

# Block of P2X7 receptors could partly reverse the delayed neuronal death in area CA1 of the hippocampus after transient global cerebral ischemia

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**Abstract** Transient global ischemia (which closely resembles clinical situations such as cardiac arrest, near drowning or severe systemic hypotension during surgical procedures), often induces delayed neuronal death in the brain, especially in the hippocampal CA1 region. The mechanism of ischemia/reperfusion (I/R) injury is not fully understood. In this study, we have shown that the P2X7 receptor antagonist, BBG, reduced delayed neuronal death in the hippocampal CA1 region after I/R injury; P2X7 receptor expression levels increased before delayed neuronal death after I/R injury; inhibition of the P2X7 receptor reduced I/R-induced microglial microvesicle-like components, IL-1 $\beta$  expression, P38 phosphorylation, and glial activation in hippocampal CA1 region after I/R injury. These results indicate that antagonism of the

P2X7 receptor and signaling pathways of microglial MV shedding, such as src-protein tyrosine kinase, P38 MAP kinase and A-SMase, might be a promising therapeutic strategy for clinical treatment of transient global cerebral I/R injury.

**Keywords** P2X7 receptor · Ischemia/reperfusion injury · Delayed neuronal death · Microvesicle · Interleukin-1 $\beta$

## Introduction

In global ischemia animal models (that most closely resembles clinical situations such as cardiac arrest, near drowning or severe systemic hypotension during surgical procedures), a transient (5–30 min) complete or nearly complete global lack of blood flow is induced. If flow is not restored within 30 min, widespread necrosis ensues, and functional recovery of the tissue is not possible [3]. However, reperfusion after transient ischemia may exacerbate brain injury, which is called cerebral ischemia/reperfusion (I/R) injury [6]. The most interesting and important phenomenon of brain I/R injury is delayed neuronal death, which may occur in the hippocampal CA1 region, cerebral cortex and striatum, particularly pyramidal neurons of the hippocampal CA1 region 48–72 h after reperfusion. The form of the delayed neuronal death in these regions was shown to be apoptosis [12, 37, 40, 55, 60, 63].

The mechanisms of delayed neuronal death are complicated. It may be due to release of excitatory amino acids [7, 26, 33, 41], intracellular calcium overload, or oxygen free radical generation [9, 21, 46, 54, 55, 76, 82]. One of the key mechanisms is the large release of glutamate, which activates ion channel receptors and induces intracellular calcium overload [21, 50]. Excitatory glutamate also acts as a neurotoxin via

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mGluR1 [53, 58]. Adenosine via the A1 receptor is beneficial to neurons or deleterious via the A2A and A3 receptors to neurons in animal ischemia models [2]. In addition to excitatory amino acids and adenosine, extracellular ATP acts as an important signaling molecule in the processes of ischemic stress and has attracted attention [18, 19, 25, 31, 38, 43, 61, 80, 27, 32, 77, 81, 42, 47]. Antagonists of P2 purinergic receptors can rescue neurons *in vitro* under different stimulations, such as excessive glutamate [74, 73] and chemical hypoxia [18, 19]. These data indicate that extracellular ATP via purinergic receptors is involved in the processes of neuron death during hypoxia and ischemia.

P2 receptors were divided into P2X and P2Y receptor families on the basis of pharmacology and molecular cloning [1, 17]. Currently, seven subtypes of ionotropic P2X receptors (non-selective cation channels) and eight subtypes of metabotropic P2Y receptors (G-protein-coupled receptors) are known to exist [15].

The P2X7 receptor is predominantly expressed on microglia and ependyma of the central nervous system (CNS) [24]. P2X7 receptors can be activated by high concentrations of ATP. Sustained stimulation of P2X7 receptors on microglia leads to activation of microglia, which produces reactive oxygen species and pro-inflammatory cytokines which are involved in neuroinflammation in many CNS disorders, including Alzheimer's disease [66], epilepsy [39], spinal cord injury [59, 79], and multiple sclerosis [68].

The P2X7 receptor has been reported to participate in cerebral ischemic injury, although the results were conflicting. Antagonism of the P2X7 receptor reduced infarct volume after transient middle cerebral artery occlusion injury [8, 49] and improved transient global cerebral I/R injury [22]. However, ischemic injury exacerbation by P2X7 receptor antagonists has also been reported [83]. In the P2X7 receptor knockout mouse, the P2X7 receptor has been reported not to be a primary mediator of experimentally induced neuronal death [45]. In order to further confirm whether inhibition of P2X7s receptor has beneficial or harmful effects or no effects in global cerebral I/R injury, we designed experiments using a widely used P2X7 receptor antagonist, Brilliant blue G [59, 79] and a selective P2X7 receptor antagonist, A-740003, to investigate the role of P2X7 receptors in a rat model of transient global cerebral I/R injury. We found that inhibition of the P2X7 receptor could partially rescue the delayed neuronal death and improve memory functions.

## Material and methods

### Animals and surgical procedures

Male Sprague–Dawley rats weighing 250–300 g were provided by the Animal Center of Second Military Medical

University. All experimental procedures were approved by the Institutional Animal Care and Use Committee at Second Military Medical University and conformed to the UK Animals (Scientific Procedures) Act 1986 and associated guidelines on the ethical use of animals.

Fifteen minutes of global cerebral ischemia was induced by the four-vessel occlusion (4-VO) method, with a slight modification [64]. Briefly, rats were anesthetized with 10 % (*w/v*) chloral hydrate (400 mg/kg, intraperitoneally (*i.p.*)), then the bilateral common carotid arteries (CCAs) were freed and both vertebral arteries were permanently electrocauterized. Rats were allowed to recover for 24 h after closing the surgical incisions. On the following day (0D), anesthesia was applied, the surgical incision in the neck was opened and both CCAs were occluded with aneurysm clips to induce global cerebral ischemia. The clips were removed for reperfusion. Rectal temperature was maintained at 36.0 to 37.0 °C throughout the procedures. Cerebral blood flow (CBF) before and after clamping the bilateral CCAs was monitored using a laser doppler flowmetry (MBF3D, Moor Instruments, Axminster, Devon, UK), and rats with a decrease in CBF of less than 80 % were excluded [78].

### Experimental groups and drug administration

To study the effect of the P2X7 receptor antagonist (BBG, Sigma-Aldrich) on delayed neuronal death in the hippocampal CA1 region after I/R injury, rats were divided into three groups: sham group (sham operated), saline group, and BBG group. To study the effect of the P2X7 receptor antagonists (A74003 and BBG) on spatial learning and memory after rat I/R injury, rats were divided into four groups: sham group (sham operated), saline group, A740003 group, and BBG group. BBG (50 mg/mL; Sigma-Aldrich) was dissolved in 0.9 % NaCl for intravenous injection. A74003 (40 mM/L, Tocris) was dissolved in DMSO for intraperitoneal injection. Rats in the sham and saline control groups received *i.v.* injection of 0.25 mL 0.9 % NaCl or DMSO. BBG at a dose of 50 mg/kg was given intravenously immediately after I/R injury and once daily for the next 2 days (three times total). A74003 at a dose of 100  $\mu$ l/kg was given intraperitoneally immediately after I/R injury and once daily for the next 2 days (three times total).

