fig. 3 Effects of Sig-1R activation on learning and memory induced by brain ischaemia/reperfusion in the Morris water maze test. **a** The escape latency in training period; **b** swimming speed in probe test; **c** exploration

time in probe test; **d** exploration distance in probe test; all results are presented as mean±SEM. $n=11-12$; [#] $p<0.05$ vs sham; ^{*} $p<0.05$, ^{**} $p<0.01$ vs BCCAO

as did the DTG ($p<0.05$; Fig. 4g, i). Co-treatment with BD1047 (1 mg/kg) reversed the effect of PRE084 ($p<0.05$; Fig. 4h, i). Furthermore, the degenerating neurons were further quantified by Western blotting. Compared with the sham group, the NeuN protein levels in BCCAO mice showed a dramatic decrease ($F_{7,16}=5.895$, $p<0.01$, post hoc, $p<0.01$; Fig. 4j). In the PRE084 (3 and 1 mg/kg)-treated group, the NeuN level was significantly increased ($p<0.01$ both; Fig. 4j). DTG also increased the NeuN level ($p<0.05$; Fig. 4j). However, this increase was totally blocked by BD1047 (1 mg/kg) ($p<0.05$ both; Fig. 4j).

The effects of Sig-1R activation on the expression of SYP, GAP-43, PSD95 and NogoA in the hippocampus

To investigate the effect of Sig-1R on synaptic plasticity in BCCAO mice, the expressions of synapse-associated proteins, synaptophysin (SYP), growth-associated protein-43 (GAP-43), postsynaptic density 95 (PSD95) and NogoA were detected. Western blot analysis showed that PRE084 (3 and 1 mg/kg) strongly increased the levels of SYP, GAP-43 and PSD95 in the hippocampus compared with BCCAO mice (SYP: $F_{7,16}=3.536$, $p<0.05$, post hoc, $p<0.05$ for both doses; GAP-43: $F_{7,16}=7.542$, $p<0.001$, post hoc, $p<0.05$ for both doses; PSD95: $F_{7,16}=4.751$, $p<0.01$, post hoc, $p<0.01$ for both doses; Fig. 5a–c), and similar results were observed in the DTG group (1 mg/kg) (SYP: $F_{7,16}=3.528$, $p<0.05$, post hoc, $p<0.01$; GAP-43: $F_{7,16}=7.542$, $p<0.001$, post hoc, $p<0.05$; PSD95: $F_{7,16}=4.751$, $p<0.01$, post hoc, $p<0.01$;

Fig. 5a–c). However, the restoration of the SYP, GAP-43 and PSD95 expressions was abolished by co-administration with BD1047 (1 mg/kg) (SYP: $F_{7,16}=3.536$, $p<0.05$, post hoc, $p<0.05$ both; GAP-43 $F_{7,16}=7.542$, $p<0.001$, post hoc, $p<0.01$ both; PSD95 $F_{7,16}=4.751$, $p<0.01$, post hoc, $p<0.01$ and $p<0.05$, respectively; Fig. 5a–c). The level of Nogo-A as an inhibitor of axonal growth showed no differences among these groups ($F_{7,24}=1.362$, $p=0.266$; Fig. 5d).

Sig-1R activation up-regulates the expression of BDNF in the hippocampus

BDNF immunoreactivity in the hippocampus CA1 region was significantly decreased in the BCCAO group compared with that in the sham group 22 days after the BCCAO ($F_{7,32}=15.572$, $p<0.001$, post hoc, $p<0.001$; Fig. 6a, b, i), whereas BDNF immunoreactivity in the PRE084 (3 and 1 mg/kg)- and DTG (1 mg/kg)-treated groups was higher than that in the BCCAO group ($p<0.001$; Fig. 6d, f, g, i). After co-administration with BD1047, the restored immunoreactivity was decreased ($p<0.001$; Fig. 6f, h, i). Western blotting analysis showed consistent results with immunohistochemical staining. Compared with the sham group, the protein levels of BDNF in the hippocampus decreased in BCCAO mice ($F_{7,16}=13.803$, $p<0.001$, post hoc, $p<0.01$; Fig. 6j). Treatments with PRE084 (3 and 1 mg/kg) or DTG (1 mg/kg) increased the hippocampal BDNF levels significantly (PRE084: $p<0.01$ for both doses; DTG: $p<0.001$; Fig. 6j).

