Inhibition of P2X4 and P2X7 receptors improves histological and behavioral outcomes after experimental traumatic brain injury in rats

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Abstract. Release of large amounts of adenosine triphosphate (ATP), a gliotransmitter, into the extracellular space by traumatic brain injury (TBI) is considered to activate the microglia followed by release of inflammatory cytokines resulting in excessive inflammatory response that induces secondary brain injury. The present study investigated whether antagonists of ATP receptors (P2X4 and/or P2X7) on microglia are beneficial for reducing the post-injury inflammatory response that leads to secondary injury, a prognostic aggravation factor of TBI. Adult male Sprague-Dawley rats were subjected to cortical contusion injury (CCI) and randomly assigned to injury and drug treatment conditions, as follows: i) No surgical intervention (naïve group); ii) dimethyl sulfoxide treatment after CCI (CCI-control group); iii) 5-BDBD (antagonist of P2X4 receptor) treatment after CCI (CCI-5-BDBD group); iv) CCI-AZ11645373 (antagonist of P2X7 receptor) treatment after CCI (CCI-AZ11645373 group); v) or 5-BDBD and AZ11645373 treatment after CCI (CCI-5-BDBD + AZ11645373 group). In the CCI-5-BDBD, CCI-AZ11645373, and CCI-5-BDBD + AZ11645373 groups, expression of activated microglia was suppressed in the ipsilateral cortex and hippocampus 3 days after the CCI. Western blotting with ionized calcium-binding adaptor molecule 1 antibody revealed that administration of CCI-5-BDBD and/or CCI-AZ11645373 suppressed expression of microglia and reduced expression of inflammatory cytokine mRNA 3 days after the CCI. Furthermore, the plus maze test, which reflects the spatial memory function and involves the hippocampal function, showed improvement 28 days after secondary injury to the hippocampus. These findings confirmed that blocking the P2X4 and P2X7 receptors, which are ATP receptors central in gliotransmission, suppresses microglial activation and subsequent cytokine expression after brain injury, and demonstrates the potential as an effective treatment for reducing secondary brain injury.

Introduction

The pathophysiology of traumatic brain injury (TBI) consists of primary brain injury and secondary brain injury. The primary injury results from the mechanical damage caused by the external force, whereas the secondary injury occurs due to various factors such as hypoxia, elevated intracranial pressure, metabolic disorder, brain edema, inflammation, etc. Therefore, the treatment of TBI focuses on the prevention or mitigation of the damage from the secondary injuries.

Astrocytes maintain the pH and the concentration of ions such as sodium and potassium in the extracellular space, supply oxygen and glucose to the nerve cells, and reabsorb the glutamate, a neurotransmitter released from nerve terminal. Consequently, astrocytes are essential for maintaining nerve cell homeostasis. On the other hand, microglia, another glial cell, is responsible for the inflammatory response in the brain. The concept of gliotransmission proposed in 1990 suggests that astrocytes may be crucial in signal transduction and constitute a long-distance signaling system in the brain (1-3). In response to an increase in the glutamate concentration in the extracellular space, including due to stress or insult such as ischemia or trauma, astrocytes increase the intracellular calcium level and release gliotransmitters, such as adenosine triphosphate (ATP), glutamate, and D-serine, into the extracellular space. When the ATP binds to P2 receptor on the cell surface of

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Abbreviations: ANOVA, one way analysis of variance; ATP, adenosine triphosphate; CCI, cortical contusion injury; DMSO, dimethyl sulfoxide; ELISA, enzyme-linked immunosorbent assay; GAPDH, glyceraldehyde-3-phosphate dehydrogenase; GFAP, glial fibrillary acidic protein; Iba-1, ionized calcium-binding adaptor molecule 1; IL, interleukin; PCR, polymerase chain reaction; TBI, traumatic brain injury; TNFα, tumor necrosis factor α

Key words: adenosine triphosphate, gliotransmitter, microglia, purinergic receptors, traumatic brain injury

adjacent astrocytes in a paracrine manner, signal transduction such as inositol trisphosphate pathway is activated and a large amount of calcium is released into the cell, and then these adjacent astrocytes are also activated as a group in a chain reaction (4). These streams are called 'calcium waves' (5,6), and also activate the microglia through the ATP receptor, namely P2 receptors. The P2 receptor is an ATP-gated ion channel receptor, classified as P2X and P2Y receptors. P2X receptor is a ligand-gated ion channel, and P2Y receptor is a G protein-coupled receptor. The P2X4 and/or P2X7 receptors on microglia are responsible for receiving ATP signals that will activate microglia to initiate several inflammatory responses such as migration to the damaged area, phagocyte brain debris, and release cytokines (7,8). Massive release of cytokines will cause excessive inflammatory response that results in secondary injury (9-13).