### Immunohistochemistry

After 12 h, 24 h, 48 h, 4 days, 7 days of I/R injury, rats were anesthetized and perfused intracardially with saline, followed by 4 % (*w/v*) paraformaldehyde in 0.1 mol/L PBS, pH7.4. Brains were removed and fixed overnight in 4 % (*w/v*) paraformaldehyde, then transferred to 25 % sucrose in PBS and kept in the solution until they sank to the bottom. Thereafter, the tissue blocks were rapidly frozen and coronal

sections (20  $\mu\text{m}$  in thickness) were cut with a Leica cryostat and floated in PBS. The following protocol was used for immunofluorescence. The sections were washed 3–5 min in PBS, and then preincubated in a blocking solution (10 % normal bovine serum, 0.2 % Triton X-100, 0.4 % sodium azide in 0.01 mol/l PBS pH 7.2) for 30 min followed by incubation with the primary antibodies (P2X7 (1:1000), Alomone, rabbit polyclonal, APR-004; NeuN (1:500), Millipore, monoclonal, clone A60; Iba-1 (1:500), Abcam, goat polyclonal, ab107159, biotin-conjugated IB4 (1:200), Sigma, L3019; GFAP (1:400), Boster (1:400), monoclonal, BM0055) at room temperature overnight. Subsequently, the sections were incubated with Cy3-conjugated donkey anti-rabbit IgG (Jackson, 711-165-152) diluted 1:400 for P2X7, FITC-conjugated donkey anti-mouse IgG (Jackson, 705-475-151)1:200 for NeuN and GFAP, FITC-conjugated donkey anti-goat IgG ((Jackson, 715-475-003)1:200 for Iba-1, Fluorescein-conjugated streptavidin (Jackson, 016-010-018)1:200 for IB4. All incubations were separated by 5–10-min washes in PBS.

#### TUNEL method

In situ labeling of DNA fragmentation (terminal deoxynucleotidyl transferase-mediated UTP nick end labeling (TUNEL)) was carried out with an in situ cell death detection kit (Roche, Mannheim, Germany) according to the manufacturer's instructions.

#### Photomicroscopy

Images were taken with a Nikon digital camera DXM1200 (Nikon, Japan) attached to a Nikon Eclipse E600 microscope (Nikon). Images were imported into a graphics package (Adobe Photoshop 5.0, USA).

#### Western blot

For Western blotting, the rats were killed after 12 h, 24 h, 48 h, 4 days, 7 days of I/R injury and their hippocampuses were removed immediately and lysed with 20 mM Tris-HCl buffer, pH 8.0, containing 1 % NP-40, 150 mM NaCl, 1 mM EDTA, 10 % glycerol, 0.1 % L-mercaptoethanol, 0.5 mM dithiothreitol, and a mixture of proteinase and phosphatase inhibitors (Sigma). Protein concentration was determined by the BCA protein assay method using bovine serum albumin (BSA) as standard. One hundred micrograms of protein samples from hippocampus was loaded per lane, separated by SDS-PAGE (12 % polyacrylamide gels) and then was electrotransferred onto nitrocellulose membranes. The membranes were blocked with 10 % nonfat dry milk in Tris-buffered saline for 1 h and incubated overnight at 4 °C with P2X7 (1:1000, Alomone), p-p38(1:1,000, CST), IL-1 $\beta$  antibody (1:200, Santa Cruz) and Tublin (1:1,000, Beyotime)

diluted in 2 % BSA in PBS. The membranes were then incubated with alkaline phosphatase-conjugated goat anti-rabbit IgG (Sigma) or goat anti-mouse IgG (Sigma) diluted 1:5000 in 2 % BSA in PBS for 1 h at room temperature. The color development was performed with 400  $\mu\text{g}/\text{ml}$  Nitro-Blue Tetrazolium, 200  $\mu\text{g}/\text{ml}$  5-bromo-4-chloro-3-indolyl phosphate and 100 mg/ml levamisole in TSM2 (0.1 mol/l Tris-HCl buffer, pH 9.5, 0.1 mol/l NaCl and 0.05 mol/l  $\text{MgCl}_2$ ) in the dark. Bands were scanned using a densitometer (GS-700; Bio-Rad Laboratories)

#### Morris water maze

At day 8, spatial learning and memory were tested with the Morris water maze [52], a circular black tank of 130 cm in diameter and 60 cm in height. The tank was filled with a depth of 30 cm water at  $25 \pm 1$  °C. The maze was divided into four equal quadrants. The trials were performed according to Vorhees' method [75]. Spatial acquisition: all rats received a training trial consisting of daily sessions of four consecutive trials for 5 days. The hidden platform (diameter 10 cm, 1.5 cm below the water surface) was positioned in the middle of the southwest (SW) quadrant for all rats. The rats were released into the tank facing the maze wall at north (N), west (W), south (S), or east (E) quadrants in a predetermined pseudorandom order. A trial was terminated as soon as the rat found the platform; if the rat did not succeed within 120 s, it was guided onto the platform with a stick. The rat was allowed to stay on the platform for 20 s before being removed. Probe trial: immediately after the final training trial, the platform was removed. Rats were released into the pool at NE position and allowed to swim freely for 2 min. The time needed to find the platform (escape latency) in the training trials and time spent in the SW quadrant in the probe trial were recorded. The mean value of four escape latencies in the daily four training trials was taken as the escape latency for the rat. Values from eight rats in the same group were averaged to generate a mean escape latency for that day.

#### Quantitative analysis

Neuronal damage, apoptosis and microvesicle-like components were quantified by counting the number of surviving neurons, positive neurons for TUNEL and microvesicle-like components with P2X7-immunoreactivity (ir) in the hippocampal CA1 region at high magnification ( $\times 400$ ). Photomicrographs of the CA1 region were taken using a Nikon digital camera DXM1200 (Nikon, Japan) attached to a Nikon Eclipse E600 microscope (Nikon). The numbers of surviving/TUNEL-positive neurons and total neurons, and P2X7-ir microvesicle-like components in the hippocampal CA1 region per 1 mm length were counted and analyzed blind by investigators. Six sections were used for each rat and the mean number of these six sections was calculated. Six rats were used for one group.

To quantify glial activation, we measured the value of the average area optical density (AAOD) for immunohistochemistry images from the CA1 region stained with antibodies against Iba-1 or GFAP using NIS-elements D3.1 system (Nikon, Japan). Pictures were taken using the same method as mentioned above. Six images ( $\times 200$ ) were randomly selected for each animal, and the mean AAOD of these six fields was considered as the AAOD of the animal.

