Neuroprotective effects of brilliant blue G on the brain following traumatic brain injury in rats

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Abstract. The P2X7 inhibitor, brilliant blue G (BBG), has been reported as a neuroprotective drug against a variety of disorders, including neuropathic pain and brain ischemia. Currently, no studies have examined the potential for BBG to provide neuroprotection in animal models of TBI. The aim of the present study was to investigate the neuroprotective effect of BBG on TBI and to determine the underlying mechanisms. The rats were subjected to a diffuse cortical impact injury caused by a modified weight-drop device, and then divided randomly into three groups: the sham-operated, BBG treatment and vehicle groups. In the BBG treatment group, 50 mg/kg brilliant blue G (BBG; 100% pure), a highly specific and clinically useful P2X7 antagonist, was administered via the tail vein 15 min prior to or up to 8 h following TBI. The co-localization of NeuN and protein kinase Cγ (PKCγ) was followed with immunofluorescent staining. The expression of P2X7, PKCγ and inflammatory cytokines was identified by western blot analysis. Wet-dry weight method was used to evaluate brain edema, and motor function outcome was examined using the neurological severity score. The present study demonstrated that the administration of BBG attenuated TBI-induced cerebral edema and the associated motor deficits. Following trauma, BBG treatment significantly reduced the levels of PKCγ and interleukin-1β in the cortex. The results provide *in vivo* evidence that BBG exerted neuroprotective effects by attenuating brain edema and improving neurological functions via reducing PKCγ and interleukin-1β levels following TBI.

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Abbreviations: TBI, traumatic brain injury; BBG, brilliant blue G; PKCγ, protein kinase Cγ; NeuN, neuron-specific nuclear protein; DAPI, 4',6-diamidino-2-phenylindole; NSS, neurological severity score; IL-1, interleukin-1

Key words: brilliant blue G, P2X7, neuroprotection, traumatic brain injury, rats

Introduction

Traumatic brain injury (TBI) is a complex and devastating clinical condition mediated at least in part by pro-inflammatory cytokines that produce neuronal loss, axonal destruction and demyelination during the secondary injury cascade (1). In contrast to primary injuries that occur at the time of impact, secondary pathological processes develop while under supervised medical care and profoundly affect patient recovery (2,3). These processes trigger an increase in ATP release and the activation of the microglia, which leads to neuronal cell death and the release of inflammatory factors (4,5). Identifying novel strategies to inhibit the upregulation of harmful factors following increased ATP release and to reduce the release of inflammatory factors, has provided a new window of opportunity for the clinical treatment of TBI.

Neuronal cell death is the main cause of neurological deficits following TBI, and glial cells have an important role in triggering the processes that lead to apoptosis. Microglia represent the most abundant time of normal central nervous system (CNS) immune cell and are widely distributed, accounting for ~20% of the total number of glial cells in the brain and spinal cord. The role of the guardian of the actor, the inlet to the role of the warning system, also taking into account the protection and restoration (6). Microglia activation is associated with a number of CNS diseases, including Alzheimer's, Parkinson's disease, amyotrophic lateral sclerosis and multiple sclerosis (7-11). In certain pathological conditions, including brain trauma, ischemia, inflammation and others, local cell damage induces the release of large amounts of ATP and degradation products, and when the concentrations of these substances increase locally, nearby microglia are activated (12). Microglial cells are sensitive to changes in the surrounding environment and a variety of physiological and pathological factors activate rapid microglia hyperplasia, induce an 'activated' amoeba-like morphology that includes increasing cell body size, shortening of processes, and also trigger the release of white blood cell interleukin-1β (IL-1β),

interleukin-6, cyclooxygenase -2 and other inflammatory factors. These factors not only cause damage to the neighboring neurons, but also convene a series of immune cells into the CNS causing further damage.

