

# Activation of GABA<sub>B</sub> Receptors Ameliorates Cognitive Impairment via Restoring the Balance of HCN1/HCN2 Surface Expression in the Hippocampal CA1 Area in Rats With Chronic Cerebral Hypoperfusion

Chang-jun Li · Yun Lu · Mei Zhou · Xian-gang Zong · Cai Li · Xu-lin Xu · Lian-jun Guo · Qing Lu

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**Abstract** Hyperpolarization-activated cyclic-nucleotide-gated cation nonselective (HCN) channels are involved in the pathology of nervous system diseases. HCN channels and  $\gamma$ -aminobutyric acid (GABA) receptors can mutually co-regulate the function of neurons in many brain areas. However, little is known about the co-regulation of HCN channels and GABA receptors in the chronic ischemic rats with possible features of vascular dementia. Protein kinase A (PKA) and TRP containing Rab8b interacting protein (TRIP8b) can modulate GABA<sub>B</sub> receptors cell surface stability and HCN channel trafficking, respectively, and adaptor-associated

kinase 1 (AAK1) inhibits the function of the major TRIP8b-interacting protein adaptor protein 2 (AP2) via phosphorylating the AP2  $\mu$ 2 subunit. Until now, the role of these regulatory factors in chronic cerebral hypoperfusion is unclear. In the present study, we evaluated whether and how HCN channels and GABA<sub>B</sub> receptors were pathologically altered and investigated neuroprotective effects of GABA<sub>B</sub> receptors activation and cross-talk networks between GABA<sub>B</sub> receptors and HCN channels in the hippocampal CA1 area in chronic cerebral hypoperfusion rat model. We found that cerebral hypoperfusion for 5 weeks by permanent occlusion of bilateral common carotid arteries (two-vessel occlusion, 2VO) induced marked spatial and nonspatial learning and memory deficits, significant neuronal loss and decrease in dendritic spine density, impairment of long-term potentiation (LTP) at the Schaffer collateral-CA1 synapses, and reduction of surface expression of GABA<sub>B</sub> R1, GABA<sub>B</sub> R2, and HCN1, but increase in HCN2 surface expression. Meanwhile, the protein expression of TRIP8b (1a-4), TRIP8b (1b-2), and AAK1 was significantly decreased. Baclofen, a GABA<sub>B</sub> receptor agonist, markedly improved the memory impairment and alleviated neuronal damage. Besides, baclofen attenuated the decrease of surface expression of GABA<sub>B</sub> R1, GABA<sub>B</sub> R2, and HCN1, but downregulated HCN2 surface expression. Furthermore, baclofen could restore expression of AAK1 protein and significantly increase p-PKA, TRIP8b (1a-4), TRIP8b (1b-2), and p-AP2  $\mu$ 2 expression. Those findings suggested that, under chronic cerebral hypoperfusion, activation of PKA could attenuate baclofen-induced decrease in surface expression of GABA<sub>B</sub> R1 and GABA<sub>B</sub> R2, and activation of GABA<sub>B</sub> receptors not only increased the expression of TRIP8b

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Chang-jun Li and Yun Lu contributed equally to this work.

C.-j. Li · Y. Lu · M. Zhou · C. Li · X.-l. Xu · L.-j. Guo (✉) · Q. Lu (✉)

Department of Pharmacology, School of Basic Medical Sciences, Tongji Medical College, Huazhong University of Science and Technology, Wuhan 430030, People's Republic of China  
e-mail: gljyl2009@163.com  
e-mail: ljguo@hust.edu.cn

X.-l. Xu · L.-j. Guo · Q. Lu  
Key Laboratory of Drug Target Research and Pharmacodynamic Evaluation, Hubei Province Wuhan 430030, China

C.-j. Li  
Neurology Department, Huanggang central hospital, Hubei Province Huanggang 438000, People's Republic of China

X.-g. Zong  
Center for Integrated Protein Science (CIPSM) and Zentrum für Pharmaforschung, Department Pharmazie, Ludwig-Maximilians-Universität München, Munich 80539, Germany

(1a-4) and TRIP8b (1b-2) but also regulated the function of TRIP8b via AAK1 and p-AP2  $\mu$ 2, which restored the balance of HCN1/HCN2 surface expression in rat hippocampal CA1 area, and thus ameliorated cognitive impairment.

**Keywords** Two-vessel occlusion · Cognitive impairment · HCN channels · GABA<sub>B</sub> receptors

## Introduction

Hyperpolarization-activated currents (I<sub>h</sub>s) have aroused continuing interest of physiologists since their initial discovery in the early 1980s [1]. The current-designated I<sub>h</sub> flowing through hyperpolarization-activated cyclic-nucleotide-gated cation nonselective (HCN) channels plays a key role in the control of neuronal rhythmicity [2, 3]. These channels are also proposed to play a fundamental role in determining the neuronal resting membrane potential [4], dendritic integration [5], synaptic plasticity [6], neurotransmitter release [7], and the periodicity of network oscillations [8]. With the development of molecular biology, a family of four mammalian genes that encode the HCN channels (HCN 1–4) are identified, and two (HCN1 and 2) are substantially expressed in rodent hippocampus [9]. In contrast, HCN3 and HCN4 are expressed at very low levels in hippocampus [3]. It has been reported that HCN1 channels constrain learning and memory by regulating dendritic integration of distal synaptic inputs to pyramidal cells [5]; the deletion of HCN1 channels causes profound motor learning and memory deficits in swimming and rotarod tasks [10]. In our previous study, we have found that HCN1 messenger RNA (mRNA) is shown to be decreased significantly in the hippocampus and neocortex in the chronic ischemic rats [11]. Besides, HCN2 channel dysfunction has been implicated in neurological disorders such as ataxia [12] and absence epilepsy [13] as well as reduced inflammatory and neuropathic pain [14]. Double immunofluorescence revealed colocalization of immunoreactivity for HCN1 and HCN2 in distal dendrites of pyramidal cells in the hippocampus and neocortex [15]. However, whether HCN2 may be altered has not yet been adequately established in chronic cerebral hypoperfusion rats with possible features of vascular dementia.

HCN channels and  $\gamma$ -aminobutyric acid (GABA) receptors can mutually co-regulate neuronal excitability in many brain areas [16–19]. In the central nervous system, three main classes of GABA receptors exist and are termed GABA<sub>A</sub>, GABA<sub>B</sub>, and GABA<sub>C</sub> receptors [20]. The GABA<sub>A</sub> and GABA<sub>C</sub> are ionotropic receptors, belonging to the Cys-loop family of ligand-gated ion channels. GABA<sub>B</sub> receptors are members of the metabotropic receptor family; these receptors couple via G proteins (Gi/o) to interact with neuronal inwardly rectifying potassium and voltage-gated calcium channels,

mediating slow synaptic inhibition by increasing potassium (GIRK) and decreasing calcium conductances. Atherton et al. have reported that HCN channels in subthalamic nucleus neurons are conformed to selectively counteract GABA<sub>A</sub> receptor-mediated inhibition arising from the globus pallidus [18]. It has been found that the genetic deletion of HCN increased the expression of  $\alpha$ 5GABA<sub>A</sub> receptors in cortical pyramidal neurons [16], but the expression of HCN1 protein is significantly reduced in the hippocampus of Gabr $\alpha$ 5<sup>-/-</sup> mice (lack the  $\alpha$ 5 GABA<sub>A</sub> subunit gene) [17]. Studies have shown that activation of GABA<sub>B</sub> receptors can inhibit I<sub>h</sub> in rat ventral tegmental neurons and substantia nigra zona compacta principal neurons [19, 21]. However, little is known about the co-regulation of GABA<sub>B</sub> receptors and HCN channels in the hippocampal CA1 area.

Cerebral ischemia can cause the imbalance of excitation–inhibition. Glutamatergic and GABAergic transmissions work by each counterbalancing the function of the other; enhancing GABAergic activity should balance excessive glutamatergic excitation, which is the pivotal event leading to cell death. Baclofen, a selective GABA<sub>B</sub> receptors agonist, has been shown to be neuroprotective in animal models of transient brain ischemia [22–27], middle cerebral artery occlusion (MCAO) [28], and oxygen-glucose deprivation (OGD) [24, 27, 29, 30]. However, the potential roles of GABA<sub>B</sub> receptors and the co-regulation of GABA<sub>B</sub> receptors and HCN channel in chronic cerebral hypoperfusion are poorly understood.

A brain-specific protein termed TPR containing Rab8b interacting protein (TRIP8b) interacts through a conserved tripeptide sequence in the COOH terminus of HCN channels [31], which is thought to play an important role in the HCN channels trafficking [32]. TRIP8b colocalizes with HCN channels in cortical and hippocampal pyramidal cells, with more than ten isoforms expressed in the brain [33]. Based on quantitative real-time PCR analysis of brain tissue, TRIP8b (1a-4) and TRIP8b (1a) represent the two most prominently expressed isoforms (account for ~30–40 and ~25–30 % of total TRIP8b mRNA, respectively), with TRIP8b (1b-2) expressed at somewhat lower levels (~10–15 % of total TRIP8b mRNA) [34]. Functional coexpression of TRIP8b with HCN protein, either in native cells or heterologous systems, results in a strong up or downregulation of HCN channels in the plasma membrane. TRIP8b (1a) prevents mislocalization of HCN1 in the axons of CA1 pyramidal neurons [33], and its physiological functions in HCN2 remain unclear. Indeed, whereas TRIP8b (1a-4) strongly increases HCN1 surface expression [34, 35], this isoform decreases the surface expression of HCN2 [36]. A study has reported that TRIP8b (1b-2) produces a potent downregulation in the surface expression of HCN1 and HCN2 [31]. In the present study, we investigated the functional role of TRIP8b (1a-4) and TRIP8b (1b-2) in chronic cerebral hypoperfusion. Studies have reported that subunits of adaptor protein 2 (AP2)

complex appeared to be the major TRIP8b-interacting proteins [37, 38]. AP2 is a member of the family of adaptor proteins (today, comprising AP1, AP2, AP3, and AP4) and is composed of four subunits, which are termed  $\alpha$ ,  $\beta$ 2,  $\mu$ 2, and  $\sigma$ 2, respectively [39]. A recent study has been reported that AP2 may function as two partially independent hemicomplexes ( $\alpha$  and  $\mu$ 2 subunits) [40]. Adaptor-associated kinase 1 (AAK1), a member of the Prk/Ark family of serine/threonine kinases, can decrease AP2-stimulated transferrin internalization via phosphorylating the AP2  $\mu$ 2 subunit [41]. Besides, activation of protein kinase A (PKA) promotes the stabilization of GABA<sub>B</sub> receptors, presumably by protecting the receptors from proteolysis at or near the cell surface [42]. Until now, the role of these regulatory factors in chronic cerebral hypoperfusion is unclear.

In the present study, a widely accepted model of chronic cerebral hypoperfusion induced by permanent occlusion of bilateral common carotid arteries (two-vessel occlusion, 2VO) in rats was used to evaluate whether and how HCN channels were pathologically altered in the hippocampal CA1 area. We also investigated the neuroprotective effects of baclofen and cross-talk networks between GABA<sub>B</sub> receptors and HCN channels in the hippocampus of 2VO rats.

## Materials and Methods

### Animals

Adult male Sprague–Dawley rats of clean grade (approval number SCXK(E)2010-0007, No. 00008512), aged 2–3 months, weighing 220–250 g, were provided from the Experimental Animal Center, Tongji Medical College, Huazhong University of Science and Technology. Animals were group-housed with free access to water and food with a 12-h light/dark cycle and a thermoregulated environment and adapted to these conditions for at least 7 days before experiments. All experiments were approved by the Review Committee for the Care and Use of Laboratory Animals of Tongji Medical College, Huazhong University of Science and Technology. All efforts were made to minimize both the suffering and number of animals used.