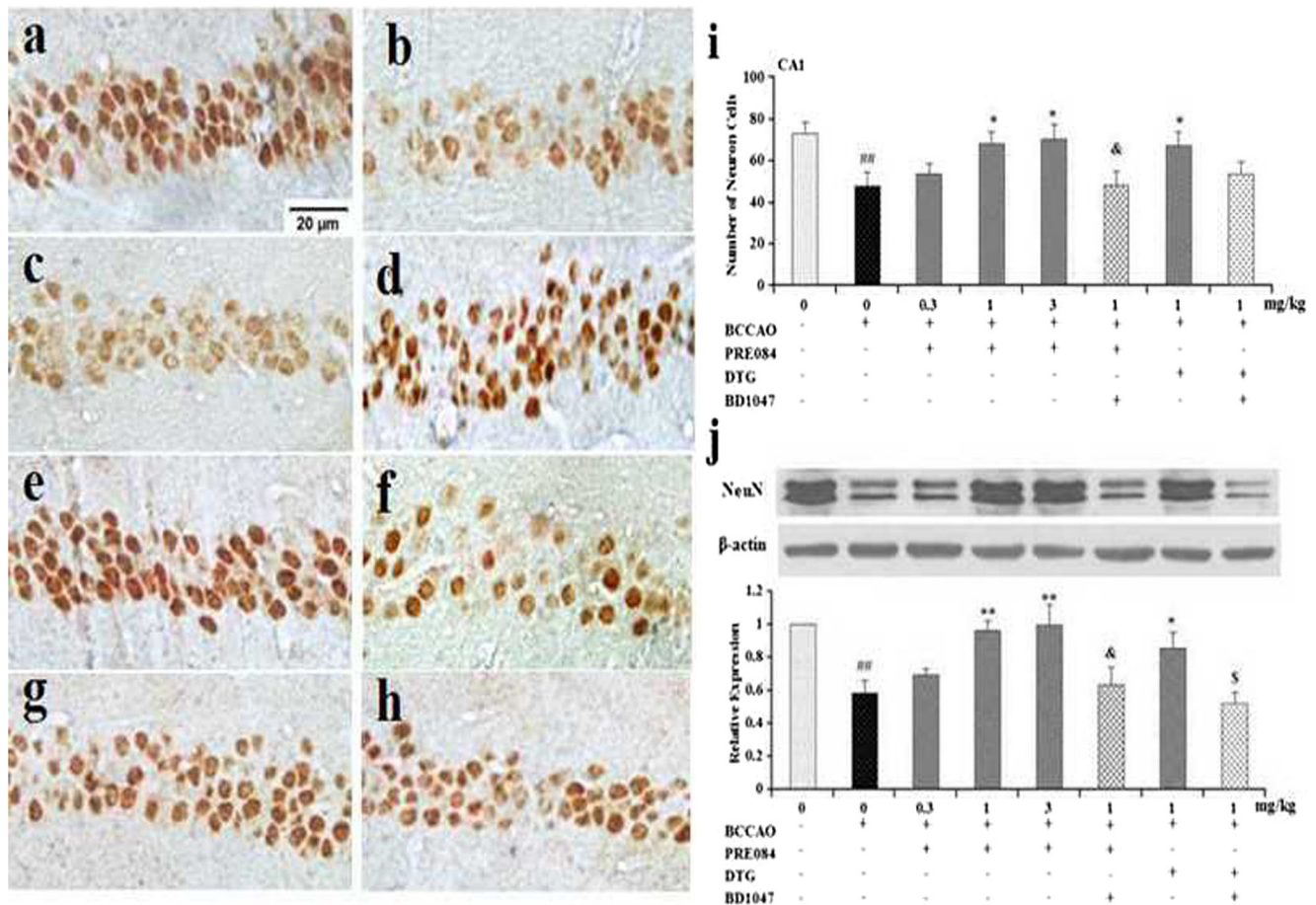


Fig. 4 Effects of Sig-1R activation on neuronal cell damage in the hippocampus induced by BCCAO. NeuN-positive cell in CA1 region of hippocampus: **a** sham, **b** BCCAO, **c** PRE084 (0.3 mg/kg), **d** PRE084 (1 mg/kg), **e** PRE084 (3 mg/kg), **f** PRE084 (1 mg/kg) + BD1047 (1 mg/kg), **g** DTG (1 mg/kg), **h** DTG (1 mg/kg) + BD1047 (1 mg/kg); **i**

graphic representation of NeuN-positive cell number, $n=5$; **j** Western blot analysis of NeuN in hippocampus, $n=3$. All results are presented as mean \pm SEM. ^{##} $p<0.01$ vs sham; ^{**} $p<0.01$ vs BCCAO; [&] $p<0.05$ vs PRE084 (1 mg/kg), [§] $p<0.05$ vs DTG (1 mg/kg)

The improvement of the agonists (PRE084 and DTG) on BDNF expression was totally blocked by the antagonist BD1047 ($p<0.05$ and $p<0.01$, respectively; Fig. 6j). The quantification of the BDNF protein levels and other behavioural data were assessed by the Pearson correlation coefficients, as presented in Table 1. The correlations are significant, suggesting a close relationship between BDNF protein levels and learning and memory behaviours.