Purinergic P2X7 receptors mediate, at least in part, the biological actions of extracellular ATP (13). Sustained activation of P2X7 with high concentrations of ATP induces the release of biologically active IL-1β (14,15), a potent pro-inflammatory cytokine. Notably, IL-1β exhibited a prolonged induction in multiple pre-clinical models of TBI (16-21), and increased IL-1β levels in the CSF and brain was positively correlated with elevated intracranial pressure (ICP) and unfavorable outcomes in TBI patients (22-24). Furthermore, it has been previously demonstrated that genetic or pharmacological inhibition of IL-1β attenuates cerebral edema and secondary injury following TBI (25-28), indicative of a deleterious role for IL-1β after head trauma. Therefore, once the role of IL-1β in TBI has been fully elucidated, it may provide opportunities for the development of novel therapeutic strategies. As a robust inflammatory response that clinically correlates with secondary neurovascular injury following TBI, it was hypothesized that activation of P2X7 mediates neurological demise following TBI via attenuation of IL-1β.

The gamma isotype of protein kinase C (PKCγ) is a member of the classical PKC (CPKC) subfamily which is activated by Ca^{2+} and diacylglycerol in the presence of phosphatidylserine (29-31). PKCγ is expressed solely in the brain and spinal cord and its localization is restricted to neurons (32). Within the brain, PKCγ levels are most abundant in the cerebellum, hippocampus and cerebral cortex, where notable neuronal plasticity occurs (33,34). Recently, Matsumoto *et al* demonstrated that the PKCγ expression was significantly increased following TBI in rats (35). Nevertheless, to the best of our knowledge, no studies have examined the potential for BBG to regulate the expression of PKCγ in neurons in an animal model.

The present study aimed to investigate the hypothesis that the P2X7 inhibitor, BBG, induced neuroprotective properties via reducing PKCγ and the levels of inflammatory cytokine IL-1β following TBI.

Materials and methods

Animals. A total of 150 Sprague-Dawley rats (obtained from Hebei United University Experimental Animal Center, Tangshan, Hebei, China), weighing 280-320 g, were housed under a 12 h light/dark cycle with regular food and water supply. All experimental procedures were conducted in conformity with the Institutional Guidelines for the Care and Use of Laboratory Animals of Hebei United University, (Shijiazhuang, China) and all procedures were performed in accordance with the National Institutes of Health Guide for the Care and Use of Laboratory Animals (NIH Publication no. 80-23, revised 1996).

Models of TBI. A rat model of TBI was established by using a modified weight-drop device, as described previously by Marmarou *et al* (36). Briefly, the rats were anesthetized with sodium pentobarbital (Nembutal, 60 mg/kg) prior to the surgery. A midline incision was performed to expose the skull between the bregma and lambda suture lines, and a steel disc (10 mm in diameter and 3 mm thickness) was adhered to the skull using dental acrylic. Following this, the rats were placed on a foam mattress underneath a weight-drop device in which a 450 g weight falls freely through a vertical tube from 1.5 m onto the steel disk. The sham-operated animals underwent the same surgical procedure without being exposed to percussion injury, but no trauma was induced. Following surgery, the rats received supporting oxygenation with 95% O_2 for no longer than 2 min and were returned to their cages. All of the rats were housed in individual cages and placed on heat pads (37˚C) for 24 h to maintain normal body temperature during the recovery period.

Group and drug administration. The rats were randomly assigned to the sham-operated group (sham; n=30), TBI treated with BBG group (BBG; n=60) and TBI received only equal volumes of 0.9% saline solution (vehicle; n=60). BBG was dissolved in 2% sucrose water and stored at 4˚C. Following the brain injuries, BBG was immediately administered to the rats of the BBG group following TBI as a tail vein injection (50 mg/kg body weight). All of the tests were blinded, and the animal codes were revealed only at the end of the behavioral and histological analyses.