### Experimental Design

The experimental design is detailed in Fig. 1a. The rats were anesthetized with chloral hydrate (350 mg/kg, intraperitoneal injection, i.p.). Bilateral common carotid arteries were exposed and freed from surrounding connective tissue through a midline cervical incision. Both common carotid arteries were ligated with 4-0 type surgical silk and cut between the double ligatures to induce cerebral ischemia. The same operation was carried out on the sham-operated rats except for

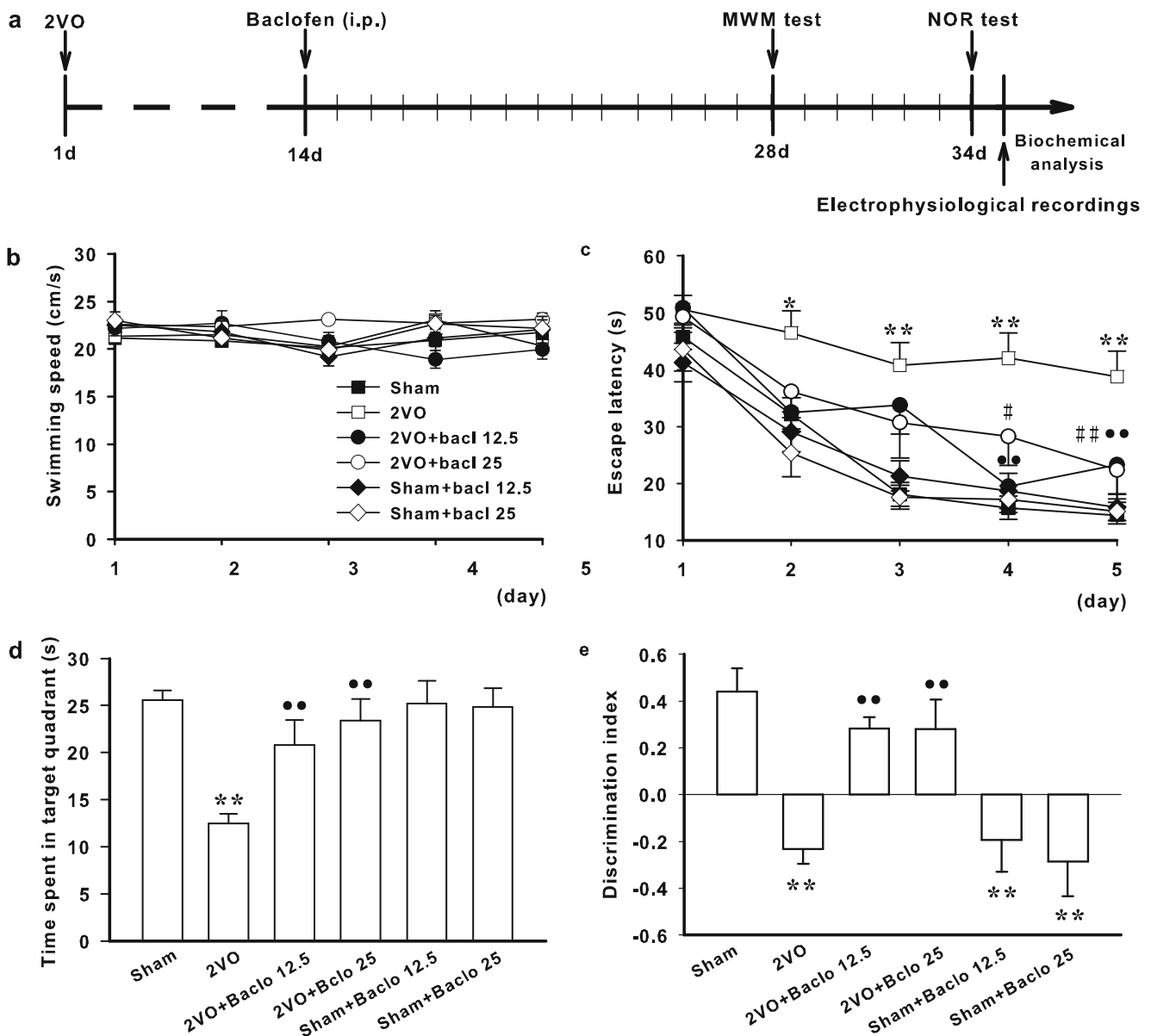
ligation and cutting of the carotids. During the surgery, their body temperature was monitored and maintained at  $37.5 \pm 0.5$  °C by means of a heating lamp until the rats recovered to thermal homeostasis. Two weeks after induction of hypoperfusion, the repeated drug treatment of 2VO animals was performed once daily at 20:00–21:00 during the last 21 days. Baclofen (Meryer Chemical Technology Co., Ltd, Shanghai, China) was dissolved in saline at concentration of 1.25 and 2.5 mg/ml [24–26]. Baclofen was administered by i.p. injection in a volume of 10 ml/kg. Sham-operated rats were given normal saline (NS), also in the same volume. Four weeks after induction of hypoperfusion, the Morris water maze (MWM) was utilized to evaluate the spatial learning and memory performances of rats. One day after the Morris water maze test, novel object recognition (NOR) test evaluated nonspatial working memory. Five weeks after 2VO, we recorded the field excitatory postsynaptic potential (fEPSP) and carried out biochemical studies as described below.

### Water Maze Task

The swimming pool named Morris water maze was a circular water tank of 150 cm in diameter and 50-cm deep. It was filled to a depth of 21 cm with water at  $23 \pm 2$  °C. A platform of 15 cm in diameter and 20 cm in height was placed inside the tank to hold the rats, the horizontal plane being 1.5 cm above the platform top. Many cues external to the maze were around the pool. Each rat received four trials everyday with an inter-trial interval of approximately 10 min. Animals were trained for five consecutive days. Latency to escape onto the hidden platform was recorded. On the 6th day, each rat was subjected to a 60-s probe trial, in which the platform was removed and quadrant dwell time in Q2 was recorded. Parameters of escape latency, swim speed provided information about the ability of learning and about the motor performance. The amount of time spent in the quadrant of the former platform position was one of the measures for the performance in spatial memory [43, 44]. We used a video camera linked to a computer-based image analyzer Morris water-maze tracking system MT-200 (Chengdu Technology and Market Co., Ltd, Chengdu, Sichuan province, China) to monitor swimming activities. Tests were carried out between 10:00 and 12:00 A.M.

### Novel Object Recognition Test [45–47]

The apparatus was formed by a gray-colored Plywood box (50×50×90 cm) with a grid floor that could be easily cleaned. The apparatus was illuminated by a 40-W lamp suspended 120 cm above the box. The objects to be discriminated were in three different shapes and colors: polyvinyl chloride cylinders (blue, 6-cm height), glass cubes (pink, 6-cm side), and tennis balls (yellow, 6.5–6.8-cm diameter). The day before testing, rats were allowed to explore the box for 5 min without objects.



**Fig. 1** Activation of GABA<sub>B</sub> receptors rescued the learning and memory deficits induced by 2VO. **a** Experimental design. **b** The average speed of rats from the 1st day to the 5th day. Swimming speed of each group did not differ significantly ( $n=10$  in sham-operated and 2VO groups,  $n=9$  in baclofen-treated groups). **c** The escape latencies to find the hidden platform from the 1st day to the 5th day. Latency to find the platform of 2VO rats was longer than sham-operated rats; after treatment of sham-operated rats with baclofen at 12.5 and 25 mg/kg, no changes in escape latencies compared to normal saline treated rats were observed. However, 2VO rats given baclofen at 12.5 and 25 mg/kg showed learning latency similar to that of sham-operated rats ( $n=10$  in sham-operated and 2VO groups,  $n=9$  in baclofen-treated groups). **d** The percentage of quadrant dwell time in

Q2 on the sixth training day. The sham-operated rats sojourned longer in Q2 than 2VO rats; treatment with baclofen at 12.5 and 25 mg/kg improved the 2VO-induced deficit of acquisition in the Morris water maze ( $n=10$  in sham-operated and 2VO groups,  $n=9$  in baclofen-treated groups). **e** Effects of baclofen on novel object recognition test. Rats with 2VO significantly reduced their discrimination ability; baclofen at 12.5 and 25 mg/kg significantly ameliorated 2VO-induced cognitive impairment; and treatment with baclofen at 12. and 25 mg/kg in sham-operated rats resulted in a significant decrease in discrimination index ( $n=10$  in sham-operated and 2VO groups,  $n=9$  in baclofen-treated groups). \* $P<0.05$  and \*\* $P<0.01$  vs sham-operated rats; \* $P<0.01$  vs 2VO rats; # $P<0.05$  and ## $P<0.01$  vs 2VO rats

On the day of the test, a session of two trials was given. The inter-trial interval was 60 min. In the first trial, two objects, cylinders and glass cubes, were presented in two opposite corners of the box, and the amount of time taken by each rat to complete 20 s of object exploration was recorded.

Exploration was considered directing the nose at a distance  $\leq 2$  cm from the object and/or touching it with the nose. During the second trial, one of the familiar objects, cylinder, was replaced by a novel object, tennis ball, and the rats were left in the box for 5 min. The time spent for the exploration of the



familiar (*F*) and the novel (*N*) object was recorded separately. In normal rats, the time spent to explore a novel object is significantly higher than that spent to explore a familiar one. A discrimination index that normalized the data excluding the individual variability was calculated ( $(N-F)/(N+F)$ ) to compare different groups. Care was taken to avoid place preference and olfactory stimuli by randomly changing the position of the two objects (familiar and novel object) during the second trial and cleaning them carefully.

#### Electrophysiological Recordings In Vivo

Extracellular recordings were described in detail previously [48]. Rats in each group were anesthetized with urethane (1.5 g/kg, i.p.), and the body temperature was maintained at 37 °C via a constant temperature water cycling system. The head was mounted in a stereotaxic frame (SN-3, Narishige, Japan), and both the skin and fascia were retracted to expose the skull under sterile conditions. The tissue was kept moist with gauze moistened with sterile saline throughout the surgical procedures. The ground electrode was inserted into subcutaneous tissue of the hind limb, and the reference electrode was placed on the scalp. A concentric bipolar stimulating electrode was placed in the Schaffer collaterals, which arose from hippocampal CA3 neurons and terminated on more proximal CA1 dendrites in stratum radiatum (SR) (3.5–4.0 mm posterior to bregma, 3.1–3.5 mm lateral to midline, and 2.2–2.5 mm ventral to dura). Recording electrode was positioned under stereotactic guidance in the CA1 stratum radiatum (3.3–3.6 mm posterior to bregma, 2.2–2.5 mm lateral to midline, 1.8–2.2 mm ventral to dura). There was a 30-min rest period after electrodes insertion. Stimulation was achieved with pulses of 0.15-ms duration delivered at 0.3 Hz (30 s intervals); evoked field responses were acquired, amplified, monitored, and analyzed with RM6240BD biology signal processing system (Chengdu Instrument Factory, China). For recordings of the slope of the fEPSP, the stimulation intensity for the tests pulses was set to elicit a response for which the fEPSP slope was 50–60 % of the maximum response so as to allow increases or decreases of the fEPSP slope to be detected. Long-term potentiation (LTP) was induced by high-frequency stimulation (HFS, four trains of 100 Hz, 1-s stimulation separated by 5 min at a stimulus intensity that evoked a fEPSP of approximately 80 % of maximum response). After HFS, the slope of fEPSP was recorded for at least 60 min. The percentage of the ratio of absolute fEPSP slope to baseline value was used to represent the fEPSP slope level. It was defined as a successful induction of LTP if the amplitude of fEPSP change exceeded 20 % [49]. After the experiment, correct placement of the electrodes was confirmed by histology.

#### Immunohistochemistry

Rats in each group were deeply anesthetized. Following the perfusion of rats by intracardiac injection of 0.9 % saline solution, followed by 4 % paraformaldehyde (PFA) in 0.1 M phosphate buffer (PB). After perfusion, brains were harvested and postfixed in PFA overnight. After fixation and dehydration with gradient ethanol, the tissues were embedded in paraffin. Five-micrometer sections were cut, and the slides were deparaffinized in xylene and then dehydrated by incubation in graded ethanol solutions. Slides were boiled in sodium citrate buffer for 20 min for antigen retrieval and then incubated sequentially with 3 % H<sub>2</sub>O<sub>2</sub> in PBS for 30 min and 5 % bovine serum albumin (BSA) for 1 h. The primary antibodies used were rabbit anti-mouse NeuN (1:400, MAB377, Millipore). The slides were incubated with the indicated antibody overnight at 4 °C, followed by sequential incubation with the secondary antibody, DyLight 488 Affinipure Goat Anti-Mouse IgG (H+L) (E032210, EarthOx, LLC) for 2 h. The sections were analyzed with Olympus FluoView 1200 confocal microscope system (Olympus Corporation, Japan), and photomicrographs of representative fields were taken. Image analysis was then performed using analySIS software (analySIS 3.0; Soft Imaging System) [50].