Sig-1R activation increases the phosphorylation of TrkB, ERK and CREB in the hippocampus

To further examine the effect of Sig-1R activation on BDNF signalling, we detected the phosphorylation of TrkB and the downstream event, the phosphorylation of ERK, with Western blotting. The ratio of phosphorylated TrkB and TrkB in BCCAO mice showed a dramatic decrease ($F_{7,16}=12.26$, $p<0.001$, post hoc, $p<0.001$; Fig. 7a) compared with the sham

group. In the PRE084 (3 and 1 mg/kg)-treated group, this ratio was significantly increased ($p<0.01$ both; Fig. 7a). DTG also increased the phosphorylated TrkB levels ($p<0.001$; Fig. 7a). However, the increase was reversed by BD1047 (1 mg/kg) ($p<0.05$ both; Fig. 7a). Compared to BCCAO mice, PRE084- (3 and 1 mg/kg) and DTG (1 mg/kg)-treated mice had improved ratios of phosphorylated ERK (p-ERK) and ERK (PRE084: $F_{7,16}=3.831$, $p<0.05$, post hoc, $p<0.01$ for 3 mg/kg, $p<0.05$ for 1 mg/kg; DTG: $p<0.05$; Fig. 7b). However, co-administration of BD1047 (1 mg/kg) blocked the improvement of PRE084 ($p<0.05$; Fig. 7b). Furthermore, the level of phosphorylated CREB (p-CREB), the main regulator of BDNF, was significantly decreased in the BCCAO mice ($F_{7,16}=8.563$, $p<0.05$, post hoc, $p<0.01$; Fig. 7c). PRE084 (3 and 1 mg/kg) and DTG increased the depressed ratio of p-CREB/CREB in the BCCAO mice (PRE: $p<0.001$ for both doses; DTG: $p<0.001$; Fig. 7c). The increase in the ratio of p-CREB/CREB by PRE084 and DTG treatments was blocked by co-administration of BD1047 ($p<0.01$; Fig. 7c).

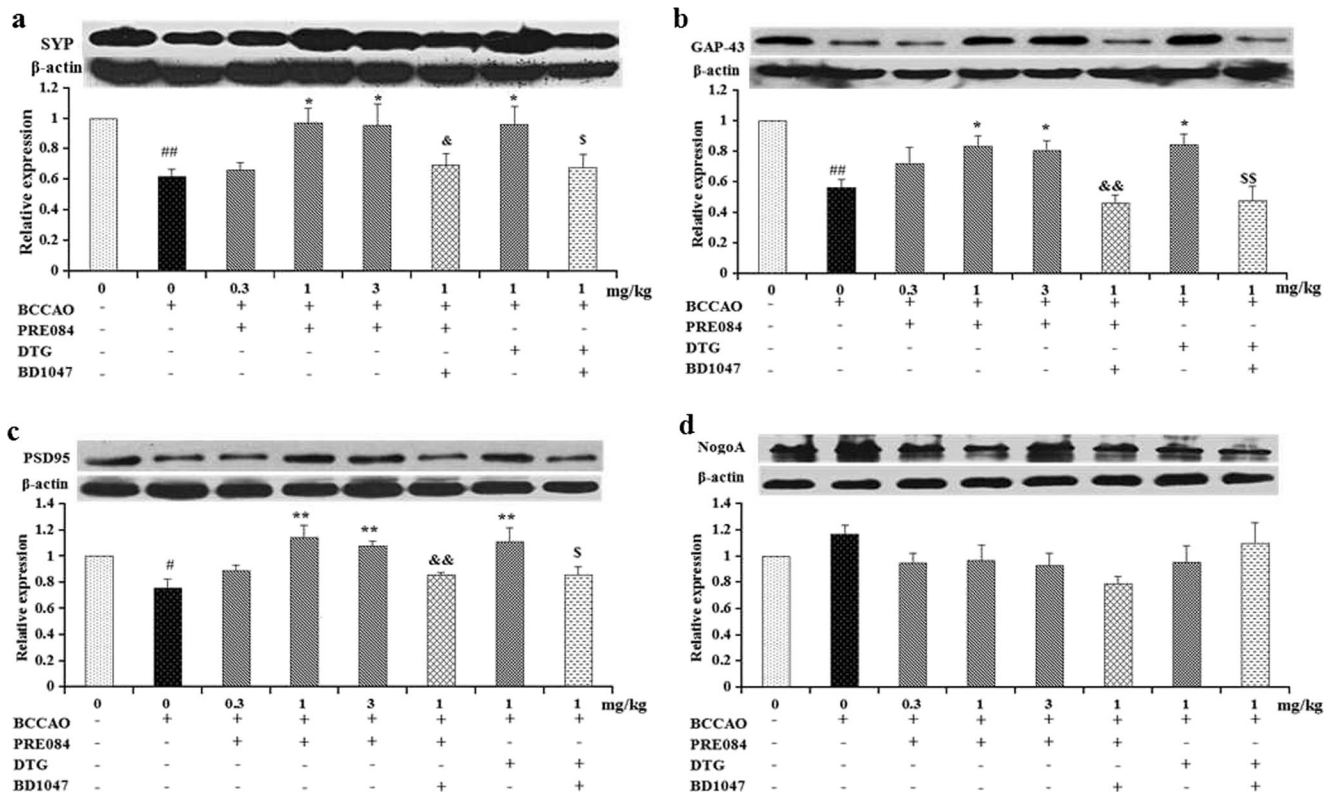


Fig. 5 Effects of Sig-1R activation on the expression of SYP, GAP-43, PSD95, NogoA in the hippocampus. Changes of the expression of SYP (a), GAP-43 (b), PSD95 (c) and NogoA (d) among the groups, shown as