#### Statistical analysis

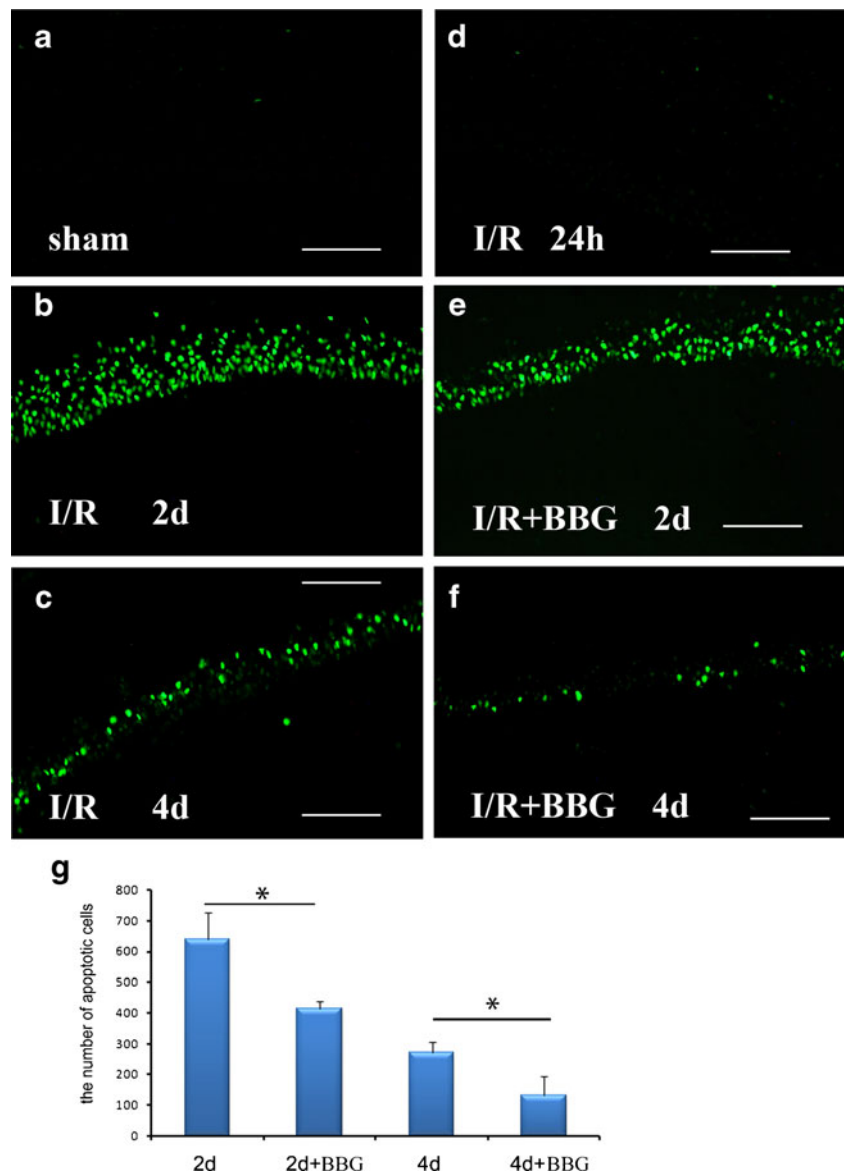
Results are expressed as mean  $\pm$  SEM. Values were analyzed using a one-way analysis of variance followed by Dunnett's post hoc test.  $p < 0.05$  was considered to be statistically significant.

## Results

### BBG reduce the number of delayed neuronal death in the hippocampal CA1 region after I/R injury

In order to determine whether the P2X7 receptor is involved in delayed neuronal death after transient global cerebral I/R injury, apoptosis and neuronal survival was analyzed in the hippocampal CA1 region. No cell apoptosis was detected by the TUNEL technique until 48 h after I/R injury (Fig. 1a, d). The vast majority of the apoptotic cells were detected in the CA1 region of the hippocampus. The peak of cell apoptosis occurrence was at 48 h after I/R injury and reduced significantly 4 days after I/R injury (Fig. 1c, g). No cell apoptosis was detected after 7 days (data not shown). BBG inhibited significantly apoptotic cell

**Fig. 1** Effect of BBG on neuronal apoptosis in the CA1 region after I/R injury. **a, b, c, d** are TUNEL staining of the CA1 region from the sham group, I/R 2d group, I/R 4d group and I/R 24-h group, respectively. **e** and **f** are TUNEL staining of the CA1 region from I/R+BBG 2d group and I/R+BBG 4d group, respectively. Note that BBG treatment significantly reduced the number of the apoptotic cells in the CA1 region. **g** summarizes the number of TUNEL-positive cells in the hippocampal CA1 region per 1-mm length in the sham, saline, and BBG-treated groups. Values are expressed as the mean  $\pm$  S.E.M. \* $p < 0.01$  BBG-treated group versus saline group at 2 days and 4 days after I/R injury. All the scale bars = 130  $\mu$ m. *BBG* brilliant blue G, *I/R* ischemia/reperfusion, *S.E.M.* standard error of the mean, *TUNEL* deoxynucleotidyl transferase-mediated UTP nick end labeling

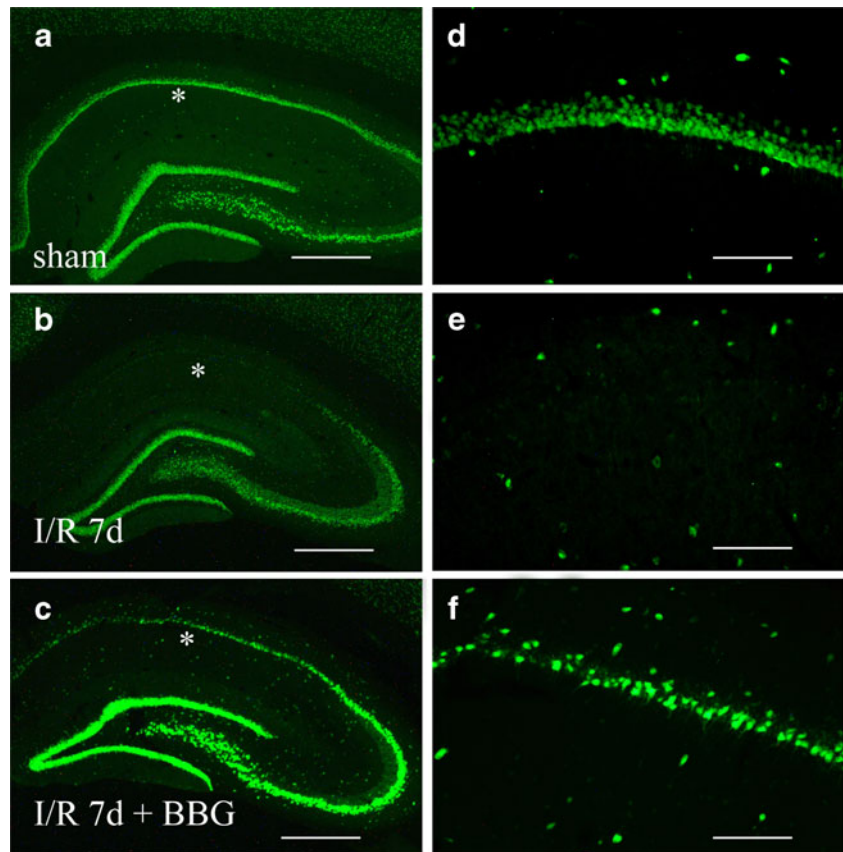


death in the CA1 region of hippocampus (Fig. 1e, f, g). The neuron survival rate was studied with NeuN (a neuronal marker) immunohistochemical method at 7 days after I/R. All the neurons were stained with the NeuN antibody in hippocampus regions of normal rats (Fig. 2a). The total number of neurons in the hippocampal CA1 region per 1 mm length was  $1,145 \pm 139$ . However, the vast majority of neurons with NeuN-ir disappeared in the CA1 region of hippocampal sections after I/R injury (Fig. 2b, g). The number of neurons that survived was  $157 \pm 29$ . The antagonist of the P2X7 receptor, BBG, significantly increased the survival rate of CA1 region neurons, although not all delayed neuronal deaths were prevented. The number of neurons that survived in the hippocampal CA1 region per 1 mm length was  $479 \pm 54$ . These data indicate that BBG reduced apoptosis of cells and increased the survival rate of CA1 region neurons after I/R injury.