#### Golgi Silver Staining

Rats in each group were deeply anesthetized with urethane and perfused intracardially with 0.9 % saline solution. The brains were removed and stained by the modified Golgi-Cox method [51, 52], and then stored for 14 days in Golgi-Cox solution, followed by 3 days in 30 % sucrose solution. Coronal sections of 50- $\mu$ m thickness of regions to be studied were obtained using a vibratome (Camden Instrument, MA752, Leicester, UK). Sections were then treated with ammonium hydroxide for 30 min, followed by 30 min in Kodak Film Fixer (Eastman Kodak Company, Rochester, NY, USA) and finally rinsed with distilled water, dehydrated, and mounted with 90 % glycerol. Golgi-impregnated pyramidal neurons in the CA1 subfield of hippocampus were studied. Spine density calculation was performed as follows: a length of dendrite (at least  $\geq 10$ - $\mu$ m long) was traced (at  $\times 1,000$ ), the exact length of the dendritic segment was calculated, and the number of spines along the length counted (to yield spines/10  $\mu$ m).

#### Western Blotting Analysis

Rats in each group were killed by decapitation under anesthesia. Brains rapidly removed and plunged into ice-cold artificial cerebrospinal fluid (ACSF). The cutting ACSF solution contained (mM) 125 NaCl, 3 KCl, 6 MgCl<sub>2</sub>, 1 CaCl<sub>2</sub>, 1.25 NaH<sub>2</sub>PO<sub>4</sub>, 25 NaHCO<sub>3</sub>, and 10.6 glucose, and was saturated with 95 % O<sub>2</sub>/5 % CO<sub>2</sub>. Coronal slices (400  $\mu$ m) of brain

tissue through the hippocampus were cut and subdivided into CA1, CA3, and dentate gyrus (DG) regions under a dissecting microscope (as shown in Fig. 2b) [53]. Samples were stored at  $-80^{\circ}\text{C}$  until required. Membrane protein extracts were prepared using a ProteoExtract Native Membrane Protein Extraction kit (71772–3, Calbiochem/Merck Biosciences). Protein concentration was determined using a BCA Protein Assay kit (Pierce). Equal amounts of protein samples (80  $\mu\text{g}$ ) were separated by 10 % SDS/PAGE gel electrophoresis and then transferred to Polyvinylidene difluoride (PVDF) membranes (03010040001, Roche). After blocking with 5 % nonfat milk powder in tris-buffered saline containing 0.1 % Tween-20 (TBST) for 1 h at room temperature, transferred membranes were incubated overnight at  $4^{\circ}\text{C}$  with primary antibodies to anti-neuronal nuclear antigen (NeuN) (1:2,000, MAB377, Millipore), anti-GABA<sub>B</sub> R1 (1:1,000, AF7000, R&D systems), anti-GABA<sub>B</sub> R2 (1:200, MABN488, Millipore), anti-HCN1 (1:800, NBP1-20250, Novus), anti-HCN2 (1:200, APC-030, Alomone labs), anti-TRIP8b (1a-4) (1:500, NeuroMab clone N212/3), anti-TRIP8b (1b-2) (1:200, NeuroMab clone N212A/34), PKA  $\alpha/\beta/\gamma$  (H-56) (1:100, sc-98951, Santa Cruz), anti-phospho-PKA (Thr197) (1:500, 4781, Cell Signaling), anti-AAK1 (H-140) (1:200, sc-134662, Santa Cruz), anti-adaptin 2 (M-16) (1:400, sc-6422, Santa Cruz), and p-AP $\mu$ 2 (pT156) (1:4,000, 3312–1, Epitomics) or GAPDH (1:5,000, cw0100, Cwbiochem). The antigen-antibody complexes were visualized with goat anti-rabbit, goat anti-mouse, mouse anti-goat or rabbit anti-sheep horseradish peroxidase (HRP)-conjugated secondary antibodies (1:5,000; Proteintech Group Inc, china) by using immobilized Western chemiluminescent HRP substrate (WBKLS0500, Millipore). The optical density of the bands was determined using NIH ImageJ software. The results were normalized to the quantity of GAPDH in each sample lane. All assays were performed at least three times.

#### Statistical Analysis

All analyses were performed using SPSS 16.0 software (SPSS Inc., USA) and data are presented as mean  $\pm$  SEM. Differences between mean values were evaluated using one- or two-way analysis of variance (ANOVA), as appropriate. The *t* test is used for testing differences between two groups.  $P < 0.05$  was considered statistically significant.

## Results

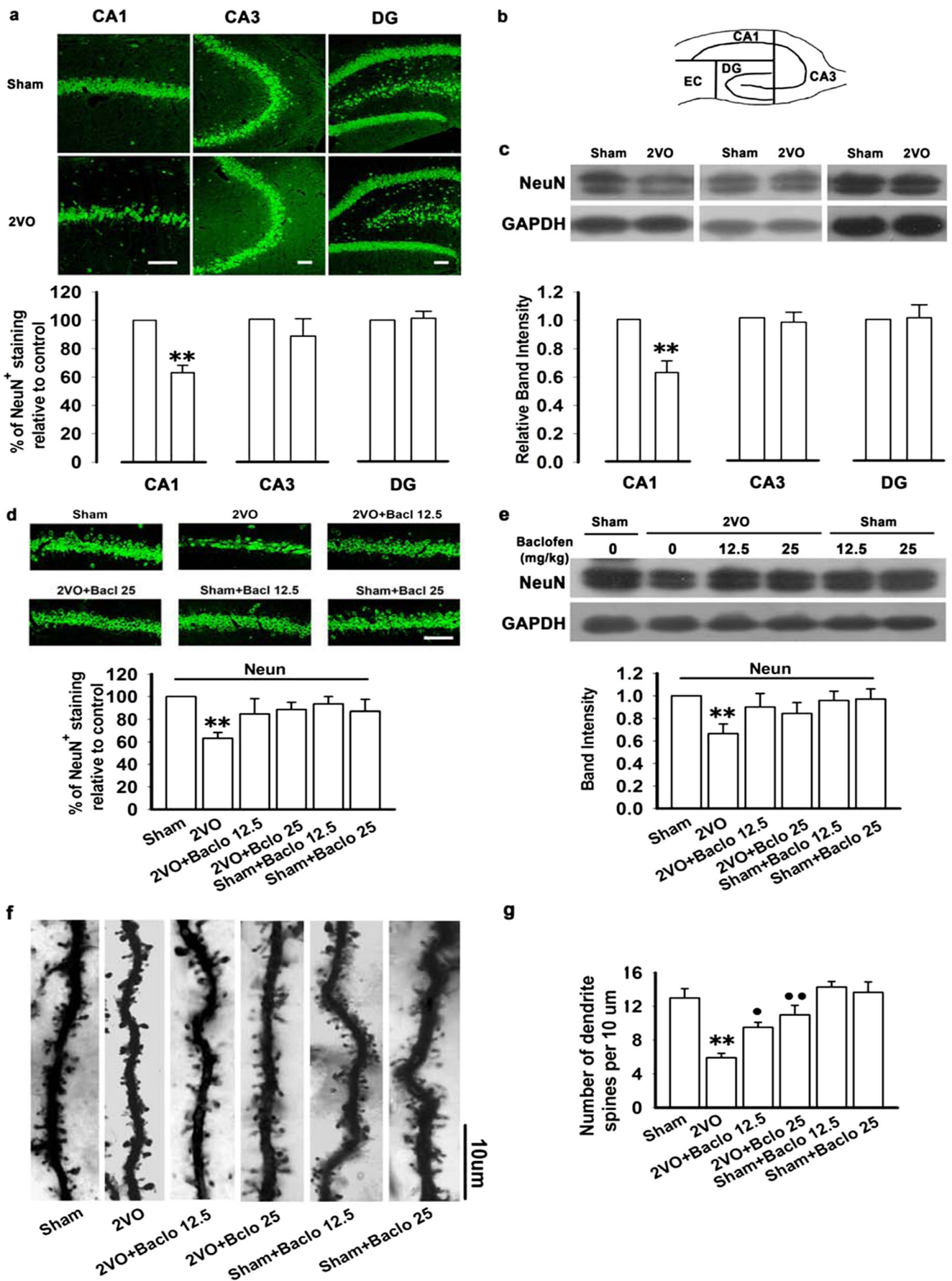
Activation of GABA<sub>B</sub> receptors reversed the learning and memory deficits induced by 2VO.

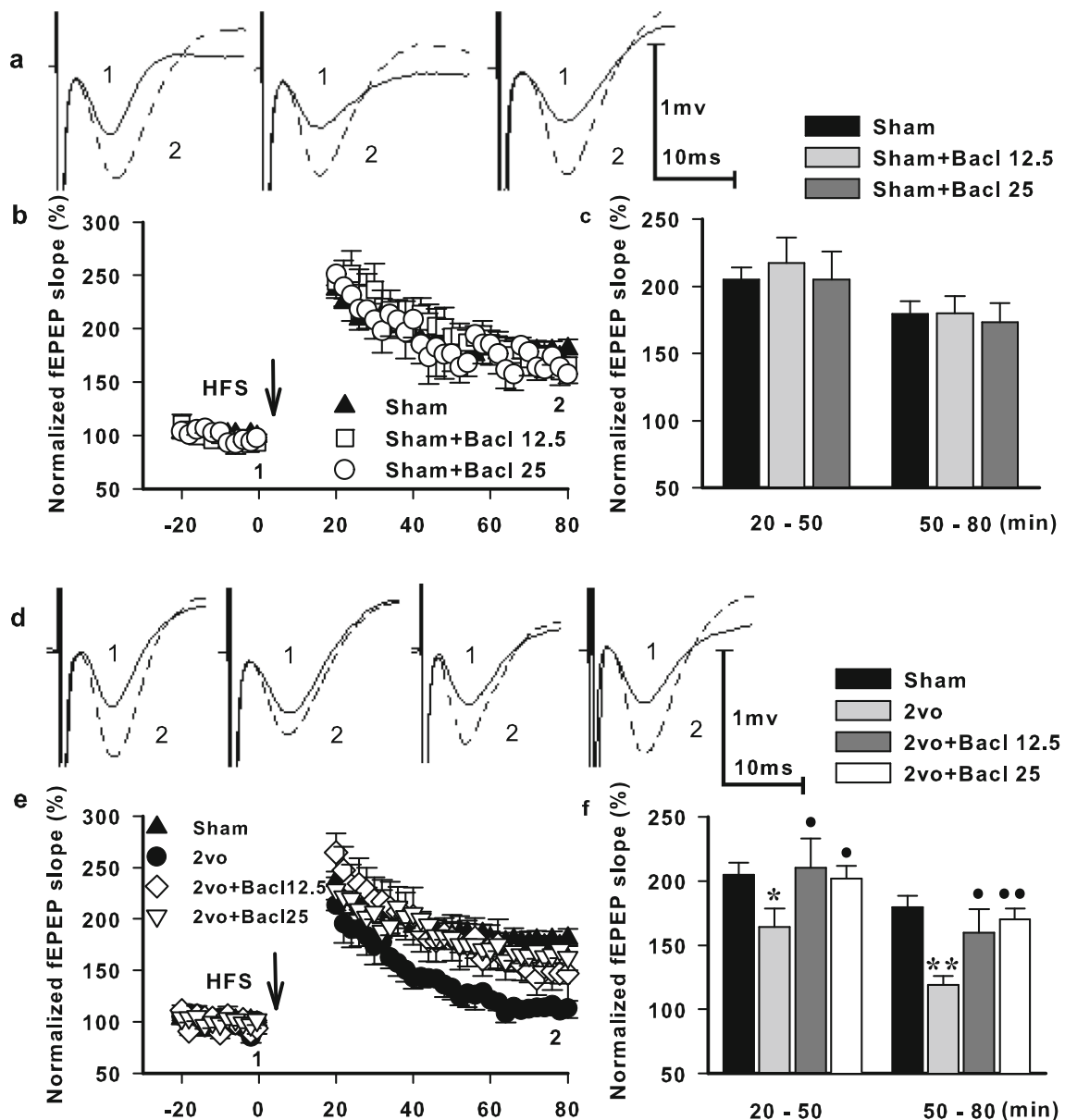
To investigate the potential protective effects against chronic cerebral hypoperfusion injury, we first examined the effect of baclofen on the water maze task for five consecutive days.