percentage of control. All results are presented as mean \pm SEM. $n=3$; # $p<0.05$, ## $p<0.01$ vs sham; * $p<0.05$, ** $p<0.01$ vs BCCAO; && $p<0.01$ vs PRE084 (1 mg/kg), $^S p<0.05$, $^{SS} p<0.01$ vs DTG (1 mg/kg)

Sig-1R targets in the NR2A-CaMKIV-TORC1 pathway

Sig-1R regulates the ratio of p-CREB and CREB to increase the transcription of BDNF and the dephosphorylation of CREB-specific co-activator transducers of regulated CREB activity 1 (TORC1), resulting in the activation of CREB and its downstream gene targets. Thus, we investigated the level of TORC1 and its upstream regulators, including NR2A and CaMKIV, with Western blotting (Fig. 8a). The level of TORC1 was reduced in BCCAO mice compared with the sham group ($F_{7, 16}=6.131$, $p<0.01$, post hoc, $p<0.01$; Fig. 8b). PRE084 (3 and 1 mg/kg) increased the reduction in mice ($p<0.01$ and $p<0.05$, respectively; Fig. 8b). DTG ($p<0.01$; Fig. 8b) also increased the reduction in mice, while the encasement was reversed by the co-administration of BD1047 in both groups ($p<0.05$ and $p<0.01$, respectively; Fig. 8b). The levels of NR2A and CaMKIV were similar to those of TORC1 (Fig. 8a–c). These results suggest that Sig-1R is involved in the NR2A-CaMKIV-TORC1 signalling pathway.

The relationship between Sig-1R activation and VEGF

As VEGF is important for ischaemia restoration, we detected the expression of VEGF in the hippocampus and found no

significant differences among the groups ($F_{7,16}=0.223$, $p=0.974$; Figs. 9 and 10).

Discussion

Abundant evidence has suggested that ischaemia/reperfusion injury in BCCAO causes delayed neuronal death in the vulnerable CA1 region of the hippocampus and extensive neuronal excitotoxicity (Penton-Rol et al. 2011). Our results show that Sig-1R agonists rescued the degenerating neurons in BCCAO mice, while the antagonist BD1047 reversed the effect of the agonists, which suggests that Sig-1R was involved in the rescue of neuronal loss in the hippocampus as a means of neuroprotection. Ischaemia/reperfusion injuries also result in the reduction of synaptic proteins and the impairment of spatial memory in mice (Yamamoto et al. 2009; Zhao et al. 2005). In this study, we found that ischaemia/reperfusion injuries resulted in spatial memory impairments in the Y maze and Morris water maze, consistent with other reports (Watanabe and Utsuki 2011; Zhang et al. 2010). In this study, the Sig-1R agonist, PRE084, and the non-selected sigma 1/2 receptor agonist, DTG, dramatically improved the learning and memory deficits in BCCAO mice. We further