Immunofluorescence. The brain tissues were fixed in 4% paraformaldehyde for 24 h, and placed into 30% sucrose solution with 0.1 mol/l phosphate-buffered saline (PBS; pH 7.4) until sinking to the bottom. The tissues were divided 200 μm apart from each section from anterior to posterior hippocampus (bregma -1.90 to -3.00 mm) from TBI rats, and then embedded in OCT. Frozen sections (15 μm) were sliced with a frozen slicer, treated with 0.4% Triton X-100 for 10 min and blocked in normal donkey serum for 1 h. For double labeling, the frozen sections were incubated with a mixture of rabbit anti-microtubule-associated protein 1 PKCγ polyclonal antibody (Santa Cruz Biotechnology, Inc., Santa Cruz, CA, USA; diluted 1:100) and mouse anti-neuron-specific nuclear protein (NeuN) polyclonal antibody (Santa Cruz Biotechnology, Inc.; diluted 1:100) overnight at 4˚C. The next day, the sections were incubated with a mixture of fluorescein-conjugated anti-rabbit IgG and anti-mouse IgG (Santa Cruz Biotechnology, Inc.; diluted, 1:1,000) for 2 h at 37˚C in the dark. All cell nuclei were counterstained by 4',6-diamidino-2-phenylindole (DAPI). The images were captured in a laser scanning confocal microscope (Olympus FV1000). Primary antibodies were replaced with PBS in the negative control group.

Western blot analysis. Briefly, rats were anesthetized and underwent intracardiac perfusion with 0.1 mol/l PBS (pH 7.4). The cortex of the brains were rapidly isolated, the total proteins were extracted and the protein concentration was determined by the BCA reagent (Solarbio, Beijing, China) method. Samples were subjected to sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE). Separated proteins on the gel were transferred onto PVDF membranes (Roche Diagnostics, Mannheim, Germany). The blots were blocked with 5% fat-free dry milk for 1 h at room temperature. Following blocking, the membrane was incubated with the indicated primary antibodies overnight at

4˚C, including rabbit anti-PKCγ polyclonal antibodies (Santa Cruz Biotechnology, Inc.; diluted 1:500), rabbit anti-P2X7 polyclonal antibodies (Santa Cruz Biotechnology, Inc.; diluted 1:500), rabbit anti-IL-1β polyclonal antibody (Santa Cruz Biotechnology, Inc.; diluted 1:500), mouse anti-β-actin monoclonal antibody (Santa Cruz Biotechnology, Inc.; diluted 1:500). Next, the membrane was incubated with horseradish peroxidase conjugated anti-rabbit IgG and anti-mouse IgG (Cell Signaling Technology, Inc., Danvers, MA, USA; diluted 1:5,000) for 2 h at room temperature. Following incubation with a properly titrated secondary antibody, the immunoblot on the membrane was visible following development with an enhanced chemiluminescence (ECL) detection system and the densitometric signals were quantified using an imaging program. Immunoreactive bands of all protein expression were normalized to the intensity of the corresponding bands for β-actin. The western blotting results were analyzed with National Institutes of Health Image 1.41 software (Bethesda, MD, USA).

Evaluation of brain edema. Brain edema was examined by analysis of brain water content as described previously (37). The rat brains were separated and weighed immediately with a chemical balance to obtain the wet weight (WW). Following drying in a desiccating oven for 24 h at 100˚C, the dry tissues were weighed again to obtain the constant dry weight (DW). The percentage of water in the tissues was calculated according to the formula: % brain water = [(WW-DW)/(WW) x 100].

Recovery of motor function. The neurobehavioral status of the rats was determined using a set of ten tasks, collectively termed the neurological severity score (NSS), which examines reflexes, alertness, coordination and motor abilities. One point is awarded for failure to perform a particular task, thus, a score of 10 reflects maximal impairment, whereas a normal rat scores 0 (38). Post-injury, NSS was evaluated at 12 and 24 h. Each animal was assessed by an observer who was blinded to the animal treatment. The difference between the initial NSS and that at any later time was calculated for each rat, and this value (ΔNSS) reflects the spontaneous or treatment-induced recovery of motor function.