**Fig. 2** Baclofen improved neuronal survival and dendritic spine density in rat hippocampal CA1 area after 5 weeks of 2VO. **a** Representative photomicrographs of immunohistochemical staining with an anti-NeuN antibody in the hippocampal CA1 ( $\times 400$ , scale bar, 100  $\mu\text{m}$ ), CA3 ( $\times 200$ , scale bar, 100  $\mu\text{m}$ ), and DG ( $\times 200$ , scale bar, 100  $\mu\text{m}$ ) areas and quantitative analysis of neuron density, which was performed by NeuN staining. Significant neuronal loss was observed in hippocampal CA1 area; however, the CA3 and DG exhibited no detectable cell loss ( $n=4$  in each group). **b** Schematic illustration of sectioning of hippocampus technique for regional analysis, which is based upon Fig. 3 of K. L. Powell et al. [53]. **c** Western blotting showed that baclofen treatment reversed the 2VO-induced reduction of NeuN protein in hippocampal CA1 area, but not in CA3 and DG area ( $n=5$  in each group). **d** Representative photomicrographs of immunohistochemical staining with an anti-NeuN antibody in the hippocampal CA1 of each group ( $\times 400$ , scale bar, 100  $\mu\text{m}$ ) and quantitative analysis of neuron density, which was performed by NeuN staining ( $n=4$  in each group). **e** Treatment with baclofen at 12.5 and 25 mg/kg markedly diminished the reduction of NeuN protein of hippocampal CA1 area ( $n=5$  in each group). **f** Representative photomicrographs of Golgi staining from each group. **g** Summary data showed that the decrease in the spine density induced by 2VO was partly reversed by baclofen ( $n=4$  in each group). \*\* $P < 0.01$  vs sham-operated rats; \* $P < 0.05$  and \*\* $P < 0.01$  vs 2VO rats

As shown in Fig. 1b, swimming speed of each group did not differ significantly. Latency to find the platform of 2VO rats was longer than sham-operated rats (Fig. 1c). It demonstrated that 2VO rats usually spent much more time to locate the platform than sham-operated rats, which found the platform in an appropriate path. After treatment of sham-operated rats with baclofen at 12.5 and 25 mg/kg, no changes in swim speed and escape latencies compared with normal saline-treated rats were observed. However, 2VO rats given baclofen at 12.5 and 25 mg/kg showed learning latency similar to that of sham-operated rats (Fig. 1c). On the sixth training day, the retention experiment with the platform removed was carried out. The percentage of quadrant dwell time in Q2 was adopted to evaluate the retention performance. The sham-operated rats sojourned longer in Q2 than 2VO rats (sham-operated rats  $25.53 \pm 1.08\%$ , 2VO rats  $12.49 \pm 1.03\%$ , Fig. 1d). Treatment with baclofen at 12.5 and 25 mg/kg improved the 2VO-induced deficit of acquisition in the Morris water maze ( $20.80 \pm 2.65$  and  $23.38 \pm 2.31\%$ , respectively, Fig. 1d).

Next, in order to evaluate nonspatial learning and memory, novel object recognition test was performed. Rats with 2VO significantly reduced their discrimination ability (discrimination index,  $-0.23 \pm 0.06$  vs.  $0.44 \pm 0.09$  compared with sham-operated rats); baclofen at 12.5 and 25 mg/kg significantly ameliorated 2VO-induced cognitive impairment ( $0.28 \pm 0.05$ ,  $0.27 \pm 0.13$ , respectively); and treatment with baclofen at 12.5 and 25 mg/kg in sham-operated rats resulted in a significant decrease in discrimination index ( $-0.19 \pm 0.14$ ,  $-0.29 \pm 0.15$ , respectively) (Fig. 1e). Our results showed that even though the GABA<sub>B</sub> receptor agonist baclofen itself reduced discrimination ability of sham-operated rats, it improved the





**Fig. 3** Baclofen partially reversed the 2VO-induced inhibitory action on LTP at the Schaffer collateral-CA1 synapses in rats in vivo. **a, d** Representative fEPSP recorded before (*solid line*) and after HFS (*dashed line*). The *downward-filled arrow* indicates HFS. **b** Treatment with baclofen at 12.5 and 25 mg/kg in sham-operated rats had no significant effect on LTP ( $n=5$  in each group). **d** LTP was significantly inhibited in 2VO rats at the

Schaffer collateral-CA1 synapses; administration of 12.5 and 25 mg/kg baclofen partially reversed the 2VO-induced inhibitory action on LTP ( $n=5$  in each group). **c, d** The change of average fEPSP slopes 30–50 and 50–80 min after HFS in different groups. \* $P<0.05$  and \*\* $P<0.01$  vs sham-operated rats; \* $P<0.05$  and \*\* $P<0.01$  vs 2VO rats; \* $P<0.05$  and \*\* $P<0.01$  vs 2VO rats

2VO-induced reduction of exploratory preference to the novel object in 2VO rats.

Baclofen partially reversed the 2VO-induced inhibitory action on LTP at the Schaffer collateral-CA1 synapses in rats in vivo.

To determine the electrophysiological basis for the changes of cognitive functions, we recorded the LTP in Schaffer collateral synapses on CA1 pyramidal cells. Long-lasting significant enhancement appears in the slope of fEPSP induced by HFS (0–

30 min after HFS,  $204.78\pm 9.25$  % of baseline values; 30–60 min,  $179.43\pm 9.30$  % of baseline values), and treatment with baclofen at 12.5 and 25 mg/kg in sham-operated rats had no significant effect on LTP (Fig. 3a–c). As shown in Fig. 3d, LTP was significantly inhibited in 2VO rats (0–30 min after HFS,  $164.29\pm 14.32$  % of baseline values; 30–60 min,  $118.92\pm 7.31$  % of baseline values). Administration of 12.5 and 25 mg/kg baclofen partially reversed the 2VO-induced inhibitory action on LTP (30–50 min after HFS,  $210.38\pm 22.84$  and  $201.92\pm 10.00$  % of



baseline values, respectively; 50–80 min,  $159.76 \pm 18.09$  and  $170.17 \pm 8.23$  % of baseline values, respectively) (Fig. 3d–f).

Baclofen improved neuronal survival and dendritic spine density in rat hippocampal CA1 area after 5 weeks of 2VO.

The most obvious signs of neurodegeneration are the loss of neuronal cell bodies and synaptic contacts. Immunohistochemical staining with an anti-NeuN antibody was performed to assess loss of neurons in hippocampus after 5 weeks of 2VO. Significant neuronal loss was observed in hippocampal CA1 area; however, the CA3 and DG exhibited no detectable cell loss (CA1,  $\times 400$ ; CA3,  $\times 200$ ; DG,  $\times 200$ ; Fig. 2a). Correspondingly, as shown in Fig. 2c, the protein levels of NeuN were significantly decreased in hippocampal CA1 area, but no significant change in hippocampal CA3 and DG areas. Treatment with baclofen at 12.5 and 25 mg/kg markedly diminished the neuronal loss of hippocampal CA1 area (Fig. 2d–e). Besides, our results revealed that the dendritic spine density was markedly decreased in 2VO rats compared with sham-operated rats, and baclofen reversed the reduction of spine density (Fig. 2f–g).

Chronic GABA<sub>B</sub> receptors agonist exposure results in a specific loss of cell surface GABA<sub>B</sub> receptors in the hippocampal CA1 area of sham-operated rats, but not in 2VO rats.

In this study, we identified the surface expression of GABA<sub>B</sub> R1 and GABA<sub>B</sub> R2 in the hippocampal CA1 area with Western blot analyses. No contamination with cytosolic protein was observed as GAPDH was not seen by Western blot in these samples (data not shown). Our results showed that, 5 weeks after induction of hypoperfusion, the surface expression of GABA<sub>B</sub> R1 and GABA<sub>B</sub> R2 was significantly decreased and intracellular expression of GABA<sub>B</sub> R1 was significantly increased; by contrast, intracellular expression of GABA<sub>B</sub> R2 was not significantly changed, and treatment of sham operated rats with baclofen accelerated the decrease in the population of surface GABA<sub>B</sub> R1 and GABA<sub>B</sub> R2 and the increase in the population of intracellular GABA<sub>B</sub> R1 and GABA<sub>B</sub> R2 (Fig. 4a–b). But, our data revealed that baclofen could attenuate 2VO-induced reduction of GABA<sub>B</sub> R1 and GABA<sub>B</sub> R2 surface expression (Fig. 4a–b). In order to further investigate possible mechanism for modulate GABA<sub>B</sub> receptor cell surface stability under chronic cerebral hypoperfusion, we examined the expression of PKA  $\alpha/\beta/\gamma$  and p-PKA  $\alpha/\beta/\gamma$  (the active form of PKA  $\alpha/\beta/\gamma$ ) in the hippocampal CA1 area. PKA  $\alpha/\beta/\gamma$  levels remained unchanged among all treatment groups. Five weeks after induction of hypoperfusion, the expression of p-PKA  $\alpha/\beta/\gamma$  was not significantly changed, but chronic treatment with baclofen significantly increased p-PKA  $\alpha/\beta/\gamma$  (Fig. 4c).

Activation of GABA<sub>B</sub> receptors restored the balance of HCN1/HCN2 surface expression in the hippocampal CA1 area in 2VO rats.

Next, we evaluated the interaction between GABA<sub>B</sub> receptors and HCN channels under chronic cerebral hypoperfusion.

We observed only a slight reduction of the surface expression of HCN1 and HCN2 in sham-operated rats which were treated with baclofen (25 mg/kg) (Fig. 5a); 5 weeks after induction of hypoperfusion, the surface expression of HCN1 was significantly reduced and intracellular expression of HCN1 was significantly increased in the hippocampal CA1 region (Fig. 5a); and however, hypoperfusion resulted in a significantly increase in HCN2 surface expression of hippocampal CA1 cells (Fig. 5b), but the intracellular HCN2 had no obvious change. Chronic treatment with baclofen significantly increased HCN1 surface expression, but reduced HCN2 surface expression; correspondingly, intracellular expression of HCN1 was reduced and that of HCN2 was increased (Fig. 5a–b). Immunoblotting of membrane protein extracts demonstrated no reactivity with anti-GAPDH antibodies, excluding the possibility of cross-contamination by cytoplasmic fractions (data not shown).

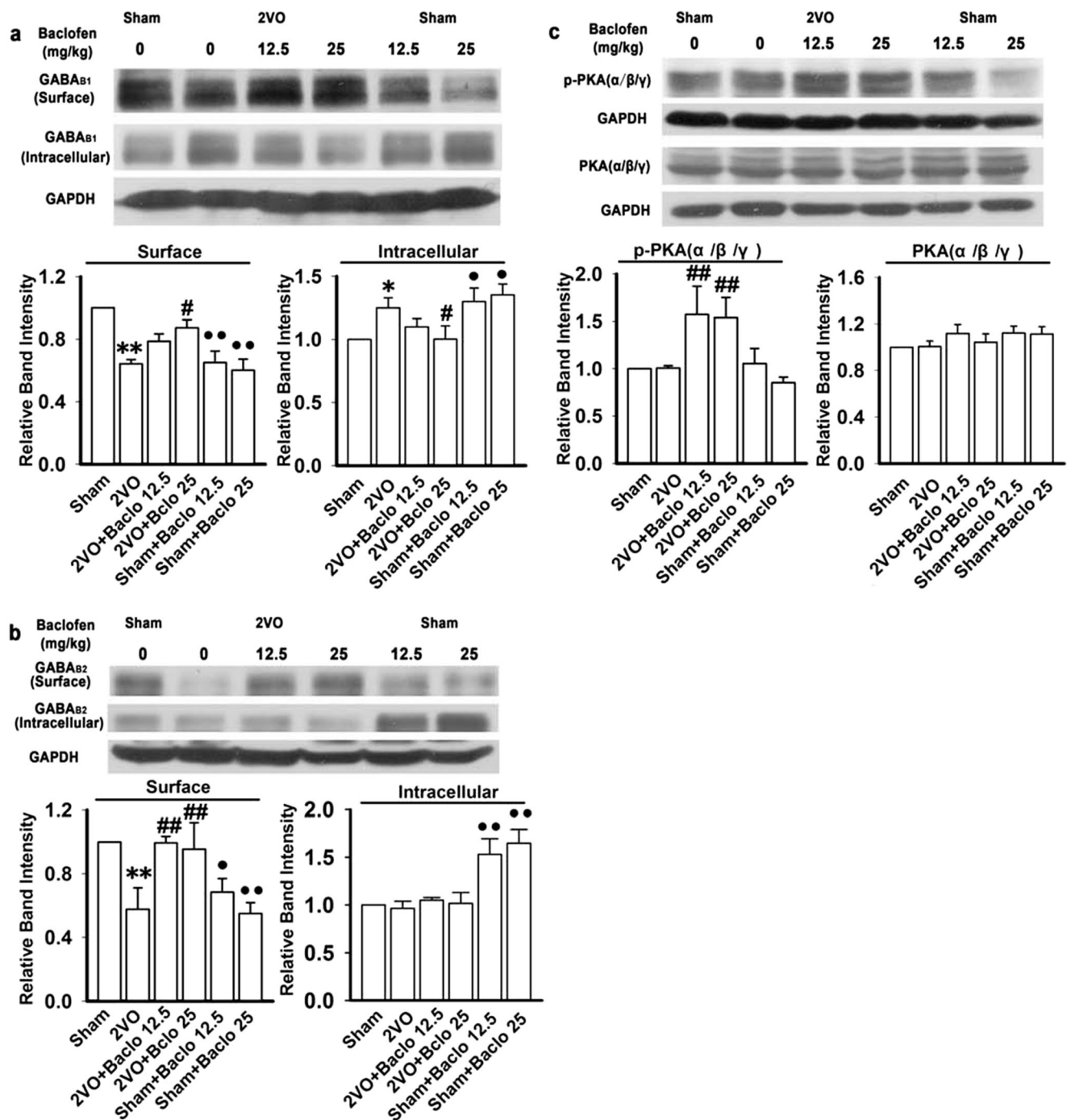
Activation of GABA<sub>B</sub> receptors could not only increase the expression of TRIP8b (1a-4) and TRIP8b (1b-2) but also regulate the function of TRIP8b via AAK1 and AP2  $\mu 2$ .