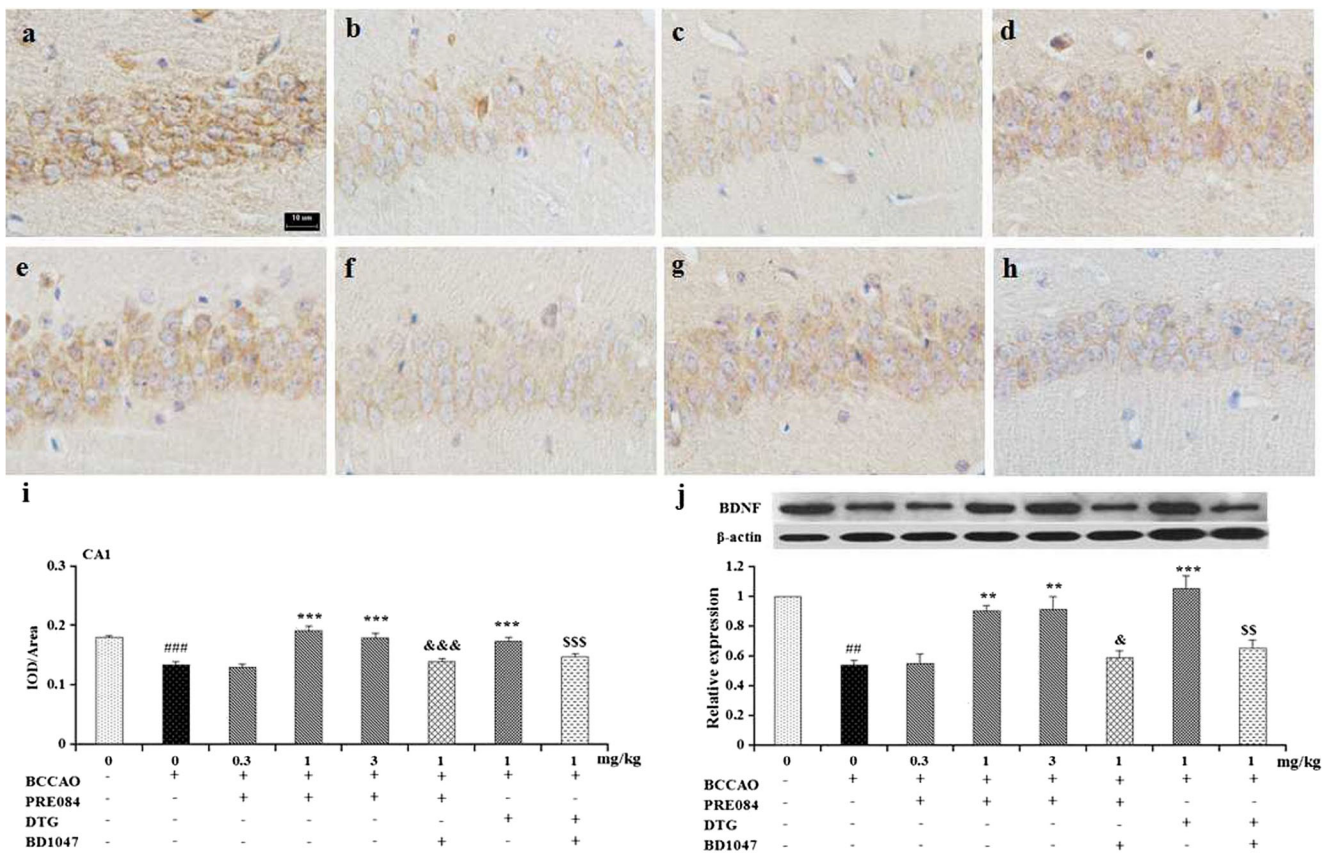


Fig. 6 Sig-1R activation up-regulates the expression of BDNF in the hippocampus. BDNF immunoreactivity in CA1 region of hippocampus: **a** sham, **b** BCCAO, **c** PRE084 (0.3 mg/kg), **d** PRE084 (1 mg/kg), **e** PRE084 (3 mg/kg), **f** PRE084 (1 mg/kg) + BD1047 (1 mg/kg), **g** DTG (1 mg/kg), **h** DTG (1 mg/kg) + BD1047 (1 mg/kg); **i** graphic

representation of BDNF immunoreactivity, $n=5$; **j** Western blot analysis of BDNF in hippocampus, $n=3$. All results are presented as mean \pm SEM. $^{###}p<0.01$, $^{####}p<0.001$ vs sham; $^{**}p<0.01$, $^{***}p<0.001$ vs BCCAO; $^{\&}p<0.05$, $^{\&\&}p<0.001$ vs PRE084 (1 mg/kg), $^{SS}p<0.01$, $^{SSS}p<0.001$ vs DTG (1 mg/kg)

found that the effects of PRE084 and DTG on the spatial memory impairments were blocked by the Sig-1R-selected antagonist BD1047. Previous studies reported that DHEA, an endogenous ligand of Sig-1R, improved spatial memory impairment from transient cerebral ischaemia in rats (Li et al. 2009). As reported, when administered during a 3- to 48-h time period after ischaemia, the sigma agonist DHEA exerts neuroprotection (Li et al. 2009), which is consistent with our 24-h therapeutic time period. Additionally, neuronal damage in the CA1 region was not seen until 12 h after ischaemia and

then developed over 48 h of recirculation. Therefore, treatment with Sig-1R agonists within 24 h after BCCAO still had a neuroprotective effect in this study (Olsson et al. 2003). These results suggest that the activation of Sig-1R can ameliorate spatial memory impairments resulting from brain ischaemia.

Table 1 The correlation analysis between BDNF and Y maze test, probe test, novel object recognition test and NR2A

BDNF	Y maze test	Probe test	Novel object recognition test	NR2A
Pearson correlation	0.973**	0.707*	0.947**	0.931**
<i>p</i>	0	0.05	0	0.001

** Correlation is significant at the 0.01 level (two-tailed)

* Correlation is significant at the 0.05 level (two-tailed)

Synaptic plasticity has traditionally been associated with higher brain functioning, including learning and memory processing. In particular, various forms of neuronal plasticity within the hippocampal formation are thought to underlie spatial learning and memory development (Bannerman et al. 2014). A loss of synaptic proteins was found in mice with brain ischaemia (Yamamoto et al. 2009; Zhao et al. 2005). In this study, we found that the Sig-1R agonists PRE084 and DTG attenuated the decrease of synaptic protein (SYP, GAP-43 and PSD95) levels in BCCAO mice, whereas the attenuation was abolished by the Sig-1R antagonist BD1047, suggesting that Sig-1R activation might rescue the synapse loss resulting from ischaemia/reperfusion.