Statistical analysis. All data are presented as the mean ± SD. SPSS 16.0 (SPSS, Inc., Chicago, IL, USA) was used for statistical analysis of the data. Statistical analysis was performed using analysis of variance (ANOVA) and followed by the Student-Newman-Keuls post hoc tests. $P<0.05$ was considered to indicate a statistically significant difference.

Results

Treatment of BBG attenuates TBI-induced cerebral edema. The wet-dry weight method was used to examine brain edema. As demonstrated in Fig. 1, BBG post-injury administration attenuated cerebral edema following TBI. In the vehicle group, the brain water content was significantly increased compared with the sham group at 12 and 24 h following injury. The tissue water content in the BBG treatment group was significantly reduced at 24 h compared with the vehicle group.

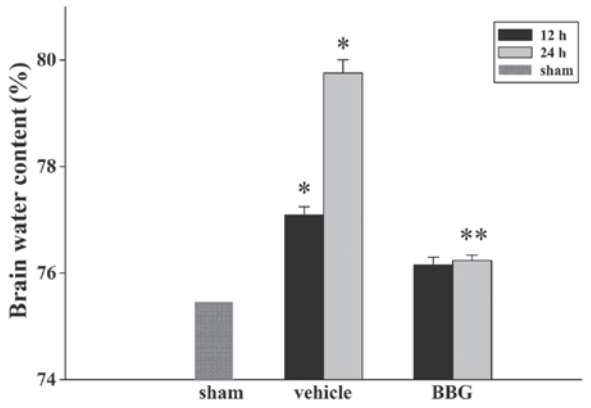

Figure 1. The effect of BBG on brain edema. Brain water content was determined at 12 and 24 h following TBI and calculated as the percentage of dry and wet weight method. Bars represent the mean ± standard deviation (n=5/group). Brain water content increased markedly at 12 and 24 h following TBI (*P<0.01 vs. sham group). Administration of BBG significantly decreased brain edema (**P<0.05 vs. TBI group) at 24 h as reflected by a decrease in brain water content. TBI, traumatic brain injury; BBG, brilliant blue G.

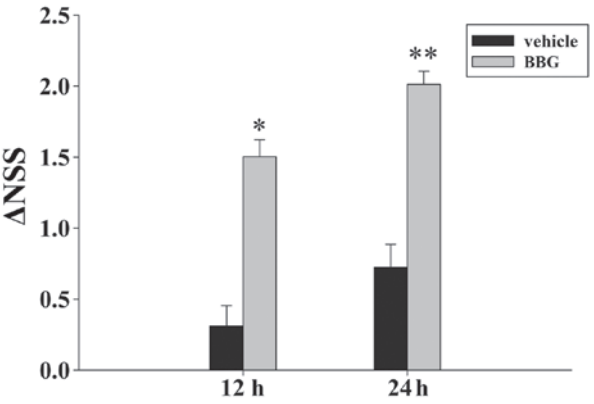

Figure 2. The effect of BBG on TBI-induced motor deficits. The temporal changes in motor recovery of the rat was determined at 12 and 24 h following TBI and calculated as the ΔNSS. Bars represent the mean ± standard deviation (n=5/group). Administration of BBG significantly improved motor function following TBI (**P<0.01 vs. TBI group). TBI, traumatic brain injury; BBG, brilliant blue G; NSS, neurological severity score.

Treatment of BBG attenuates TBI-induced motor deficits. Fig. 2 depicts the temporal changes in functional recovery of the rat, expressed as ΔNSS. It is evident that post-injury administration of BBG improved the motor function recovery of the trauma rats at 12 and 24 h following TBI.

Treatment of BBG inhibits P2X7 expression in the cortex following TBI. The expression levels of P2X7 in the rat cortex at 12 and 24 h were measured by western blot analysis (Fig. 3A). As demonstrated in Fig. 3B, the P2X7 levels in the sham rat cortex at the two time points following injury were consistently presented in a low background. In the vehicle group, all measured P2X7 levels exhibited significant increases following injury. Administration of BBG produced significant reductions in the injury-induced upregulation of P2X7.