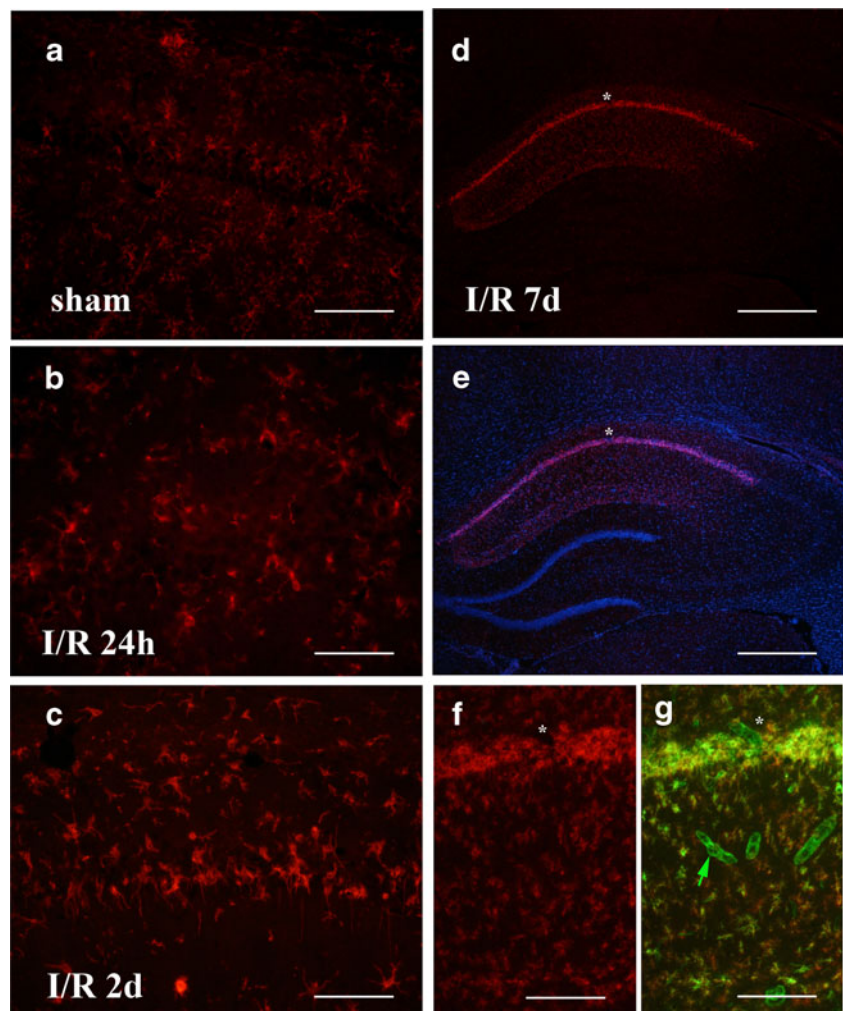
Expression of P2X7 receptors in the hippocampus after I/R injury

The cellular expression pattern of P2X7 receptors, the cellular target of the action of BBG, was next assessed within the rat hippocampus. Immunohistochemical analysis revealed that P2X7 receptors were mainly expressed in ramified microglia-like cells in normal rat hippocampus (Fig. 3a). The depth of immunostaining of P2X7 receptor on microglia increased gradually after I/R injury. At 48 h after I/R injury, the depth of immunostaining, number and cell volume of P2X7 receptors on microglia significantly increased. Afterwards, the depth of immunostaining depth, number and cell volume of P2X7 receptors on microglia increased continuously (Fig. 3); significant increased expression was confined to the CA1 region of the rat hippocampus (Fig. 3d, e, f, g).

**Fig. 2** Effect of BBG on neuronal survival rate in the CA1 region after I/R injury. **a, b, c** are NeuN immunostaining of rat hippocampus from the sham group, I/R+saline group, and I/R+BBG group at +7 days after I/R, respectively. **d, e, and f** are higher magnifications of **a, b, c** in an area indicated by a *star*. Note that BBG treatment increased significantly the number of survival neurons with NeuN-ir in the CA1 region. **g** summarizes the number of survival neurons with NeuN-ir in the hippocampal CA1 region per 1 mm length in the sham, saline, and BBG-treated groups at 7 days after I/R injury. Values are expressed as mean  $\pm$  S.E.M. \* $p < 0.01$  BBG-treated group versus saline group. All *scale bars* = 130  $\mu$ m. *BBG* brilliant blue G, *I/R* ischemia/reperfusion, *S.E.M.* standard error of the mean, *TUNEL* deoxynucleotidyl transferase-mediated UTP nick end labeling



**Fig. 3** Expression of P2X7 receptors detected by an immunohistochemical method in the rat hippocampal CA1 region after I/R injury. **a, b, c, and d** are P2X7 receptor immunostaining of the rat hippocampus CA1 region from the sham group and the I/R+saline group at +24 h, 2 days and 7 days after I/R, respectively. **e** is the merged image from **d** and its DAPI counter-stained image. Note that enhanced P2X7 receptor expression is confined to the CA1 region. **f** is the higher magnification of **d** in an area indicated by a star. **g** is the merged image of **f** and IB4 immunostaining in the same field of **f**. Note that all the P2X7 receptor-ir cells were also immunoreactive for IB4, in contrast epithelial cells of small vessels were immunoreactive for IB4, but not for P2X7 receptors (indicated by an arrow). Scale bars in **a, b, c, f** and **g**=130  $\mu$ m, scale bars in **d** and **e**=1300  $\mu$ m. *BBG* Brilliant blue G, *I/R* ischemia/ reperfusion, *IB4* isolectin-B4, *DAPI* 4',6-diamidino-2-phenylindole



Double labeling immunofluorescence revealed that almost all the cells with P2X7 receptor-ir in the hippocampus also labeled for IB4 (a common marker of microglia) (Fig. 3f, g). Western blot also revealed a similar change pattern of P2X7 receptor expression in the hippocampus (Fig. 4). These data indicate that P2X7 receptor expression levels increase before 48 h after I/R injury, when delayed neuronal death in CA1 region begins to occur.