BDNF has emerged as an important regulator of synaptic plasticity and learning and memory (Korte et al. 1995; Cunha

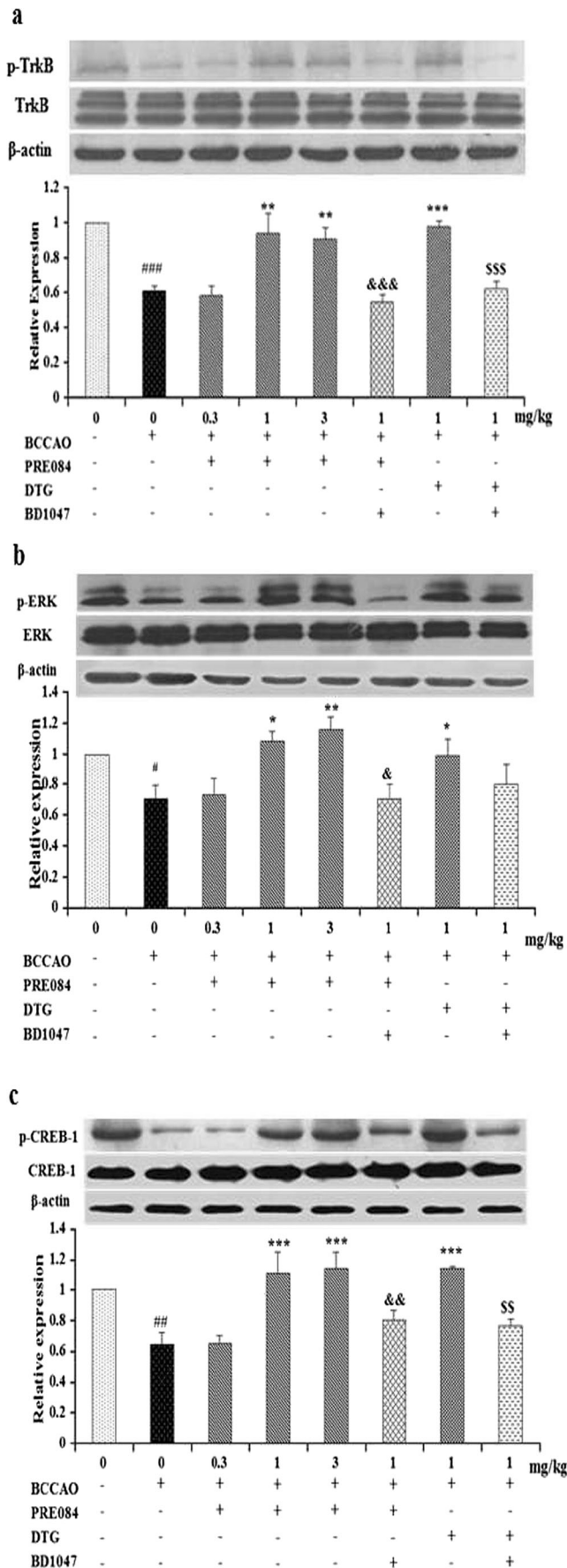


Fig. 7 Sig-1R activation increases phosphorylated of TrkB, ERK and CREB in the hippocampus. Changes of the ratio of p-TrkB/TrkB (a), p-ERK/ERK (b) and p-CREB-1/CREB-1 (c), among the groups, shown as percentage of control. All results are presented as mean±SEM. *n*=3; ##*p*<0.01, ###*p*<0.001 vs sham; **p*<0.05, ***p*<0.01, ****p*<0.001 vs BCCAO; &*p*<0.05, &&*p*<0.01, &&&*p*<0.001 vs PRE084 (1 mg/kg), ^{ss}*p*<0.01, ^{sss}*p*<0.001 vs DTG (1 mg/kg)

et al. 2010). The binding of BDNF to TrkB results in the phosphorylation of tyrosine residues in the cytoplasmic domain of the receptor, triggering the activation of three major signalling cascades, including mitogen-activated protein