Treatment of BBG suppresses inflammatory cytokine levels in the cortex following TBI. The levels of IL-1β in the cortex at

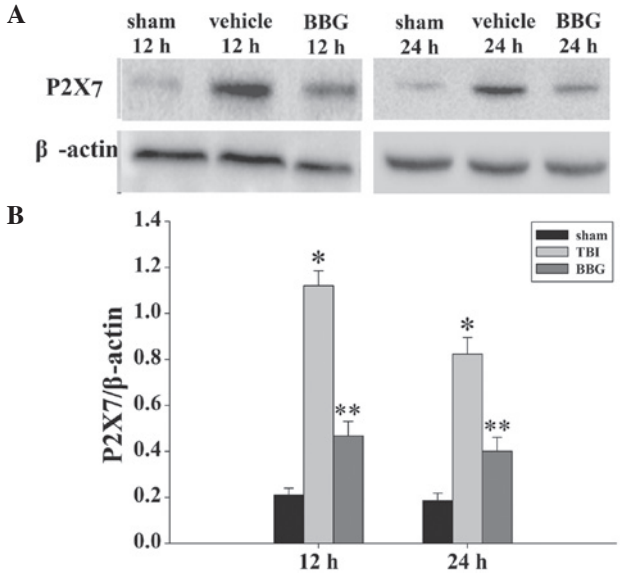

Figure 3. (A) Western blot analysis demonstrating the expression levels of P2X7 in the cortex of rats at 12 and 24 h following TBI or sham operation. (B) The quantitative results of P2X7 are expressed as the ratio of densitometries of P2X7 to β-actin bands. Bars represent the mean ± standard deviation (n=5/group). The results demonstrated a significantl increase of P2X7 expression in the TBI group (*P<0.01 vs. sham group). Treatment with BBG caused a significant downregulation of P2X7 expression at 12 and 24 h (**P<0.01 vs. vehicle group). TBI, traumatic brain injury; BBG, brilliant blue G.

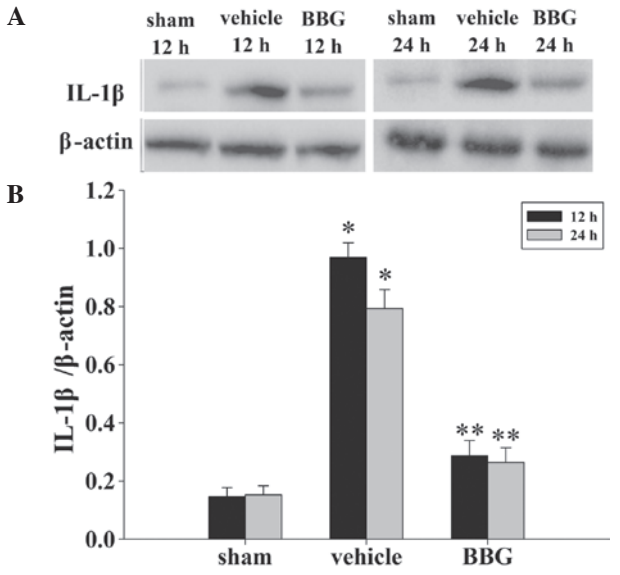

Figure 4. (A) Western blot analysis demonstrating the expression levels of IL-1β in cortex of rats at 12 and 24 h following TBI or sham operation. (B) The quantitative results of IL-1β were expressed as the ratio of densitometries of IL-1β to β-actin bands. Bars represent the mean ± standard deviation (n=5/group). The results demonstrated a significant increase in the IL-1β expression in the vehicle group (*P<0.01 vs. sham group). Treatment with BBG caused a significant downregulation of IL-1β expression at 12 and 24 h (**P<0.05 vs. vehicle group). TBI, traumatic brain injury; BBG, brilliant blue G; IL-1β, interleukin-1β.