We further explored the possible mechanisms of association between GABA<sub>B</sub> receptors and HCN channel in chronic cerebral hypoperfusion. The results showed that TRIP8b (1a-4) and TRIP8b (1b-2) protein levels were significantly reduced in the hippocampal CA1 region in 2VO rats. The expression of TRIP8b (1a-4) and TRIP8b (1b-2) were increased in baclofen-treated rats, especially in 2VO rats (Fig. 6a). AP2  $\alpha$  levels remained unchanged among all treatment groups (Fig. 6b); the expression of p-AP  $\mu 2$  was not significantly changed in 2VO rats, but chronic treatment with baclofen significantly increased p-AP  $\mu 2$  (Fig. 6b). Under chronic cerebral hypoperfusion, AAK1 levels were significantly reduced in the hippocampal CA1 region; baclofen restored the hippocampal AAK1 expression to the control level (Fig. 6c).

## Discussion

In the present study, we have demonstrated for the first time that activation of GABA<sub>B</sub> receptors has neuroprotective effects in chronic cerebral hypoperfusion by attenuating the decrease of surface expression of GABA<sub>B</sub> R1 and GABA<sub>B</sub> R2 and restoring the balance of HCN1/HCN2 surface expression.

Our results indicated that chronic administration of GABA<sub>B</sub> receptors agonist baclofen markedly alleviated the 2VO-induced deficits in the Morris water maze. Although some reports have demonstrated that baclofen injected 15–90 min prior to behavioral testing impairs spatial learning of normal animals [54–57], we did not observe any influence of chronic treatment with baclofen on the acquisition in the Morris water maze test in sham operated rats. We may suggest

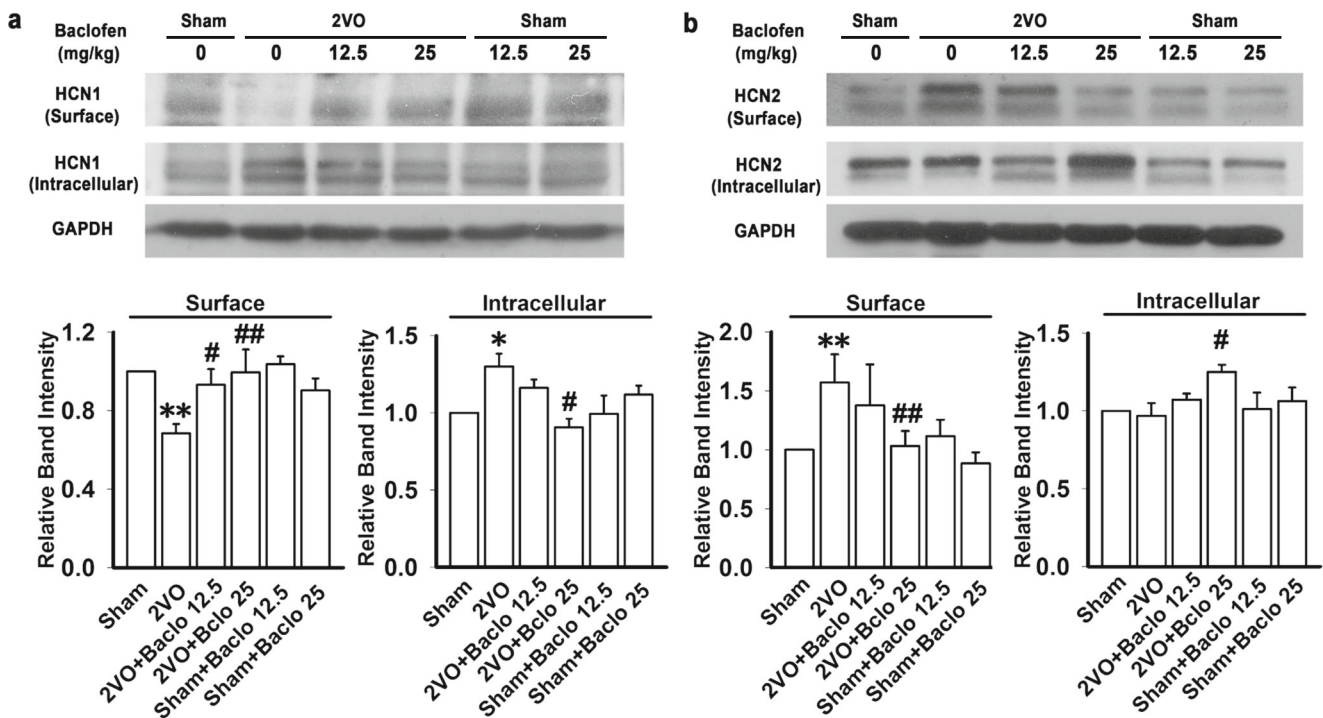


**Fig. 4** Chronic GABA<sub>B</sub> receptors agonist exposure results in a specific loss of cell surface GABA<sub>B</sub> receptors in the hippocampal CA1 area of sham-operated rats, but not in 2VO rats. **a, b.** Five weeks after induction of hypoperfusion, the surface expression of GABA<sub>B</sub> R1 was significantly decreased, and intracellular expression of GABA<sub>B</sub> R1 was significantly increased; the surface expression of GABA<sub>B</sub> R1 and GABA<sub>B</sub> R2 was also significantly decreased, but intracellular expression of GABA<sub>B</sub> R2 was not significantly changed; baclofen could attenuate 2VO-induced reduction of membrane GABA<sub>B</sub> R1 and GABA<sub>B</sub> R2 expression; treatment of

sham operated rats with baclofen dose-dependently accelerated the decrease in the population of surface GABA<sub>B</sub> receptors and the increase in the population of intracellular GABA<sub>B</sub> receptors ( $n=5$  in each group); **c** PKA  $\alpha/\beta/\gamma$  levels remained unchanged among all treatment groups; and 5 weeks after induction of hypoperfusion, the expression of p-PKA  $\alpha/\beta/\gamma$  was not significantly changed, but chronic treatment with baclofen significantly increased p-PKA  $\alpha/\beta/\gamma$  ( $n=5$  in each group). \* $P<0.05$  and \*\* $P<0.01$  vs sham-operated rats; # $P<0.05$  and ## $P<0.01$  vs 2VO rats; \* $P<0.05$  and \*\* $P<0.01$  vs sham-operated rats

that the influence of baclofen on the acquisition process in the Morris maze depends also on the time of administration. In a

nonspatial learning paradigm, our results showed that discrimination ability of SD rats was significantly reduced after



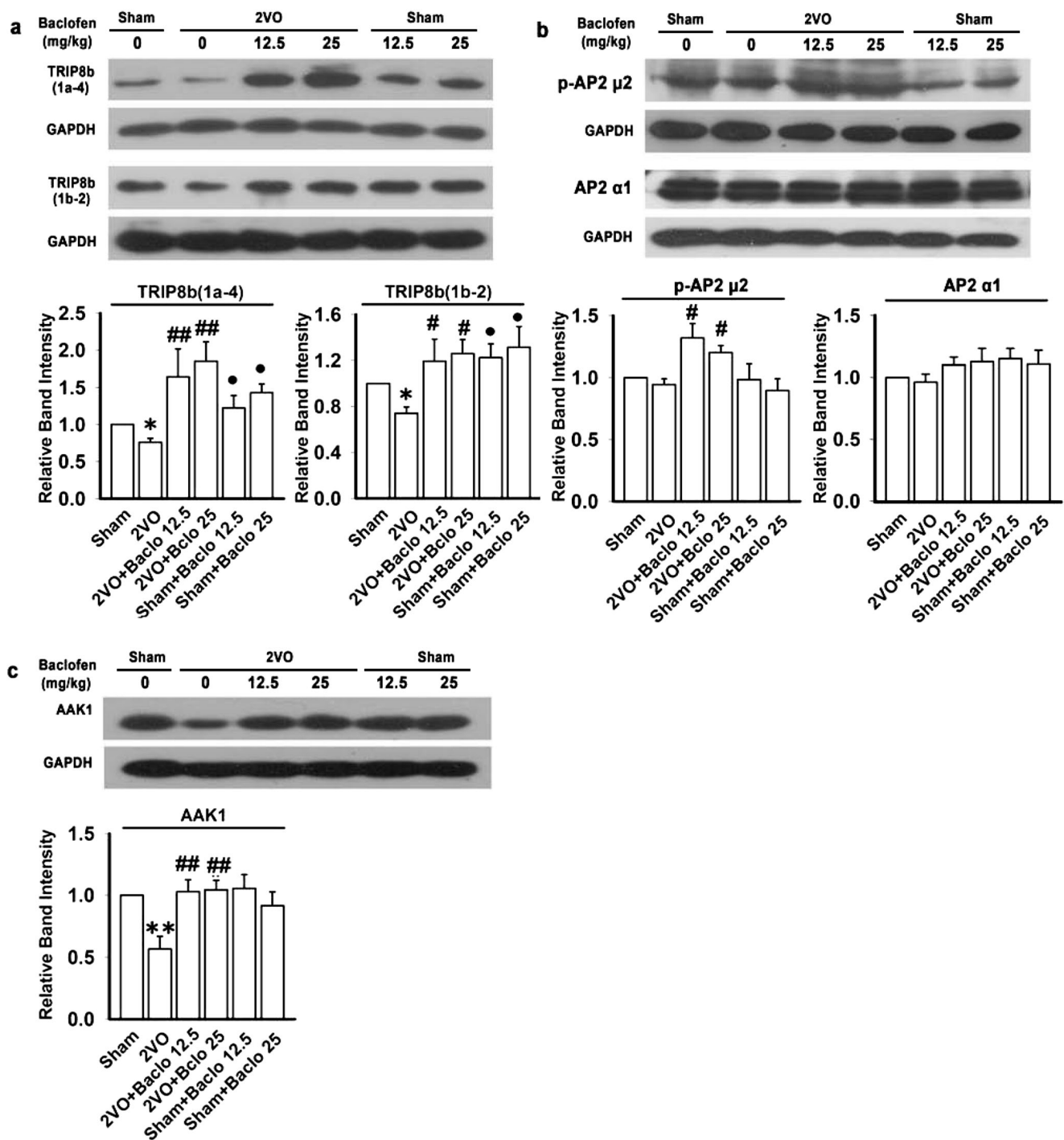
**Fig. 5** Activation of GABA<sub>B</sub> receptors restored the balance of HCN1/HCN2 surface expression in the hippocampal CA1 area in 2VO rats. **a, b** The surface expression of HCN1 was significantly reduced, and intracellular expression of HCN1 was significantly increased in the hippocampal CA1 region 5 weeks after induction of hypoperfusion; hypoperfusion significantly increased HCN2 in the surface of hippocampal CA1 cells but not in the intracellular of it; chronic treatment with

baclofen significantly increased surface expression of HCN1, but reduced surface expression of HCN2; correspondingly, intracellular expression of HCN1 was reduced and that of HCN2 was increased; and treatment of sham-operated rats with baclofen had no effect on the surface and intracellular expression of HCN1 and HCN2 ( $n=5$  in each group). \* $P<0.05$  and \*\* $P<0.01$  vs sham-operated rats; # $P<0.05$  and ## $P<0.01$  vs 2VO rats

5 weeks of 2VO. It has been reported that Wistar rats performed as well as the controls after 30 days of 2VO in the object recognition test [58]. The reason for this inconsistent result may be that the process of memory impairments induced by 2VO varies according to the strain of rat involved [59]. In the present study, we found that baclofen could reduce the discrimination ability of sham-operated rats in the object recognition, which might be associated with the nitroergic system [60] and the decrease in the population of surface GABA<sub>B</sub> receptors as described below. Nevertheless, baclofen improved the 2VO-induced deficit of acquisition the object recognition test.