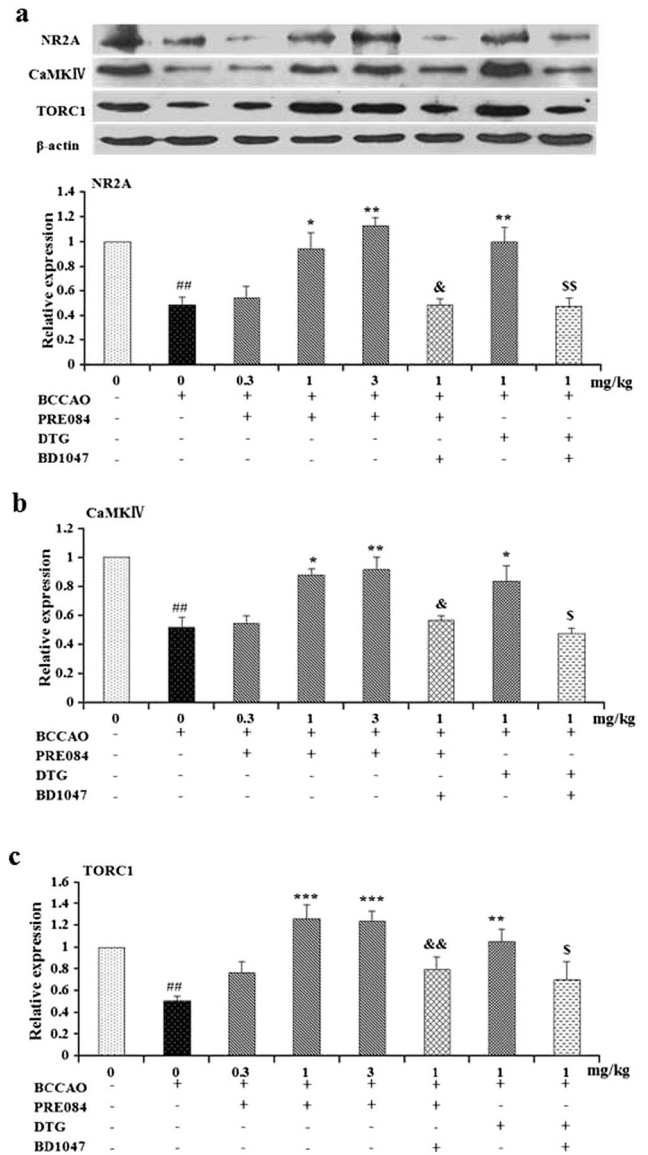


Fig. 8 Sig-1R activation involved in NR2A-CaMKIV-TORC1 pathway through Western blotting. **a** Western blotting bands of NR2A, CaMKIV and TORC1 in hippocampus and the analysis of NR2A, **b** statistic analysis of CaMKIV, **c** statistic analysis of TORC1. All results are presented as mean±SEM, *n*=3; ##*p*<0.01 vs sham; **p*<0.05, ***p*<0.01, ****p*<0.001 vs BCCAO; &*p*<0.05, &&*p*<0.01 vs PRE084 (1 mg/kg), ^s*p*<0.05, ^{ss}*p*<0.01 vs DTG (1 mg/kg)

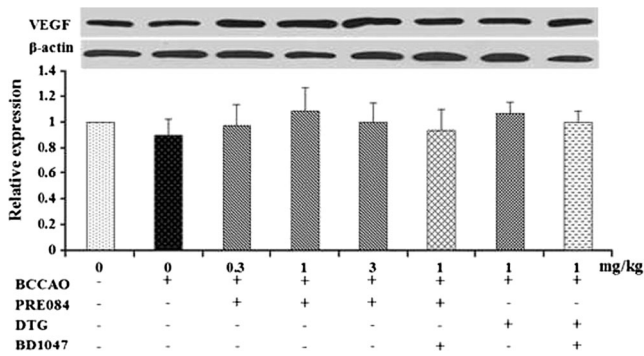


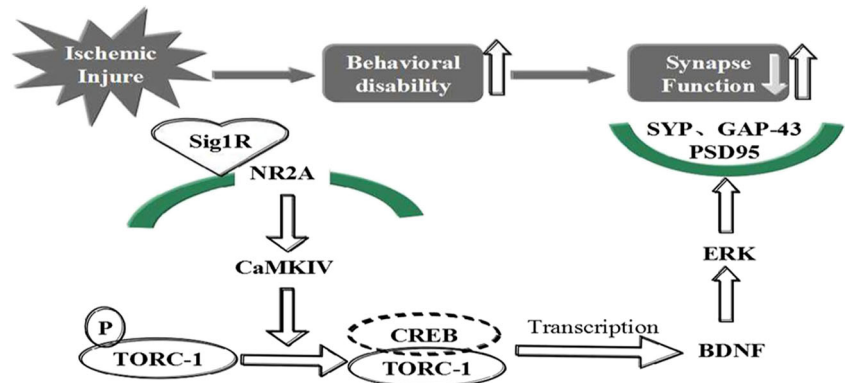
Fig. 9 Western blot analysis of the expression of VEGF in hippocampus. All results are presented as mean±SEM. n=3