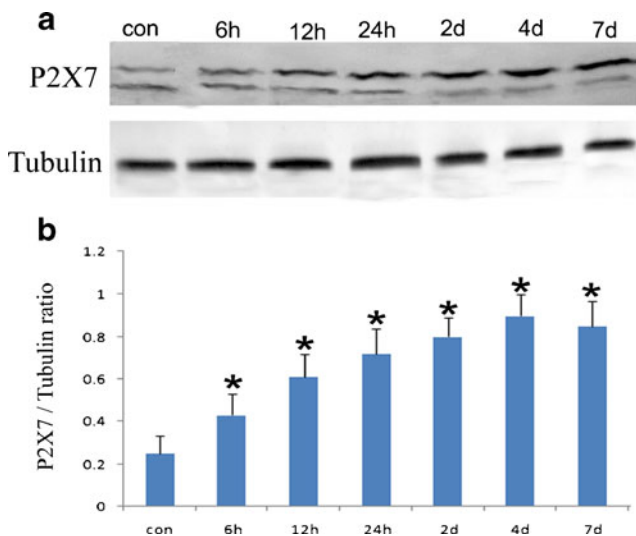
#### Inhibition of the P2X7 receptor reduces I/R-induced microglial microvesicle-like components

At the time when neuronal death occurred in the CA1 region, P2X7 receptor-ir microvesicle-like components were detected at high magnification. These components were round or oval, with a diameter of about 0.5–2.0  $\mu$ m (Fig. 5). Double labeling immunofluorescence revealed that the majority of microvesicle-like components with P2X7 receptor-ir were also immunoreactive for Iba-1 (a microglia marker) (Fig. 5a, b, d). The highest density of these components was found at 48 h after I/R injury, but not at earlier stages, before neuronal death occurred in the

CA1 region, and not after 7 days of I/R injury [Fig. 5e]. BBG reduced significantly the number of these components in the CA1 region of I/R injured rats. (Fig. 5c, e).

#### P2X7 receptor mediates IL-1 $\beta$ expression after I/R injury

The P2X7 receptor is involved in secretion of IL-1 $\beta$  from macrophages and microglia [29]. After P2X7 receptor activation, a src-protein tyrosine kinase interacts with the P2X7 receptor c-terminus and promptly phosphorylates P38 MAP kinase. P38 phosphorylation, in turn, induces translocation of acid sphingomyelinase to the outer leaflet of plasma membrane, where it generates ceramide and induces MVs shedding [11]. Biologically mature IL-1 $\beta$  expression was detected in the sham rat hippocampus at a low level. After I/R injury, the level of expression of IL-1 $\beta$  increased. 48 h after I/R injury, the level of expression of P2X7 receptors reached a peak. Treatment with BBG significantly reduced the expression of biologically mature IL-1 $\beta$  within the hippocampus at 48 h after I/R injury, as assessed by Western blotting (Fig. 6a, c). I/R injury increased IL-1 $\beta$  expression



**Fig. 4** Expression of P2X7 receptors detected by Western blotting in the rat hippocampal CA1 region after I/R injury. **a** is the result of Western blots (top panel) of P2X7 receptor protein in the hippocampus of rats from the sham group and 6h, 12h, 24h, 48h, 4d, 7d after I/R injury. Blots were normalized to tubulin to control for equal protein loading between lanes. Data are representative of 5 rats per group. The ratio of P2X7 receptor protein/tubulin was analyzed with one-way ANOVA followed by Dunnett's post hoc test (\* $p < 0.05$  vs. sham operated rats)

by 52.72 % compared to sham-operated rats at 48 h ( $p < 0.01$  vs. sham). Post-treatment with BBG reduced I/R injury-induced expression of IL-1 $\beta$  to 15.23 % compared to sham ( $p < 0.01$  vs. I/R injury) at 48 h (Fig. 6b, c).

The p38 signaling pathway has been identified as being involved in microvesicle shedding and IL-1 $\beta$  secretion from macrophages and microglia. Phosphorylated p38 was detected in the sham rat hippocampus at a low level. After I/R injury, the level of phosphorylated p38 increased. At 48 h after I/R injury, the level of phosphorylated p38 reached a peak (Fig. 7a, c). Treatment with BBG significantly reduced the level of phosphorylated p38 within the hippocampus at 48 h after I/R injury, as assessed by Western blotting (Fig. 7b, c). I/R injury increased phosphorylated p38 by 90.38 % compared to sham-operated rats at 48 h ( $p < 0.01$  vs. sham). Post-treatment with BBG reduced I/R injury-induced expression of phosphorylated p38 to 41.12 % compared to sham ( $p < 0.05$  vs. I/R injury) at 48 h. These data suggest that the p38 pathway may be involved in vivo in microvesicle shedding and IL-1 $\beta$  secretion from microglia of I/R injured rat hippocampus.

#### BBG and A74003 improve neurobehavioral outcomes after I/R injury

The Hippocampus is one of the most important brain regions involved in learning and memory functions. Neuronal death in the CA1 region after I/R injury is related to dysfunction of learning and memory. Spatial memory was evaluated using the Morris water maze. During the 5-day hidden platform trial,

escape latency of sham and the three experimental groups decreased in a day-dependent pattern. However, the sham group took significantly less time to find the platform than the saline group on all five days. In addition, the saline group required significantly more time to find the platform than the BBG and A74003 groups after the ninth day. In the probe trial, the saline group spent significantly less time than the other three groups in the SW quadrant. There was no significant difference among the sham, BBG and A74003 groups (Fig. 8).

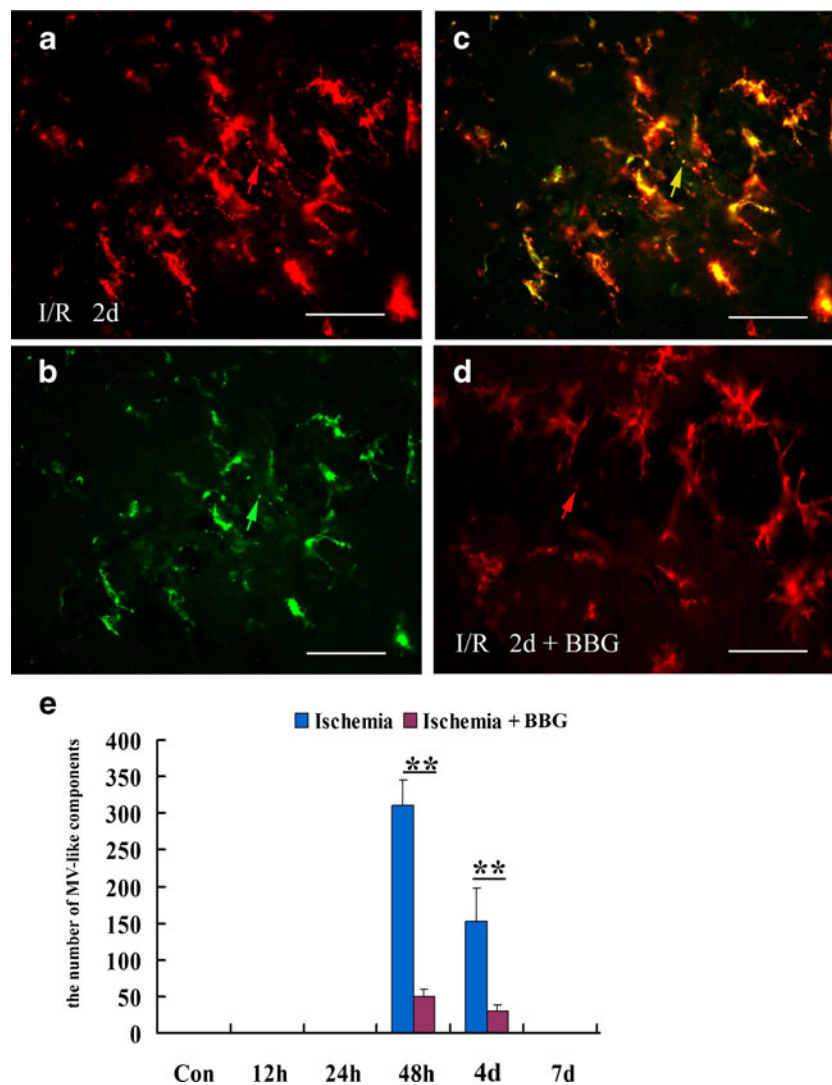
#### Inhibition of the P2X7 receptor reduces I/R-induced glial activation