12 and 24 h were measured by western blot analysis(Fig. 4A). As demonstrated in Fig. 4B, the IL-1β levels in the sham rat cortex at the two time points following injury were in a low background. The IL-1β expression levels were significantly increased in the vehicle group. By contrast, in the BBG group,

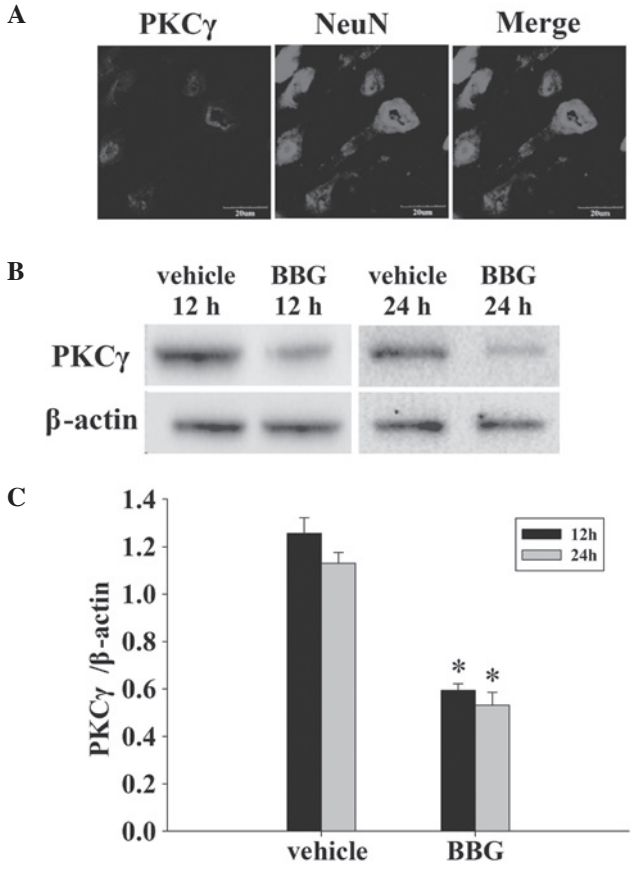

Figure 5. (A) Co-localization of NeuN and PKCγ at 24 h following TBI was determined by immunofluorescent staining (magnification, x400). (B) Western blot analysis demonstrates the expression levels of PKCγ and β-actin in the hippocampus at 12 and 24 h following TBI. (C) Densitometry of PKCγ band associated with β-actin band. Bars represent the mean ± standard deviation (n=5/group). The results demonstrated that administration of BBG significantly decreased the level of PKCγ expression at 12 and 24 h following TBI (*P<0.05 vs. vehicle group). TBI, traumatic brain injury; BBG, brilliant blue G; PKCγ, protein kinase Cγ; NeuN, neuron-specific nuclear protein.

treatment with BBG produced marked reductions in the injury-induced upregulation of IL-1β expression.

Treatment of BBG attenuates PKCγ in neurons following TBI. The co-localization of NeuN and PKCγ was examined with immunofluorescent staining at 24 h. As demonstrated in Fig. 5A, the vast majority of PKCγ expression following TBI was localized to neurons. Following this, whether BBG attenuated the expression of PKCγ was determined by western blot analysis (Fig. 5B). As demonstrated in Fig. 5C, at 12 and 24 h following TBI, administration of BBG significantly attenuated the PKCγ protein expression in rat cortex compared with the vehicle group.

Discussion

TBI most commonly results in chronic neurological abnormalities, including cognitive deficits, emotional disturbances and motor impairments, and represents a severe international health concern. Microglia activation and secretion of inflammatory factors in the post-traumatic brain promotes clinical deterioration and worsens long-term outcomes, at least in part,

by inflammatory cytokine production, infiltration of immune cells into the CNS, and by increasing the manifestation of neurological impairments, including headaches, anxiety, depression, sleep disturbances, cognitive dysfunction and appetite loss (39,40). Therefore, elucidation of the cellular mechanisms of neurological injury may permit the development of efficacious therapeutics to improve patient outcomes after TBI.