More recently, extracellular ATP concentration was shown to immediately increase after cortical contusion injury (CCI) in rats using microdialysis technique (9). The source of this rapid increase is the damaged brain cells which leak intracellular ATP and subsequent activation of neuronal cells and astrocytes that release ATP into the extracellular space, but is significantly attenuated by the selective P2Y1 receptor blocker, MRS2179, or store-operated calcium channels. Furthermore, in our previous study (14,15), in situ administration of MRS2179 successfully suppressed microglial activation in a rat CCI model, which can inhibit the initiation of inflammation. In this study, we focused on the ATP receptors, P2X4 and P2X7, which are more involved in microglial activation, and investigated whether antagonists of P2X4 and/or P2X7 could be beneficial for the post-injury inflammatory response that leads to secondary injury, a prognostic aggravation factor of TBI (16,17). As mentioned above, astrocytes are responsible for maintaining nerve cell homeostasis, so the receptors of microglia were targeted without suppressing these functions of astrocytes.

Materials and methods

Ethics. All experimental procedures were conducted according to the animal experimental protocol manuals at Nihon University School of Medicine. For example, rats with loss of postoperative weight to below 80% of the preoperative weight, or rats which did not exercise, eat or drink, or showing any signs of infection were considered to be hyperinvasive and were euthanized. However, there were no such rats in the present study.

Injury model. Male Sprague-Dawley rats (weighing 270 to 330 g) were used for the CCI model as previously described (18). Briefly, after induction of general anesthesia with 4% isoflurane in oxygen at 1.5 l/min, rats were fixed in a stereotaxic frame and anesthesia was maintained with 2% isoflurane during surgery. The body temperatures were maintained at 37.0˚C with a thermostatically controlled heating pad monitored by a rectal probe. Local anesthesia was applied to the scalp using subcutaneous injection of 0.1 ml 1% lidocaine (3.3 mg/kg), then the scalp was sterilized by repeated (three times) cleaning with Betadine (Dynarex Corp., Orangeburg, NY) followed by 70% ethanol. A midline skin incision was then made and the pericranium was spread bilaterally. A 6.0 mm diameter circular craniotomy was made, 3.5 mm lateral (left) and 3.0 mm posterior from the bregma. CCI was made using a 4.0 mm diameter flap-tip impactor with fixed magnitude such as depth (2.0 mm), velocity (3.5 m/sec), and dwell time (100 msec). This intensity is known to induce cortical brain contusion and delayed hippocampus cell death, but will not mechanically damage the hippocampus (15,19). Immediately after CCI, an Alzet Brain Infusion Kit 2 (Alzet 8663; DURECT Corp., Cupertino, CA) was implanted 2.0 mm deep from the brain surface at the center of contusion, connected with an osmotic pump (Alzet Mini-Osmotic Pump Model 2001; DURECT Corp.) and implanted subcutaneously. Surgical osmotic pump implantation was completed within 15 min in all cases, and continuous drug administration was initiated. After surgery, rats were placed in a heated recovery box until ambulatory and then kept in individual cages.

Treatment. The P2X4 receptor antagonist 5-BDBD (SML0450; Sigma-Aldrich, St. Louis, MO), P2X7 receptor antagonist AZ11645373 (A7231; Sigma-Aldrich) or dimethyl sulfoxide (DMSO) as a control drug was administered from the implanted osmotic pump. Both 5-BDBD and AZ11645373 were prepared to 1.0 mM in DMSO and given at 1.0 μl/h flow rate. Drugs were injected for 3 days [1 μl/h (total 72 μl) over 3 days, containing 0.085 mg/kg of 5-BDBD or 0.111 mg/kg of AZ11645373], because cytokine releases are prominent in this period according to the previous reports (20). AZ11645373 is a highly selective and potent antagonist of the human P2X7 receptor, but is less active in rats (less than 50% inhibition at 10 mM), as evaluation using HEK cells and THP-1 monocytes (human cells derived from monocytic leukemia that differentiate into macrophages) found lower activity against rat macrophages (21). Macrophages invade the central nervous system after trauma-induced disruption of the BBB (22). The present study focused on microglia and used AZ11645373 with lower activity on rat macrophages.

All animals were randomly assigned to five groups: no surgical intervention (naïve group), DMSO treatment after CCI (CCI-control group), 5-BDBD treatment after CCI (CCI-5-BDBD group), CCI-AZ11645373 treatment after CCI (CCI-AZ11645373 group), and 5-BDBD and AZ11645373 treatment after CCI (CCI-5-BDBD + AZ11645373 group).