Reactive gliosis was assessed by immunohistochemical analysis expression of Iba-1 (a microglia marker) and GFAP (an astrocyte marker) in the CA1 region at 7 days after I/R injury. Scattered ramified microglial cells with Iba-1-ir were observed in the CA1 region of sham rat hippocampus. 7 days after I/R injury, the immunostaining and the number of microglial cells with Iba-1-ir in this region were significantly increased in the saline group. The majority of the microglial cells with Iba-1-ir transformed to amoeboid-like cells. Treatment with BBG also markedly attenuated the increase of immunostaining density and the number of Iba-1-ir cells when compared to the saline group (Fig. 9a, b, c, g). The scattered GFAP-ir cells with thin and long processes were detected in the CA1 region of the sham rat hippocampus. However, both the immunostaining density and the number of GFAP-ir cells significantly increased in the saline group. Treatment with BBG markedly attenuated the increase of immunostaining density and the number of GFAP-ir cells when compared to the saline group (Fig. 9d, e, f, g).

#### Discussion

The main finding of this study is that the P2X7 receptor antagonist, BBG, reduced neuron apoptotic cell death and increased the survival rate of neurons in the CA1 region after I/R injury. P2X7 receptor expression was up-regulated on microglial cells during I/R injury. Inhibition of the P2X7 receptor reduced I/R-induced microglial microvesicle-like components, IL-1 $\beta$  expression, P38 phosphorylation, and glial activation in hippocampal CA1 region after I/R injury.

Purinergic receptors have important actions in the CNS [14]. P2X7 receptor is predominantly expressed by microglial and ependymal cells in the CNS [24], although in more recent studies P2X7 receptors have been claimed to be present on presynaptic nerve terminals, astrocytes and oligodendrocytes [14, 16]. This receptor comprises two transmembrane domains, an extracellular loop, and N-terminal and C-terminal domains. Transient stimulation with ATP opens a channel permeable to small cations, whereas sustained stimulation leads to conductance of moieties of up to 900 Da [15]. The



**Fig. 5** Microvesicle (MV)-like components in the CA1 region of the rat hippocampus after I/R injury. **a** shows microglial cells and MV-like components with P2X7 receptor-ir at high magnification in the CA1 region from the saline group at 2 days after I/R injury. Note that numerous MV-like components with P2X7 receptor-ir were detected. An *arrow* indicates a typical MV-like component. **b** shows microglial cells and MV-like components with Iba-1-ir in the same field of **a**. An *arrow* indicates a typical MV-like component. **c** is the merged image of **a** and **b**. An *arrow* indicates one of the double labeled MV-like components. **d** shows microglial cells and MV-like components with P2X7

receptor-ir at high magnification from the BBG-treated group 2 days after I/R injury. Note that BBG significantly reduced the number of MV-like components compared to **a**. An *arrow* indicates an MV-like component. **e** shows quantitative analysis of MV-like components with P2X7 receptor-ir after I/R injury. The number of MV-like components is expressed as mean ± S.E.M. ( $n=5$ ). Note that MV-like components were only detected from 2 to 4 days after I/R injury, BBG reduced significantly the number of MV-like components.  $**p<0.01$  BBG-treated rats vs. saline-treated rats ( $n=5$ )

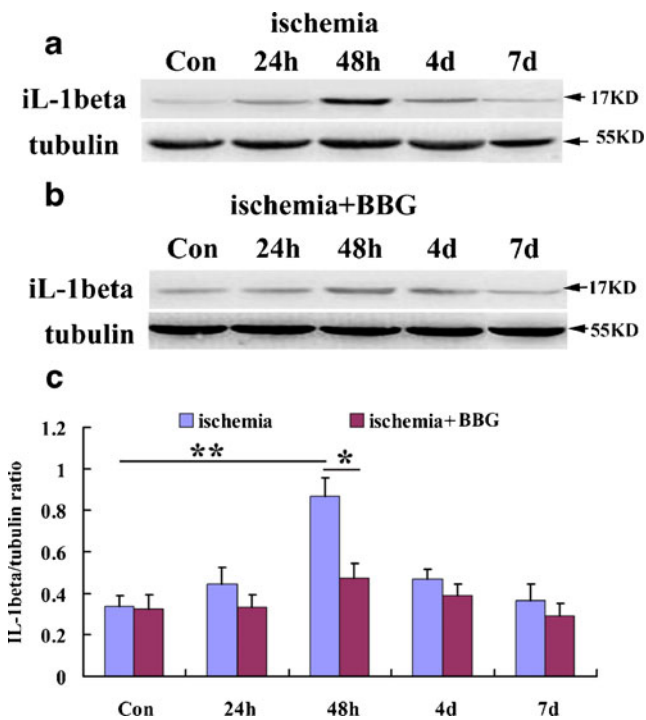
P2X7 receptor has been reported to play a role in cell-to-cell communication [20], cell proliferation [10], cytokine release [29, 44, 67], and cell death [71], and microglial activation and morphological transformation [51].

Increasing evidence shows that the P2X7 receptor plays a key role in neuroinflammation [69]. It is activated by ATP and regulates the release of the pro-inflammatory cytokine interleukin-1 (IL-1) [29, 44, 67]. IL-1 is a key mediator of experimentally induced neurodegeneration [4, 69]. Blockade of the action of IL-1 or deletion of the IL-1 gene was shown to

significantly mitigate neuronal injury induced by cerebral ischemia and excitotoxicity [4, 13]. These data indicate that IL-1 $\alpha$ , and  $\beta$  play important roles in the neuronal injury induced by ischemia.