2VO-induced, permanent neuronal damage was correlated with the memory failure [61]. Besides, dendritic spines play a crucial role in information transmission [62]. The morphology of dendritic spines is an important component of synaptic plasticity which is vital in learning and memory. Changes in dendritic spines have been shown to occur with synaptic plasticity and cognitive function [63]. In this study, chronic administration of baclofen attenuated chronic hypoperfusion-induced neuronal damage and the reduction of spine density. Furthermore, baclofen also reversed 2VO-induced suppression of LTP at the Schaffer collateral-CA1 synapse.

Changes in the GABA<sub>B</sub> receptor expression and function during hypoxia and ischemia play an important role in central nervous system damage [26, 29, 64]. It has been reported that baclofen stimulation may provide some level of neuroprotection against OGD-induced GABA<sub>B</sub> R2 degradation [29]. Our data revealed that baclofen could attenuate 2VO-induced reduction of GABA<sub>B</sub> R1 and GABA<sub>B</sub> R2 surface expression. However, treatment of sham-operated rats with baclofen accelerated the decrease in the population of surface GABA<sub>B</sub> receptors, which was in agreement with previous results [42]. It has been reported that the number of cell surface GABA<sub>B</sub> receptors is modulated by chronic agonist exposure via a mechanism that is independent of agonist-mediated phosphorylation and arrestin recruitment, and activation of PKA promotes the stabilization of GABA<sub>B</sub> receptors, presumably by protecting the receptors from proteolysis at or near the cell surface [42]. Our results showed that treatment with baclofen could increase the levels of p-PKA (Thr-197) under chronic cerebral hypoperfusion, which was not observed in sham operated rats. Together, our observations indicated that baclofen might indirectly enhance PKA activity, and then attenuated 2VO-induced reduction of GABA<sub>B</sub> R1 and GABA<sub>B</sub> R2 surface expression.



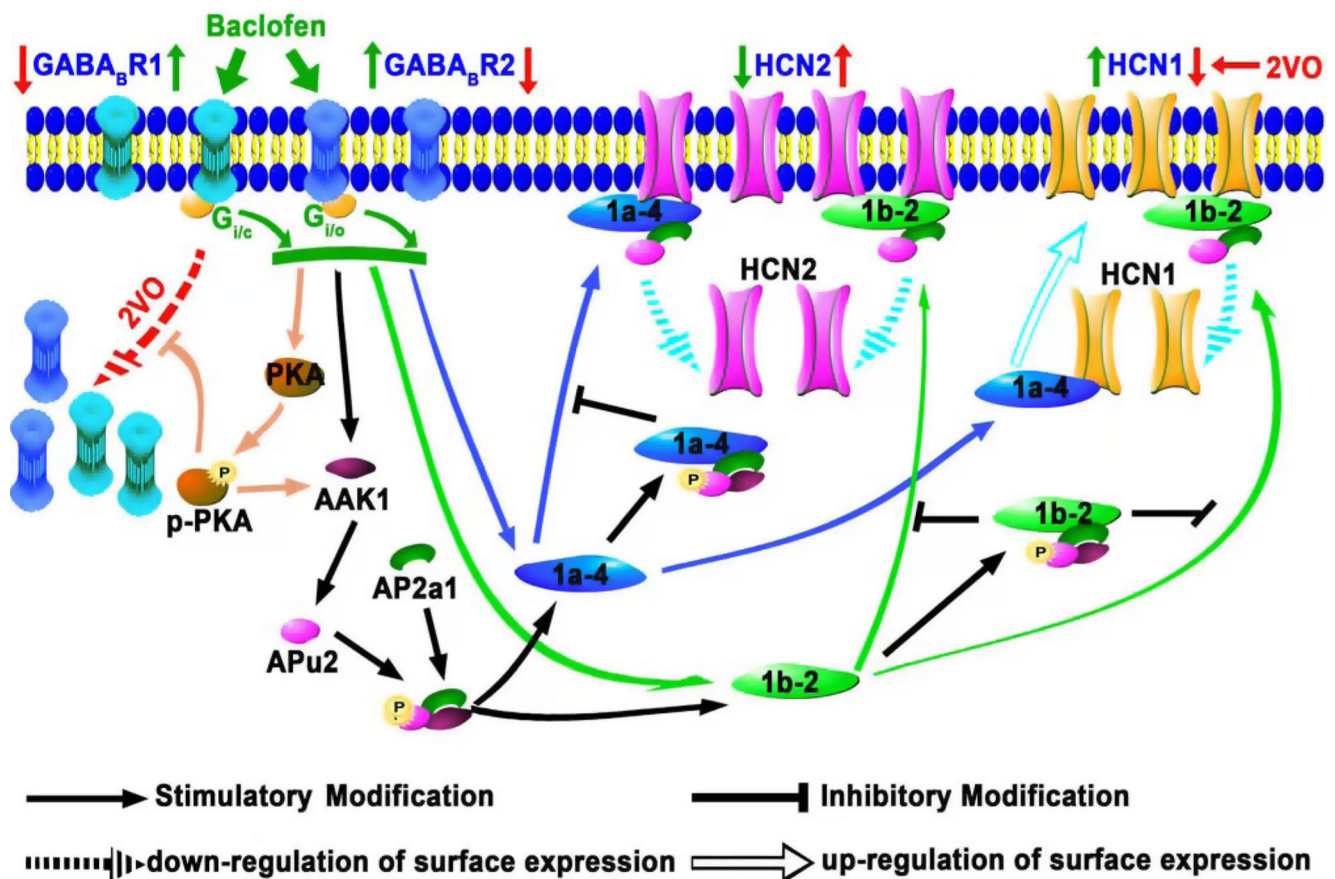
**Fig. 6** Activation of GABA<sub>B</sub> receptors could regulate not only the expression of TRIP8b (1a-4) and TRIP8b (1b-2) but also the function of TRIP8b via AAK1 and p-AP2  $\mu$ 2. **a** TRIP8b (1a-4) and TRIP8b (1b-2) protein levels were significantly reduced in the hippocampal CA1 region in 2VO rats; the expression of TRIP8b (1a-4) and TRIP8b (1b-2) were increased in baclofen-treated rats, especially in 2VO rats ( $n=5$  in each group). **b** The expression of p-AP  $\mu$ 2 was not significantly changed in

2VO rats, but chronic treatment with baclofen significantly increased p-AP  $\mu$ 2; and AP2  $\alpha$  levels remained unchanged among all treatment groups ( $n=6$  in each group). **c** AAK1 levels was significantly reduced in the hippocampal CA1 region, and baclofen restored the hippocampal AAK1 expression to the control level ( $n=5$  in each group). \* $P<0.05$  and \*\* $P<0.01$  vs sham-operated rats; # $P<0.05$  and ### $P<0.01$  vs 2VO rats; \* $P<0.05$  vs sham-operated rats

In hippocampus and cortical pyramidal neurons of mice, GABA<sub>A</sub> receptors and HCN1 channels are mutually regulated [16, 17]. But we did not observe a similar phenomenon

between GABA<sub>B</sub> receptors and HCN1/HCN2 channels in sham-operated rats: after treatment of sham operated rats with baclofen, the surface expression of GABA<sub>B</sub> R1 and GABA<sub>B</sub>





**Fig. 7** A mechanistic explanation for the co-regulation of GABA<sub>B</sub> receptors and HCN channels under chronic cerebral hypoperfusion. Five weeks after 2VO, hypoperfusion significantly reduced surface expression of GABA<sub>B</sub> R1, GABA<sub>B</sub> R2, and HCN1, but increased HCN2 surface expression (red arrow). Baclofen attenuated 2VO-induced reduction of surface expression of GABA<sub>B</sub> R1 and GABA<sub>B</sub> R2 via activation of PKA (orange arrow). Besides, baclofen increased the expression of TRIP8b (1a-4) (blue arrow) and TRIP8b (1b-2) (green arrow) in 2VO rats. TRIP8b (1a-4) strongly increases HCN1 surface expression, and TRIP8b (1b-2) produces a potent downregulation in the surface expression of HCN1. Baclofen can enhance PKA activity and restored the hippocampal

AAK1 expression to the control level under chronic cerebral hypoperfusion. Enhancement of PKA activity can promote the action of AAK1 on phosphorylation of AP2  $\mu$ 2 subunit (black arrow), which results in a decrease in TRIP8b (1b-2)-mediated HCN1 internalization. The combined effect of these on HCN1 channel is that activation of GABA<sub>B</sub> receptors attenuated the decrease of surface expression of HCN1 induced by chronic cerebral hypoperfusion. TRIP8b (1b-2) produces a potent downregulation in the surface expression of HCN2; however, TRIP8b (1a-4) also decreases the surface expression of HCN2. Increased phosphorylation of AP2  $\mu$ 2 subunit (black arrow) may prevent HCN2 surface expression from downregulating exceedingly

R2 was significantly decreased; however, no significant changes in the surface expression of HCN1 and HCN2 were observed. Our results showed that, 5 weeks after induction of hypoperfusion, the surface expression of GABA<sub>B</sub> R1 and GABA<sub>B</sub> R2 was significantly decreased, accompanying with the reduction of HCN1 surface expression and increase in HCN2 surface expression. In hippocampal CA1 neurons, I<sub>h</sub> is likely to be comprised of both HCN1 and HCN2 [65]. As described for other members of the voltage-gated cation channel family, four HCN subunits assemble to form a channel [66]. Homomeric HCN1 channels conduct fast kinetic currents with modest cAMP gating, consistent with currents recorded in hippocampal pyramidal cells where HCN1 expression is high [67]. By contrast, homomeric HCN2 channels conduct I<sub>h</sub> currents with slower kinetics and robust cAMP-evoked shifts in voltage dependence [68]. In naive rat

hippocampus, amounts of HCN1 protein are over eightfold higher than those of HCN2, so that most HCN1 channel molecules are likely to interact with other HCN1 subunits and form homomeric channels [69]. Chronic cerebral hypoperfusion-induced, an increased HCN2/HCN1 ratio should increase the stochastic probability of the HCN2 isoform's interaction with HCN1, which might augment HCN1/HCN2 heteromerization [69]. Because the properties of heteromeric channels are distinct from those of homomeric ones, their contribution should significantly modify the properties of the neuronal I<sub>h</sub>. The resulting changes in the I<sub>h</sub> properties of individual neurons, a form of intrinsic neuronal plasticity, may contribute to long-term pathological alteration of the hippocampal network. Our results suggested that activation of GABA<sub>B</sub> receptors restored the balance of

HCN1/HCN2 surface expression in the hippocampal CA1 area in 2VO rats.