Immunohistological staining. Immunostaining was performed to evaluate the expression of microglia. Three days after CCI, rats of the naïve group (n=2), CCI-control group (n=2), CCI-5-BDBD group (n=2), CCI-AZ11645373 group (n=2), and CCI-5-BDBD + AZ11645373 group (n=2) were deeply anesthetized with intraperitoneal injection of lethal dose pentobarbital (100 mg/kg). Brains were removed after transcardiac perfusion of 200 ml physiological saline followed by 200 ml of 4% paraformaldehyde. After fixing in the same fixative for 24 h, brains were immersed in 10, 20, and 30% gradient sucrose solution (24 h each) for cryoprotection. Brains were flash frozen and sliced to 20 μm thickness by a cryostat (CM1850; Leica Biosystems, Nussloch, Germany) from anteroposterior 1.0 to 6.0 mm relative to bregma. After blocking with 2% goat or horse serum, sections were reacted with 20,000 time diluted anti-Iba-1 antibody (019-19741; Wako Pure Chemical

Industries, Osaka, Japan) in 4˚C, overnight. The secondary antibody reaction was performed using VECTASTAIN Elite ABC-HRP Kit (PK-6101, 6102; Vector Laboratories, Newark, CA). After staining, the tissues were placed on a coated slide glass, dehydrated and cover slipped. Resting (ramified) and activated (amoeboid) microglia were distinguished according to previously reported procedures (21).

Western blotting. Western blotting was performed to assess the expression level of microglia and astrocytes. On the third day after CCI, rats of the naïve group (n=4), CCI-control group (n=5), CCI-5-BDBD group (n=7), CCI-AZ11645373 group (n=7), and CCI-5BDBD + AZ11645373 group (n=6) were deeply anesthetized with 5% isoflurane and decapitated. Initially, seven rats were assigned to each group, but some rats died after anesthesia or after trauma (contusion), resulting in uneven numbers of rats in each group. The numbers of rats in the naïve group were minimized for ethical reasons. Brains were removed and sliced to 2 mm thickness from anteroposterior 0.0 to 6.0 mm relative to bregma. Then the brains were dissected into 5 regions: peri-contusional cortex (cortex within 2 mm from the contusion border), ipsilateral distal cortex (cortex other than peri-contusional cortex), ipsilateral hippocampus, contralateral cortex, and contralateral hippocampus. Total protein concentration of the peri-contusional cortex was measured by RC DC Protein assay Kit (5000122JA; Bio-Rad Laboratories, Hercules, CA). Samples were prepared with Laemmli sample buffer (1610737; Bio-Rad Laboratories) and beta-mercaptoethanol (1610710; Bio-Rad Laboratories). Then a 15 μg protein sample was loaded on to polyacrylamide gel (567-1095; Bio-Rad Laboratories) and electrophoresis was performed at 120 V, 400 mA for 70 min. Bands were electrotransferred to polyvinylidene fluoride membrane by iBlot Dry Blotting System (IB1001; Thermo Fisher Scientific, Waltham, MA). Membrane was incubated overnight in 4˚C with 1,000 time diluted anti-Iba-1 antibody (019-19741), 10,000 time diluted anti-glial fibrillary acidic protein (GFAP) antibody (GTX108711; GeneTex, Irvine, CA), or 20,000 time diluted anti-β-actin antibody (GTX109639; GeneTex). After anti-rabbit immunoglobulin G secondary antibodies (AP182p; Millipore, Burlington, MA) reaction, blotted protein bands were visualized with using enhanced chemiluminescence. The expression levels were measured and the results were displayed as target/β-actin.