Recent evidence showed that MV shedding represents the major mechanism mediating secretion of inflammatory cytokines, such as IL-1 $\beta$ , from reactive microglial cells. P2X7 receptors play a key role in MV shedding and IL-1 $\beta$  secretion from reactive microglial cells [72]. Although the molecular mechanisms of P2X7 receptor-dependent MV shedding and

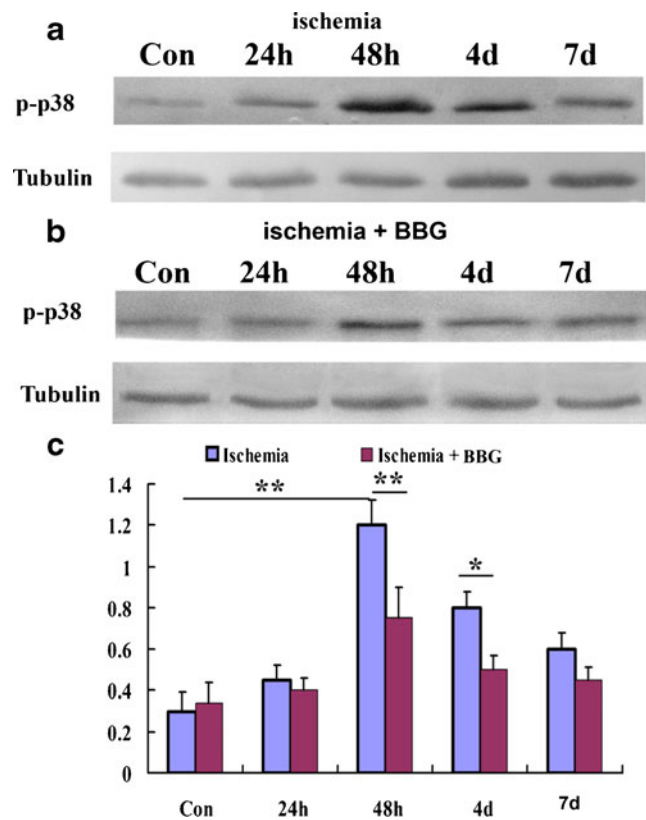




**Fig. 6** Effect of BBG on the expression of IL-1 $\beta$  in the rat hippocampus after I/R injury. **a** sham control (Con), 24h, 48h, 4d, 7d represent the expression levels of IL-1 $\beta$  and tubulin in the control and 24h, 48h, 4d, and 7d I/R injury groups, respectively. **b** sham control (Con), 24h, 48h, 4d, 7d represent the expression levels of IL-1 $\beta$  and tubulin in the control and 24h, 48h, 4d, and 7d I/R+BBG-treated groups, respectively. **c** Ratio of IL-1 $\beta$  and tubulin immunostaining signals in control (Con), I/R injury and I/R+BBG-treated groups. The ratio of IL-1 $\beta$  and tubulin in the Western blot is expressed as mean  $\pm$  S.E.M. ( $n=5$ ). A significant difference in IL-1 $\beta$  expression between sham and I/R injured rats, I/R injury and I/R-treated rats was detected at 48 h after I/R injury (\*\* $p<0.01$ , \* $p<0.05$ )

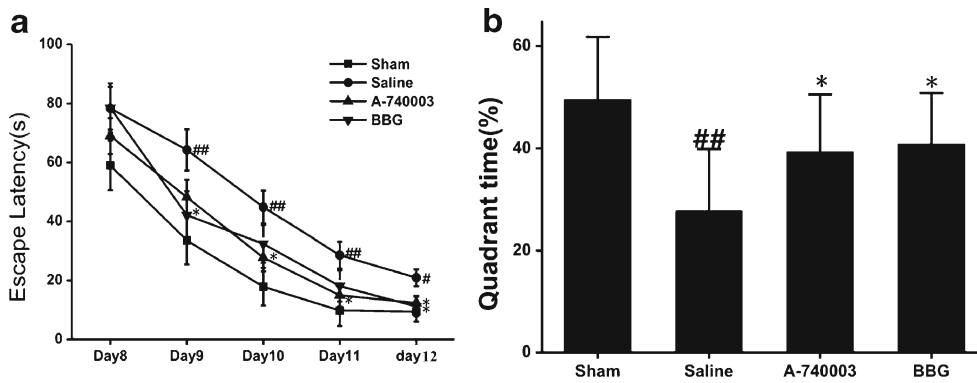
IL-1 $\beta$  secretion have not been well clarified, several key molecules such as ROCK and P38 MAPkinase, and acid sphingomyelinase (A-SMase) were shown to be involved [5, 11, 56, 62]. Following P2X7 receptor activation, a src-protein tyrosine kinase interacts with the P2X7 receptor C-terminus and promptly phosphorylates P38 MAPkinase. P38 phosphorylation, in turn, induces translocation of A-SMase to the plasma membrane outer leaflet, where it generates ceramide, thereby inducing budding of MVs [72]. These key molecules (src-protein tyrosine kinase, P38 MAP kinase, A-SMase and P2X7 receptors) might be future therapeutic targets of neuronal injury after I/R.

Massive release of purines occurs after metabolic stress and trauma, and high levels of ATP persist in the injured zone for an extended time [23, 30, 79]. ATP is an important signaling molecule mediating interactions among various cell types in the brain [30, 34, 36]. A low level of extracellular ATP is maintained in physiological conditions [84], but in pathological conditions, high concentrations of ATP leak into the extracellular fluid from injured cells



**Fig. 7** Effect of BBG on the expression of phosphorylated P38 in the rat hippocampus after I/R injury. **a** sham control (Con), 24h, 48h, 4d, 7d represent the expression levels of phosphorylated P38 and tubulin in the control and 24h, 48h, 4d, and 7d I/R injury groups, respectively. **b** sham control (Con), 24h, 48h, 4d, 7d represent the expression levels of phosphorylated P38 and tubulin in the control and 24h, 48h, 4d, and 7d I/R+BBG-treated groups, respectively. **c** Ratio of phosphorylated P38 and tubulin immunostaining signals in control (Con), I/R injury and I/R+BBG-treated groups. The ratio of phosphorylated P38 and tubulin in the Western blot is expressed as mean  $\pm$  S.E.M. ( $n=5$ ). Significant differences in phosphorylated P38 expression between sham and I/R injured rats, I/R injury and I/R-treated rats were detected at 48 h and 4 days after I/R injury (\*\* $p<0.01$ , \* $p<0.05$ )