The biological actions of ATP are mediated, at least in part, by the activation of either metabotropic P2Y receptors or ionotropic P2X receptors (13). Among the purine receptor family members, P2X7 is a low-affinity receptor that preferentially responds to sustained elevations in ATP, including those which occur following trauma, which suggests that P2X7 possesses the optimal biophysical properties for mediating the detrimental actions of ATP after brain injury. Observations from a study by Wang *et al* demonstrated that spinal cord injury was associated with prolonged purinergic receptor activation, which results in excitotoxicity-based neuronal degeneration. Furthermore, P2X7R antagonists inhibited this process, reducing the histological extent and functional consequences of acute spinal cord injury (41). The present study used BBG on a rat model of TBI, and demonstrated that BBG inhibition of P2X7 reduced secondary brain injury and improved the functional outcomes following moderate TBI in mice. Therefore, it was hypothesized that the inhibitor also has an markedly important role in the CNS recovery following TBI. Similar neuroprotective effects of BBG for TBI have been reported by Kimbler *et al*, that demonstrated that BBG reduced post-traumatic cerebral edema with an extended therapeutic window. Their study focussed on the effects of P2X7 expression of aquaporin aqp4, including reducing edema and increased intracranial pressure following TBI (42). These results are consistent with the earlier evidence, and the data of the present study also reported that BBG significantly reduced the expression of PKCγ in neurons and the levels of the inflammatory factor IL-1β.

IL-1β is an important pro-inflammatory cytokine that mediates a variety of host defense responses to tissue injury and exogenous antigens. Accumulative evidence demonstrates the presence of P2X7 receptors on microglia and the activation of these receptors by ATP triggers microglia release of IL-1β (43). Blockade of P2X7R has accordingly been demonstrated to attenuate microglial activation and inflammation in spinal cord injury (6). In the present study, it was observed that administration of the P2X7R inhibitor BBG significantly reduced the expression of IL-1β without any evident toxicity. These results are consistent with the previous findings of Peng *et al* (44) and it is therefore conceivable to hypothesize that the mechanism underlying the neuroprotective effect of BBG on TBI may be associated with the attenuation of the levels of inflammatory cytokine IL-1β.

PKCγ is predominantly expressed in the CNS. Staurosporine, a PKCγ inhibitor, has been demonstrated to reduce ischemic cell death *in vivo* (45). Hypothermia and substances that diminish PKC translocation to cell membranes are known to be neuroprotective (46). In the present study, it was identified that PKCγ expression in the injured rat hippocampus of brain was suppressed by BBG. It may therefore be one of the mechanisms that underlies the neuroprotective effects of BBG following TBI.

Of note, BBG is a derivative of a commonly used blue food color (FD&C blue no. 1), which crosses the blood-brain barrier. Systemic administration of BBG may thus comprise a readily feasible approach by which to treat TBI in humans. However, in the present study, several considerations must be further considered. Although BBG is considered as a highly selective P2X7 antagonist, it also inhibits both P2X2 and P2X5, albeit less potently than that of P2X7 (47). In the present study, while BBG demonstrated protective and potential therapeutic effects in a rat model of TBI, the possibility that off-target effects on receptors other than P2X7 mediated the beneficial actions of BBG may not be excluded. Therefore, further studies are required to identify more specific inhibitors of P2X7, or to develop a $P2X7^{(-/-)}$ rat.

In conclusion, the present study demonstrated that BBG was able to attenuate secondary brain edema and improve cognitive function following TBI. The upregulation of PKCγ and IL-1β was also attenuated by post-injury treatment of BBG in rats. These findings emphasize that BBG administration immediately following TBI may be neuroprotective, and that this effect may be associated with the attenuation of PKCγ and IL-1β expression. The present study provides a viable therapeutic window for the development of novel clinical treatment strategies for TBI.

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