Alternative mechanisms for hypoperfusion-driven enhanced HCN1/HCN2 heteromerization may involve activity-evoked changes in the intracellular trafficking. In this report, we found that TRIP8b (1a-4) and TRIP8b (1b-2) protein levels were significantly reduced in the hippocampal CA1 region in 2VO rats. Reduction of TRIP8b (1a-4) inhibited HCN1 channel trafficking to the plasma membrane, and reduction of TRIP8b (1b-2) inhibited HCN1 channel trafficking from the plasma membrane. Given that expression of TRIP8b (1a-4) is much more abundant than TRIP8b (1b-2), TRIP8b (1a-4) may play a more significant role in HCN1 channel trafficking than TRIP8b (1b-2) under chronic cerebral hypoperfusion. Thus, the reduction of HCN1 surface expression might be caused by the combined effect of a decrease in expression of both TRIP8b (1a-4) and TRIP8b (1b-2). Similarly, reduction of TRIP8b (1a-4) and TRIP8b (1b-2) inhibited HCN2 channel trafficking from the plasma membrane, which induced retention of HCN2 channels. During chronic cerebral hypoperfusion, a significant decrease in protein expression of AAK1 was not accompanied by a remarkable reduction of p-AP2  $\mu$ 2, which might be attributed to enhanced activity AAK1. Our results indicated that the expression of TRIP8b (1a-4) and TRIP8b (1b-2) was increased in baclofen-treated rats, especially 2VO rats. Although treatment of sham-operated rats with baclofen caused slight but significant increase in TRIP8b (1a-4) and TRIP8b (1b-2), no significant changes in the surface expression of HCN1 and HCN2 were observed (as mentioned above). Possible causes include the following: the degree of increase in TRIP8b is not enough to make significant changes in the surface expression of HCN channels, and other hippocampal TRIP8b isoforms are also involved in the trafficking of HCN channels. However, during chronic cerebral hypoperfusion, activation of GABA<sub>B</sub> receptors could regulate the surface expression of HCN1 and HCN2 proteins via possible mechanisms as follows: increase in protein expression of TRIP8b (1a-4) increased HCN1 surface expression, but decreased HCN2 surface expression; TRIP8b (1b-2), which was also increased in baclofen treatment groups, decreased HCN1 and HCN2 surface expression simultaneously. In addition, baclofen could regulate the function of TRIP8b via AAK1 and p-AP2  $\mu$ 2 under chronic cerebral hypoperfusion: baclofen restored the hippocampal AAK1 expression to the control level, and baclofen-induced enhancement of PKA activity (as mentioned previously) could further promote the action of AAK1 on phosphorylation of AP2  $\mu$ 2 subunit [41, 70], which resulted in a decrease in TRIP8b (1b-2)-mediated HCN1 internalization. The combined effect of these on HCN1 channel was that activation of GABA<sub>B</sub> receptors attenuated the decrease of surface expression of HCN1 induced by chronic cerebral hypoperfusion. Coincidentally, increased phosphorylation of AP2  $\mu$ 2 subunit might prevent HCN2

surface expression from downregulating exceedingly. Together, activation of GABA<sub>B</sub> receptors could not only increase the expression of TRIP8b (1a-4) and TRIP8b (1b-2) but also regulate the function of TRIP8b via AAK1 and p-AP2  $\mu$ 2, which restored the balance of HCN1/HCN2 surface expression. Similarly, we also did not exclude the effects of other TRIP8b isoforms on the trafficking of HCN channels under chronic cerebral hypoperfusion.

Although the present study has yielded some preliminary findings, several limitations to this pilot study need to be acknowledged. Firstly, in order to eliminate the interactions with fluctuating female hormones that influence memory, we only used the male rat model, but it also limited the findings to a single sex. More studies clarifying gender differences in the drug treatment of chronic cerebral hypoperfusion-induced memory impairment are needed. Secondly, it remains to be seen precisely whether and how other HCN channels and TRIP8b isoforms were pathologically altered in chronic cerebral hypoperfusion. Thirdly, neuroprotective effects of GABA<sub>B</sub> receptors activation on chronic cerebral hypoperfusion should be further evaluated in nonhuman primates, which appear to represent the best model for the study of vascular dementia, because they have well-developed white matter and vascular architectures which closely resemble those in human brains [71].

In conclusion, our present results demonstrated that, under chronic cerebral hypoperfusion, GABA<sub>B</sub> receptor agonist baclofen attenuated the decrease of surface expression of GABA<sub>B</sub> R1 and GABA<sub>B</sub> R2 possibly by activation of PKA, and activation of GABA<sub>B</sub> receptors restored the balance of HCN1/HCN2 surface expression possibly via not only increasing the expression of TRIP8b (1a-4) and TRIP8b (1b-2) but also regulating the function of TRIP8b via AAK1 and p-AP2  $\mu$ 2 (Fig. 7), which reversed the learning and memory deficits induced by 2VO.

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**Conflict of Interest** The authors declare that they have no conflict of interest.

## References

- Halliwel JV, Adams PR (1982) Voltage-clamp analysis of muscarinic excitation in hippocampal neurons. *Brain Res* 250(1):71–92
- Wahl-Schott C, Biel M (2009) HCN channels: structure, cellular regulation and physiological function. *Cell Mol Life Sci* 66(3):470–494. doi:10.1007/s00018-008-8525-0
- Biel M, Wahl-Schott C, Michalakis S, Zong X (2009) Hyperpolarization-activated cation channels: from genes to function. *Physiol Rev* 89(3):847–885. doi:10.1152/physrev.00029.2008
- Doan TN, Kunze DL (1999) Contribution of the hyperpolarization-activated current to the resting membrane potential of rat nodose sensory neurons. *J Physiol* 514(Pt 1):125–138

5. Nolan MF, Malleret G, Dudman JT, Buhl DL, Santoro B, Gibbs E, Vronskaya S, Buzsaki G, Siegelbaum SA, Kandel ER, Morozov A (2004) A behavioral role for dendritic integration: HCN1 channels constrain spatial inputs to distal dendrites memory and plasticity at of CA1 pyramidal neurons. *Cell* 119(5):719–732. doi:10.1016/j.cell.2004.11.020
6. Huang CC, Hsu KS (2003) Reexamination of the role of hyperpolarization-activated cation channels in short- and long-term plasticity at hippocampal mossy fiber synapses. *Neuropharmacology* 44(7):968–981. doi:10.1016/S0028-3908(03)00098-4
7. Mellor J, Nicoll RA, Schmitz D (2002) Mediation of hippocampal mossy fiber long-term potentiation by presynaptic Ih channels. *Science* 295(5552):143–147. doi:10.1126/science.1064285
8. Leresche N, Lightowler S, Soltesz I, Jassik-Gerschenfeld D, Crunelli V (1991) Low-frequency oscillatory activities intrinsic to rat and cat thalamocortical cells. *J Physiol* 441:155–174
9. Bender RA, Brewster A, Santoro B, Ludwig A, Hofmann F, Biel M, Baram TZ (2001) Differential and age-dependent expression of hyperpolarization-activated, cyclic nucleotide-gated cation channel isoforms 1–4 suggests evolving roles in the developing rat hippocampus. *Neuroscience* 106(4):689–698. doi:10.1016/S0306-4522(01)00314-1
10. Nolan MF, Malleret G, Lee KH, Gibbs E, Dudman JT, Santoro B, Yin DQ, Thompson RF, Siegelbaum SA, Kandel ER, Morozov A (2003) The hyperpolarization-activated HCN1 channel is important for motor learning and neuronal integration by cerebellar Purkinje cells. *Cell* 115(5):551–564. doi:10.1016/S0092-8674(03)00884-5
11. Li S, He Z, Guo L, Huang L, Wang J, He W (2010) Behavioral alterations associated with a down regulation of HCN1 mRNA in hippocampal cornu ammon 1 region and neocortex after chronic incomplete global cerebral ischemia in rats. *Neuroscience* 165(3):654–661. doi:10.1016/j.neuroscience.2009.10.053
12. Postea O, Biel M (2011) Exploring HCN channels as novel drug targets. *Nat Rev Drug Discov* 10(12):903–914. doi:10.1038/Nrd3576
13. Ludwig A, Budde T, Stieber J, Moosmang S, Wahl C, Holthoff K, Langebartels A, Wotjak C, Munsch T, Zong X, Feil S, Feil R, Lancel M, Chien KR, Konnerth A, Pape HC, Biel M, Hofmann F (2003) Absence epilepsy and sinus dysrhythmia in mice lacking the pacemaker channel HCN2. *EMBO J* 22(2):216–224. doi:10.1093/emboj/cdg032
14. Emery EC, Young GT, Berrococo EM, Chen L, McNaughton PA (2011) HCN2 ion channels play a central role in inflammatory and neuropathic pain. *Science* 333(6048):1462–1466. doi:10.1126/science.1206243
15. Notomi T, Shigemoto R (2004) Immunohistochemical localization of Ih channel subunits, HCN1–4, in the rat brain. *J Comp Neurol* 471(3):241–276. doi:10.1002/cne.11039
16. Chen X, Shu S, Schwartz LC, Sun C, Kapur J, Bayliss DA (2010) Homeostatic regulation of synaptic excitability: tonic GABA(A) receptor currents replace Ih in cortical pyramidal neurons of HCN1 knock-out mice. *J Neurosci* 30(7):2611–2622. doi:10.1523/JNEUROSCI.3771-09.2010
17. Bonin RP, Zurek AA, Yu JY, Bayliss DA, Orser BA (2013) Hyperpolarization-activated current (I-h) is reduced in hippocampal neurons from Gabra5<sup>-/-</sup> mice. *PloS One* 8(3):e58679. doi:10.1371/journal.pone.0058679
18. Atherton JF, Kitano K, Baufreton J, Fan K, Wokosin D, Tkatch T, Shigemoto R, Surmeier DJ, Bevan MD (2010) Selective participation of somatodendritic HCN channels in inhibitory but not excitatory synaptic integration in neurons of the subthalamic nucleus. *J Neurosci* 30(47):16025–16040. doi:10.1523/Jneurosci.3898-10.2010
19. Watts AE, Williams JT, Henderson G (1996) Baclofen inhibition of the hyperpolarization-activated cation current, Ih, in rat substantia nigra zona compacta neurons may be secondary to potassium current activation. *J Neurophysiol* 76(4):2262–2270
20. Chebib M, Johnston GA (2000) GABA-Activated ligand gated ion channels: medicinal chemistry and molecular biology. *J Med Chem* 43(8):1427–1447
21. Jiang ZG, Pessia M, North RA (1993) Dopamine and baclofen inhibit the hyperpolarization-activated cation current in rat ventral tegmental neurones. *J Physiol* 462:753–764
22. Sternau LL, Lust WD, Ricci AJ, Ratcheson R (1989) Role for gamma-aminobutyric acid in selective vulnerability in gerbils. *Stroke* 20(2):281–287
23. Lal S, Shuaib A, Ijaz S (1995) Baclofen is cytoprotective to cerebral ischemia in gerbils. *Neurochem Res* 20(2):115–119
24. Zhang F, Li C, Wang R, Han D, Zhang QG, Zhou C, Yu HM, Zhang GY (2007) Activation of GABA receptors attenuates neuronal apoptosis through inhibiting the tyrosine phosphorylation of NR2A by Src after cerebral ischemia and reperfusion. *Neuroscience* 150(4):938–949. doi:10.1016/j.neuroscience.2007.09.070
25. Xu J, Li C, Yin XH, Zhang GY (2008) Additive neuroprotection of GABA A and GABA B receptor agonists in cerebral ischemic injury via PI-3 K/Akt pathway inhibiting the ASK1-JNK cascade. *Neuropharmacology* 54(7):1029–1040. doi:10.1016/j.neuropharm.2008.01.014
26. Zhou C, Li C, Yu HM, Zhang F, Han D, Zhang GY (2008) Neuroprotection of gamma-aminobutyric acid receptor agonists via enhancing neuronal nitric oxide synthase (Ser847) phosphorylation through increased neuronal nitric oxide synthase and PSD95 interaction and inhibited protein phosphatase activity in cerebral ischemia. *J Neurosci Res* 86(13):2973–2983. doi:10.1002/jnr.21728
27. Tuttolomondo A, Di Sciaccia R, Di Raimondo D, Amao V, Renda C, Pinto A, Licata G (2009) Neuron protection as a therapeutic target in acute ischemic stroke. *Curr Top Med Chem* 9(14):1317–1334
28. Jackson-Friedman C, Lyden PD, Nunez S, Jin A, Zweifler R (1997) High dose baclofen is neuroprotective but also causes intracerebral hemorrhage: a quantal bioassay study using the intraluminal suture occlusion method. *Exp Neurol* 147(2):346–352. doi:10.1006/exnr.1997.6637
29. Cimarosti H, Kantamneni S, Henley JM (2009) Ischaemia differentially regulates GABA(B) receptor subunits in organotypic hippocampal slice cultures. *Neuropharmacology* 56(8):1088–1096. doi:10.1016/j.neuropharm.2009.03.007
30. Costa C, Leone G, Saulle E, Pisani F, Bernardi G, Calabresi P (2004) Coactivation of GABA(A) and GABA(B) receptor results in neuroprotection during in vitro ischemia. *Stroke* 35(2):596–600. doi:10.1161/01.STR.0000113691.32026.06
31. Santoro B, Wainger BJ, Siegelbaum SA (2004) Regulation of HCN channel surface expression by a novel C-terminal protein-protein interaction. *J Neurosci* 24(47):10750–10762. doi:10.1523/JNEUROSCI.3300-04.2004
32. Zerial M, McBride H (2001) Rab proteins as membrane organizers. *Nat Rev Mol Cell Biol* 2(2):107–117. doi:10.1038/35052055
33. Piskorowski R, Santoro B, Siegelbaum SA (2011) TRIP8b splice forms act in concert to regulate the localization and expression of HCN1 channels in CA1 pyramidal neurons. *Neuron* 70(3):495–509. doi:10.1016/j.neuron.2011.03.023
34. Santoro B, Piskorowski RA, Pian P, Hu L, Liu HY, Siegelbaum SA (2009) TRIP8b splice variants form a family of auxiliary subunits that regulate gating and trafficking of HCN channels in the brain. *Neuron* 62(6):802–813. doi:10.1016/j.neuron.2009.05.009
35. Lewis AS, Schwartz E, Chan CS, Noam Y, Shin M, Wadman WJ, Surmeier DJ, Baram TZ, Macdonald RL, Chetkovich DM (2009) Alternatively spliced isoforms of TRIP8b differentially control h channel trafficking and function. *J Neurosci* 29(19):6250–6265. doi:10.1523/JNEUROSCI.0856-09.2009
36. Zolles G, Wenzel D, Bildl W, Schulte U, Hofmann A, Muller CS, Thumfart JO, Vlachos A, Deller T, Pfeifer A, Fleischmann BK, Roeper J, Fakler B, Klocker N (2009) Association with the auxiliary subunit PEX5R/Trip8b controls responsiveness of HCN Channels to