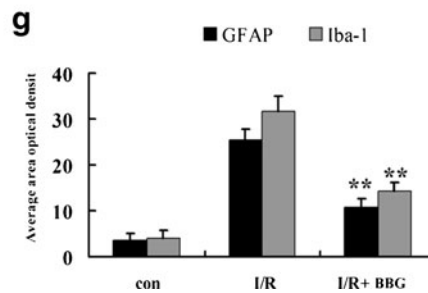
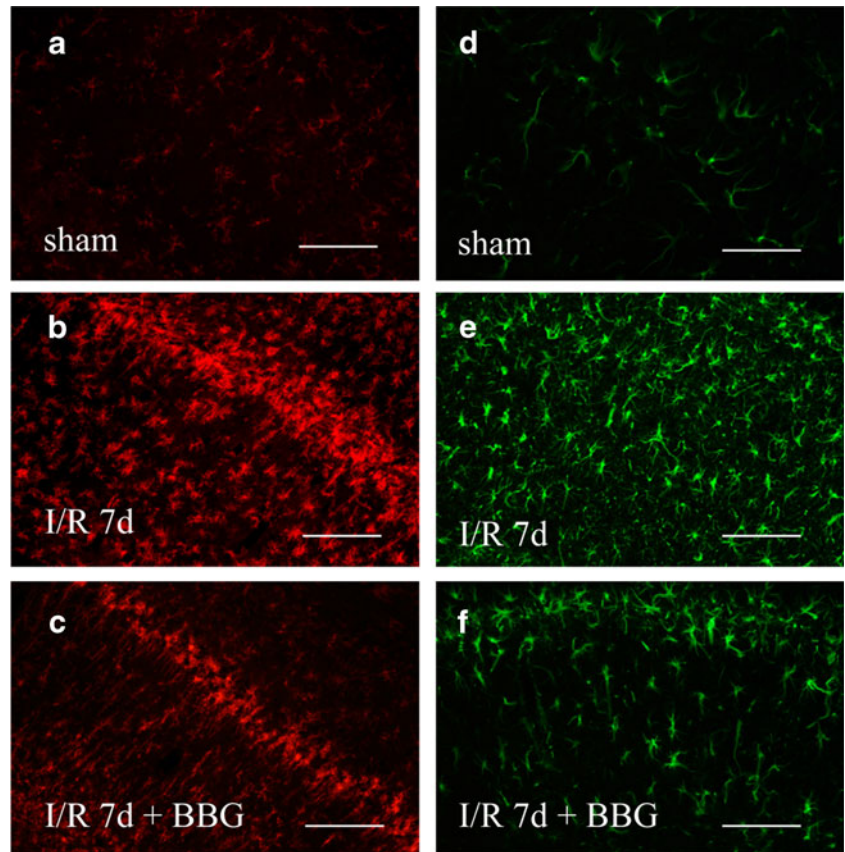
[23, 30, 79]. The leaked ATP is not rapidly degraded during inflammation or oxidative stress conditions, as the enzymes for extracellular ATP degradation are inhibited [65]. High extracellular concentrations of ATP act on the nerve cells in the local regions via P2 receptors. MV shedding from microglial cells is P2X7-dependent and high concentrations of extracellular ATP are needed to activate microglial cells and induce MV shedding [72]. This phenomenon suggests that at 48 h after I/R injury extracellular ATP in the CA1 region might be high enough to induce microglial cells to shed MVs because of the low affinity of PX7 receptors to ATP [28]. In this study, numerous MV-like cell components with P2X7 receptor-ir were detected in the CA1 region at 48 h after I/R injury, suggesting that there was a high concentration of extracellular ATP in this region at the time, although the sources of ATP need to be resolved.



**Fig. 8** Effect of A74003 and BBG on spatial learning and memory in the water maze. **a** shows escape latency to find the platform during the 5 days of the training trial from the 8th to the 12th day after I/R injury. **b** shows time spent during the probe trial in the quadrant where the platform had been located. Values are expressed as mean ± S.E.M.

##  $p < 0.01$  versus the relevant value of the sham group on the same day; #  $p < 0.05$  versus the relevant value of the sham group on the same day; \*  $p < 0.05$  BBG- or A74003-treated group versus the relevant value of the saline group on the same day. *BBG* Brilliant blue G

**Fig. 9** Effect of BBG on microgliosis and astrogliosis in the CA1 region after I/R injury. **a, b, c** show microglial cells with P2X7 receptor-ir in control, I/R injury 7d and I/R+BBG 7d groups, respectively. **d, e, f** show astrocytes with GFAP-ir in control, I/R injury 7d and I/R+BBG 7d groups, respectively. Microgliosis and astrogliosis were observed after I/R injury and BBG significantly inhibited these responses. **g** shows the quantitative analysis of average area optical density (AAOD) of microglial cells with P2X7 receptor-ir and astrocytes with GFAP-ir. Data are expressed as mean ± S.E.M ( $n=5$ ). \*\*  $p < 0.01$ , BBG-treated group versus saline group



MV shedding represents the major mechanism mediating secretion of IL-1 $\beta$  from reactive microglial cells [72]. The P2X7 antagonist, BBG, significantly reduced the number of MV-like cell components and the level of IL-1 $\beta$  and p-P38 expression of the hippocampus at 48 h after I/R injury, suggesting in vivo ATP via P2X7 receptors might be involved in MV-like component shedding and ATP via P2X7 receptors plays an important role in neuronal damage after I/R injury.

In this study, up-regulation of P2X7 receptor protein on microglial cells in the CA1 region during I/R injury was detected by both immunohistochemistry and Western blotting. The expression of P2X7 receptor protein was up-regulated before the delayed neuronal death in the CA1 region at 48 h after I/R injury, suggesting that up-regulation of P2X7 receptor protein on microglial cells at the early stages of I/R injury was not induced by delayed neuronal death.

Microglial activation is an integral part of neuroinflammation associated with many neurodegenerative conditions, such as Alzheimer disease, Parkinson's disease, and multiple sclerosis [48, 70]. Increased expression of P2X7 receptors was detected in activated microglia in neuroinflammatory foci [48, 57]. Recent evidence showed that over-expression alone of the P2X7 receptor is sufficient to drive the activation of microglia in rat primary hippocampal cultures [51]. In this study, we found enhanced P2X7 receptor expression on microglial cells in the rat hippocampal CA1 region before delayed neuronal death occurred. Thus, we speculate that the increased P2X7 receptor expression on microglial cells could be one of the factors that cause the activation of microglial cells.

If the enhanced expression of P2X7 receptors on microglial cells in the CA1 region after I/R injury is one of the causes that drive microglial cell activation and induce delayed neuronal death, then it would be important to find out the causes of the up-regulation. Previous evidence showed that interferon- $\gamma$  (IFN- $\gamma$ ) and tumor necrosis factor  $\alpha$  (TNF- $\alpha$ ) synergistically induce P2X7 mRNA and functional responses in the human THP-1 monocytic cell line in a dose-dependent manner [35]. Therefore, inhibition of IFN- $\gamma$  and TNF- $\alpha$  production or their receptors in microglial cells could be a way of decreasing P2X7 receptor expression.

In summary, this study has shown that P2X7 receptor antagonists partially rescue the delayed neuronal death in cerebral I/R injury and improve memory functions. IL-1 $\beta$  carried by MV components might be an important contributor to delayed neuronal death of the hippocampus CA1 region following transient global cerebral I/R injury. These results indicate that blockade of the P2X7 receptor and signaling pathways of microglial MV shedding, such as src-protein tyrosine kinase, P38 MAP kinase and A-SMase, might be a promising therapeutic strategy for clinical treatment of transient global cerebral I/R injury.

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**Conflict of interest** There are no conflicts of interest.

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