kinase kinases (MEKs)-extracellular signal-regulated kinases (ERKs), phospholipase Cγ1 (PLCγ1)-inositol-1,4,5-triphosphate (IP3)/diacylglycerol (DAG) and phosphoinositide 3-kinase (PI3K)-Akt pathways (Nagahara and Tuszynski 2011). BDNF directly participated in synaptogenesis by increasing the number and size of dendritic spines in the hippocampal neurons (El-Husseini et al. 2000; Tyler and Pozzo-Miller 2003). Similarly, studies have demonstrated that the expression of synaptic proteins SYP, GAP-43 and PSD95 is dependent on the binding of BDNF to TrkB receptors and the subsequent activation of the ERK and phosphatidylinositol-3 kinase (PI3K) pathways (Li and Keifer 2012; Robinet and Pellerin 2011). In this study, immunocytochemistry and Western blot analysis revealed that Sig-1R activation attenuated the decrease in BDNF levels, and TrkB and ERK activation in the hippocampus of BCCAO mice. These results suggest that increased BDNF by Sig-1R agonists may increase the phosphorylation of TrkB and ERK, thus improving synaptic plasticity and learning and memory. BDNF could regulate affective behaviours through diverse mechanisms, including the modulation of neurogenesis, synaptic functioning and structural plasticity in the hippocampus (Pattwell et al. 2012; Bath et al. 2012). We further found significant correlations between the quantitation of BDNF protein levels and performance in the Y maze as well as the index of preference in new object organisation. These results suggest that Sig-1R

activation might rescue the loss of neurons and synaptic proteins resulting from brain ischaemia/reperfusion and thus improving the learning and memory deficit through regulating BDNF signalling. Other factors, such as vascular endothelial growth factor (VEGF), might also regulate the level of p-ERK (Kilic et al. 2006). We investigated whether VEGF contributed to the decrease of p-ERK in the model group and the increase of p-ERK in the Sig-1R agonists treated groups. However, there were no changes in the VEGF level, suggesting that the decrease of hippocampal p-ERK in the BCCAO mice might not be related to VEGF.

Sig-1R activation increased the hippocampal BDNF levels, possibly contributing to the restoration of synaptic loss and spatial memory deficits resulting from brain ischaemia. How Sig-1R controlled the expression of BDNF is unclear. CREB is activated by phosphorylation and plays a central role in BDNF transcription (Tao et al. 1998). Stroke-surviving neurons have sustained concentrations of phospho-CREB and elevated concentrations of BDNF (Kokaia et al. 1995; Walton and Dragunow 2000). In this study, we found that ischaemia/reperfusion resulted in a decrease in the levels of phospho-CREB, accompanied by a decrease of BDNF expression. This can be explained by the entry of calcium through extrasynaptic NMDA receptors, triggered by bath glutamate exposure or hypoxic/ischaemic conditions, activated a general and dominant CREB shut-off pathway that blocked the induction of BDNF expression (Hardingham et al. 2002; Hardingham and Bading 2010). In contrast, synaptic NMDA receptors have an opposite effect on extrasynaptic NMDA receptors, CREB functioning and gene regulation (Hardingham et al. 2002; Peng et al. 2006; Liu et al. 2007). Calcium entry through synaptic NMDA receptors induced CREB activity and BDNF gene expression (Hardingham et al. 2002). In hippocampal neurons, extrasynaptic NMDA receptors are composed predominantly of NMDA receptor 1 (NR1) and NMDA receptor 2B (NR2B) subunits, whereas synaptic NMDA receptors also contain NMDA receptor 2A (NR2A) subunits (Hardingham and Bading 2010). Intracellular calcium neurons via NR2A-containing NMDA receptors enhance the activity of

Fig. 10 The proposed effects of Sig-1R activation in brain ischaemia injury



CaMKIV. Activated CaMKIV phosphorylates SIK2, which dephosphorylates TORC1, leading to its translocation into the nucleus. Then, TORC1 induces CREB target genes, such as BDNF (Hardingham et al. 2002; Hardingham and Bading 2010). Sig-1R serves as an inter-organelle signalling modulator locally at the mitochondrion-associated ER membrane (MAM) (Hayashi and Su 2007) and remotely at the plasmalemma/plasma membrane to regulate functional proteins (Su et al. 2010). In our study, Sig-1R activation attenuated a decrease in the level of hippocampal NR2A receptors, suggesting that Sig-1R activation may restore or increase synaptic NMDA receptors in mice with brain ischaemia. We also found that Sig-1R activation increases the levels of CAMKIV, TORC1 and phospho-CREB with an increase of NR2A expression in mice with brain ischaemia. The Pearson correlation is significant between BDNF and NR2A. Taken together, these data suggest that the NR2A-CaMKIV-TORC1-CREB pathway is possibly involved in the regulation of Sig-1R on BDNF expression.

In summary, we identified that Sig-1R activation attenuated the learning and memory impairment caused by brain ischaemia/reperfusion and up-regulated the expression of synaptic proteins. These results suggest that the NR2A-TORC1-CREB pathway may contribute to the increase of BDNF levels through Sig-1R activation. However, the causal relationship between BDNF induction and the increase in NR2A-CaMKIV-TORC1 protein levels needs to be evaluated. In future studies, we will block the NR2A receptor or silence the NR2A gene to validate the causal relationship. These findings might provide a new target for the development of novel therapeutics in dementia induced by ischaemia.

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Conflict of interest The authors declare no competing financial interests.

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