- cAMP and adrenergic stimulation. *Neuron* 62(6):814–825. doi:10.1016/j.neuron.2009.05.008
37. Popova NV, Plotnikov AN, Ziganshin R, Deyev IE, Petrenko AG (2008) Analysis of proteins interacting with TRIP8b adapter. *Biochemistry (Mosc)* 73(6):644–651
  38. Santoro B, Hu L, Liu HY, Saponaro A, Pian P, Piskrowski RA, Moroni A, Siegelbaum SA (2011) TRIP8b regulates HCN1 channel trafficking and gating through two distinct C-terminal interaction sites. *J Neurosci* 31(11):4074–4086. doi:10.1523/Jneurosci.5707-10.2011
  39. Kirchhausen T (1999) Adaptors for clathrin-mediated traffic. *Annu Rev Cell Dev Biol* 15:705–732. doi:10.1146/annurev.cellbio.15.1.705
  40. Gu M, Liu Q, Watanabe S, Sun L, Hollopeter G, Grant BD, Jorgensen EM (2013) AP2 hemicomplexes contribute independently to synaptic vesicle endocytosis. *Elife* 2:e00190. doi:10.7554/eLife.00190
  41. Conner SD, Schmid SL (2002) Identification of an adaptor-associated kinase, AAK1, as a regulator of clathrin-mediated endocytosis. *J Cell Biol* 156(5):921–929. doi:10.1083/jcb.200108123
  42. Fairfax BP, Pitcher JA, Scott MG, Calver AR, Pangalos MN, Moss SJ, Couve A (2004) Phosphorylation and chronic agonist treatment atypically modulate GABAB receptor cell surface stability. *J Biol Chem* 279(13):12565–12573. doi:10.1074/jbc.M311389200
  43. Morris R (1984) Developments of a water-maze procedure for studying spatial learning in the rat. *J Neurosci Methods* 11(1):47–60
  44. Block F (1999) Global ischemia and behavioural deficits. *Prog Neurobiol* 58(3):279–295
  45. Benice TS, Raber J (2008) Object recognition analysis in mice using nose-point digital video tracking. *J Neurosci Methods* 168(2):422–430. doi:10.1016/j.jneumeth.2007.11.002
  46. Antunes M, Biala G (2012) The novel object recognition memory: neurobiology, test procedure, and its modifications. *Cogn Process* 13(2):93–110. doi:10.1007/s10339-011-0430-z
  47. Okuda S, Roozendaal B, McGaugh JL (2004) Glucocorticoid effects on object recognition memory require training-associated emotional arousal. *Proc Natl Acad Sci U S A* 101(3):853–858. doi:10.1073/pnas.0307803100
  48. Li CJ, Zhou M, Li HG, Lv Q, Xu XL, Guo LJ (2013) Clonidine suppresses the induction of long-term potentiation by inhibiting HCN channels at the Schaffer collateral-CA1 synapse in anesthetized adult rats. *Cell Mol Neurobiol* 33(8):1075–1086. doi:10.1007/s10571-013-9974-z
  49. Bliss TV, Collingridge GL (1993) A synaptic model of memory: long-term potentiation in the hippocampus. *Nature* 361(6407):31–39. doi:10.1038/361031a0
  50. Desestret V, Riou A, Chauveau F, Cho TH, Devillard E, Marinescu M, Ferrera R, Rey C, Chanal M, Angoulvant D, Honnorat J, Nighoghossian N, Berthezene Y, Nataf S, Wiart M (2013) In vitro and in vivo models of cerebral ischemia show discrepancy in therapeutic effects of M2 macrophages. *PLoS One* 8(6):e67063. doi:10.1371/journal.pone.0067063
  51. Flores G, Alquicer G, Silva-Gomez AB, Zaldivar G, Stewart J, Quirion R, Srivastava LK (2005) Alterations in dendritic morphology of prefrontal cortical and nucleus accumbens neurons in post-pubertal rats after neonatal excitotoxic lesions of the ventral hippocampus. *Neuroscience* 133(2):463–470. doi:10.1016/j.neuroscience.2005.02.021
  52. Alquicer G, Morales-Medina JC, Quirion R, Flores G (2008) Postweaning social isolation enhances morphological changes in the neonatal ventral hippocampal lesion rat model of psychosis. *J Chem Neuroanat* 35(2):179–187. doi:10.1016/j.jchemneu.2007.10.001
  53. Powell KL, Ng C, O'Brien TJ, Xu SH, Williams DA, Foote SJ, Reid CA (2008) Decreases in HCN mRNA expression in the hippocampus after kindling and status epilepticus in adult rats. *Epilepsia* 49(10):1686–1695. doi:10.1111/j.1528-1167.2008.01593.x
  54. Arolfo MP, Zanudio MA, Ramirez OA (1998) Baclofen infused in rat hippocampal formation impairs spatial learning. *Hippocampus* 8(2):109–113. doi:10.1002/(SICI)1098-1063(1998)8:2<109::AID-HIPO2>&gt;3.0.CO;2-G
  55. McNamara RK, Skelton RW (1996) Baclofen, a selective GABAB receptor agonist, dose-dependently impairs spatial learning in rats. *Pharmacol Biochem Behav* 53(2):303–308
  56. Deng PY, Xiao Z, Yang C, Rojanathammanee L, Grisanti L, Watt J, Geiger JD, Liu R, Porter JE, Lei S (2009) GABA(B) receptor activation inhibits neuronal excitability and spatial learning in the entorhinal cortex by activating TREK-2 K<sup>+</sup> channels. *Neuron* 63(2):230–243. doi:10.1016/j.neuron.2009.06.022
  57. Nakagawa Y, Takashima T (1997) The GABA(B) receptor antagonist CGP36742 attenuates the baclofen- and scopolamine-induced deficit in Morris water maze task in rats. *Brain Res* 766(1–2):101–106
  58. Sarti C, Pantoni L, Bartolini L, Inzitari D (2002) Persistent impairment of gait performances and working memory after bilateral common carotid artery occlusion in the adult Wistar rat. *Behav Brain Res* 136(1):13–20
  59. Kim SK, Cho KO, Kim SY (2008) White matter damage and Hippocampal neurodegeneration induced by permanent bilateral occlusion of common carotid artery in the rat: comparison between wistar and Sprague–Dawley strain. *Korean J Physiol Pharmacol* 12(3):89–94. doi:10.4196/kjpp.2008.12.3.89
  60. Pitsikas N, Rigamonti AE, Cella SG, Muller EE (2003) The GABAB receptor and recognition memory: possible modulation of its behavioral effects by the nitrenergic system. *Neuroscience* 118(4):1121–1127
  61. Farkas E, Luiten PG, Bari F (2007) Permanent, bilateral common carotid artery occlusion in the rat: a model for chronic cerebral hypoperfusion-related neurodegenerative diseases. *Brain Res Rev* 54(1):162–180. doi:10.1016/j.brainresrev.2007.01.003
  62. von Bohlen, Halbach O (2009) Structure and function of dendritic spines within the hippocampus. *Ann Anat* 191(6):518–531. doi:10.1016/j.aanat.2009.08.006
  63. Jia H, Zhang XM, Zhang BA, Liu Y, Li JM (2012) Dendritic morphology of neurons in medial prefrontal cortex and hippocampus in 2VO rats. *Neuro Sci* 33(5):1063–1070. doi:10.1007/s10072-011-0898-4
  64. Anju TR, Jayanarayanan S, Paulose CS (2011) Decreased GABAB receptor function in the cerebellum and brain stem of hypoxic neonatal rats: role of glucose, oxygen and epinephrine resuscitation. *J Biomed Sci* 18:31. doi:10.1186/1423-0127-18-31
  65. Santoro B, Chen S, Luthi A, Pavlidis P, Shumyatsky GP, Tibbs GR, Siegelbaum SA (2000) Molecular and functional heterogeneity of hyperpolarization-activated pacemaker channels in the mouse CNS. *J Neurosci* 20(14):5264–5275
  66. Santoro B, Baram TZ (2003) The multiple personalities of h-channels. *Trends Neurosci* 26(10):550–554. doi:10.1016/j.tins.2003.08.003
  67. Brewster A, Bender RA, Chen YC, Dube C, Eghbal-Ahmadi M, Baram TZ (2002) Developmental febrile seizures modulate hippocampal gene expression of hyperpolarization-activated channels in an isoform- and cell-specific manner. *J Neurosci* 22(11):4591–4599
  68. Robinson RB, Siegelbaum SA (2003) Hyperpolarization-activated cation currents: from molecules to physiological function. *Annu Rev Physiol* 65:453–480. doi:10.1146/annurev.physiol.65.092101.142734
  69. Brewster AL, Bernard JA, Gall CM, Baram TZ (2005) Formation of heteromeric hyperpolarization-activated cyclic nucleotide-gated (HCN) channels in the hippocampus is regulated by developmental seizures. *Neurobiol Dis* 19(1–2):200–207. doi:10.1016/j.nbd.2005.12.015
  70. Ricotta D, Conner SD, Schmid SL, von Figura K, Honing S (2002) Phosphorylation of the AP2 mu subunit by AAK1 mediates high affinity binding to membrane protein sorting signals. *J Cell Biol* 156(5):791–795. doi:10.1083/jcb.200111068



71. Feinklestein SP, Fisher M, Furland AJ, Goldstein LB, Gorelick PB, Kaste M, Lees KR, Traystman RJ, Albers GW, Anwer UE, Ashwood T, Barone FC, Basta SL, Bogousslavsky J, Buchan AM, Cady WJ, Chan PH, Clemens JA, Cox BF, Craddock RE, Cramer SC, del Zoppo GJ, Dielrich WD, Elliott P, Faden AI, Feuerstein GZ, Ginsberg MD, Gold M, Greene WL, Hall ED, Hsu CY, Hunter AJ, Lai M, Lesko LM, Levy DE, Li FH, Locke KW, Lodge D, Lowe D, Marcoux FW, McCulloch J, McDermott J, Meibach R, Messersmith EK, Moseley M, Moskowitz MA, Mueller AL, Munro F, Nudo RJ, Oeda J, Ohlstein EH, Parsons A, Patmore L, Poole RM, Pschorn U, Pulsinelli WA, Sacco RL, Saeki S, Salazar-Gruesso E, Sandage BW, Schallert T, Schielke GP, Sharkey J, Sotak CH, Steiger B, Storall S, Takahashi Y, Tumas D, Van Bruggen N, Versavel M, Vornov J, Walker MD, Wallin B, Wang J, Warach S, Wells DS, Witcher JA, Round STAI (1999) Recommendations for standards regarding preclinical neuroprotective and restorative drug development. *Stroke* 30(12):2752–2758