Polymerase chain reaction (PCR). PCR was performed to assess the levels of interleukin-1beta (IL-1β), interleukin-6 (IL-6), and tumor necrosis factor alpha (TNFα). Tissue samples were collected simultaneously with western blotting samples and quickly stored into RNA*later* stabilization solution (AM7024; Thermo Fisher Scientific). The mRNA expressions of IL-1β, IL-6, and TNFα were evaluated by reverse transcription PCR. RNA was purified with RNeasy Lipid Tissue Mini Kit (74804; Qiagen, Venlo, The Netherlands) and total RNA concentration was measured with microvolume spectrophotometer (NanoDrop Lite; Thermo Fisher Scientific). The mRNA was reversely transferred to cDNA using SuperScript IV Reverse Transcriptase Kit (18090010; Thermo Fisher Scientific) and used for PCR (T100 Thermal Cycler; Bio-Rad Laboratories). Electrophoresis was performed with 2% agarose gel containing Gel Red (41003; Biotium, Fremont, CA). Tracklt 50 bp DNA Ladder (10488043; Thermo Fisher Scientific) was used as a marker. The bands were measured and analyzed by a detector (ChemiDoc XRS; Bio-Rad Laboratories). Glyceraldehyde-3-phosphate dehydrogenase (GAPDH) was used as an internal control. The following primers were used in this experiment: IL-1β forward, 5'-GGA TGATGACGACCTGC-3' and reverse, 5'-CTTGTTGGCTTA TGTTCTG-3'; IL-6 forward, 5'-AAGTCGGAGGCTTAA TTACACATGT-3' and reverse, 5'-AAGTGCATCATCGTT GTTCATACA-3'; TNFα forward, 5'-CTTATCTACTCCCAG GTTCTCTTCAA-3' and reverse, 5'-GAGACTCCTCCCAGG TACATGG-3'; and GAPDH forward, 5'-AAGAAGGTGGTG AAGCAGGC-3' and reverse, 5'-TCCACCACCCTGTTGCTG TA-3'. Annealing temperature and cycle number were: IL-1β, 54˚C, 32 cycles; IL-6, 62˚C, 32 cycles; TNFα, 57˚C, 31 cycles; GAPDH, 63˚C, 23 cycles. The expression levels are displayed as target/GAPDH.

Enzyme-linked immunosorbent assay (ELISA). In addition to PCR, ELISA was used for quantitative analysis of IL-1β. ELISA with IL-1β Rat ELISA Kit (BMS630; Thermo Fisher Scientific) was done according to the manufacturer's protocol. After visualization, color intensity was read at 450 nm.

Behavioral tests. The following four behavioral tests were conducted to evaluate the treatment effects (apart from the rats described above). Rats only from the CCI-control group (n=5) and CCI-5-BDBD + AZ11645373 group (n=6) underwent behavior testing, because the CCI-5-BDBD + AZ11645373 group showed the most remarkable effects in biological assay. Starting 7 days before the behavior test, rats were handled for 15 min every day in order to get used to the investigator. The osmotic pumps had an internal volume of 200 μl, which allowed continuous administration for about one week. To exclude factors such as anesthesia and brain injury associated with removal, the pump was left in place for 28 days for the behavioral tests.

i) Corner turn test. Corner turn test is used to detect unilateral sensorimotor dysfunction in rodents (10,22) and was conducted at 1, 3, 7, and 14 days after CCI. Briefly, two boards (200 mm height and 300 mm length) were placed on a flat floor with edge of two boards attached at a 30˚ angle. Rats are placed between the two boards facing the corner. After entering deep into the corner, rats will turn back to face the open side. Naïve rats will turn either left or right with the same frequency, but rats with hemiparesis preferentially turn toward the non-impaired side. Trials were recorded for 10 times and the ratio of right turns per all turns was calculated.

ii) Cylinder test. Cylinder test is another test to detect unilateral sensorimotor dysfunction in rodents (10,23). This test was conducted at 1, 3, 8, 15, and 28 days after CCI. Briefly, rats placed in the transparent cylinder will explore by rearing and touching the cylinder wall with forelimb paws for postural support. Hemiparesis rats rely mainly on the unaffected forelimb paw to support posture, resulting in less contact with the affected paw. During a 5-min trial, number of touches with right, left, or both forelimbs were counted.

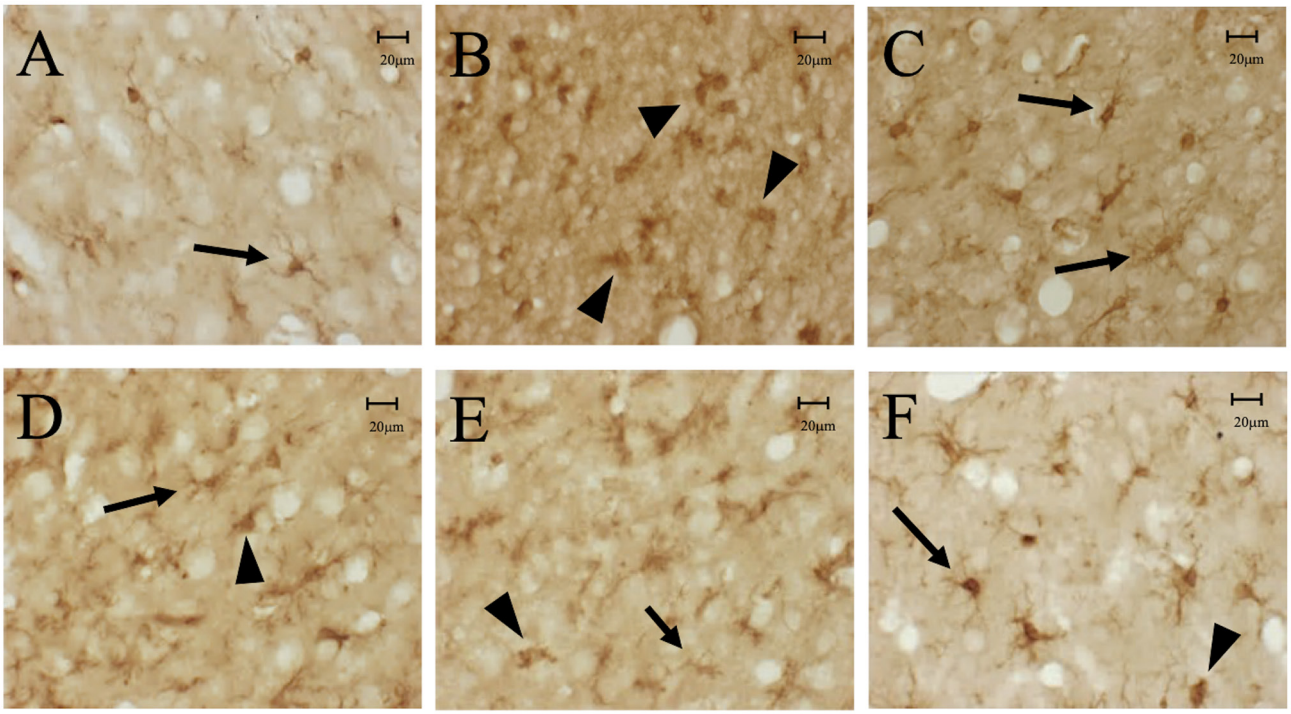

Figure 1. (A) Iba-1-positive cells were observed throughout the brain including the cerebral cortex and hippocampus in the naïve group, with morphology of resting (ramified) microglia. (B) In the CCI-control group, increased numbers of Iba-1-positive cells in the peri-contusional cortex and ipsilateral distal cortex were observed, with morphology of activated (amoeboid) microglia, (C) whereas in the contralateral CCI cortex, morphology was resting (ramified) microglia 3 days after the CCI. By contrast, in the (D) CCI-5-BDBD, (E) CCI-AZ11645373 (F) and CCI-5-BDBD + AZ11645373 groups, fewer Iba-1-positive cells were found in the peri-contusional cortex and ipsilateral distal cortex with morphology of amoeboid microglia, compared with the CCI-control group. Resting microglia are indicated with arrows and activated microglia are indicated with arrowheads. Scale bar=20 μm. Iba-1, ionized calcium-binding adaptor molecule 1; CCI, cortical contusion injury.

Asymmetry score was calculated as: (right touch/total touch)-(left touch/total touch).

iii) Grid walking test. Grid walking test is used to evaluate sensorimotor coordination of four limbs in various rodent disease models (24). This test was conducted at 1, 3, 8, and 15 days after CCI. The rat was placed on a 20x20 mm wire mesh grid and freely walked for 2 min. Number of foot faults, a paw slipping through an opening of the grid, was counted. The foot fault index was calculated as: (contralateral faults-ipsilateral faults)/(total steps). Score '0' represents no asymmetry in sensorimotor coordination and positive score represents unilateral sensorimotor deficit due to the brain injury.

iv) Plus maze test. Plus maze test is used to evaluate the spatial memory function (25,26). Plus maze test was conducted at 14 and 28 days after CCI. The maze was made of four black Plexiglas arms (120 mm height wall, 250 mm length, and 100 mm width) positioned in a plus shape and connected to an open central space (250 mm diameter). Rats were placed into the center of the maze and allowed to explore freely for 20 min. Rats will normally spontaneously alternate all arms of the maze using spatial working memory to retain knowledge of previously entered arms of the maze. The number of arm entries and sequence of entries into each arm were recorded. Arms were considered 'entered' when both hind limbs passed the halfway line of the arm. Spatial working memory was evaluated by calculating the percent four/five alternation score (25,26).

Statistical analysis. SPSS Statistics (version 21; IBM, Armonk, NY) was used for statistical analysis. One way analysis of variance (ANOVA) was used for comparisons between three or more groups. Subsequent tests with the Tukey method were performed when significant differences were found in the factors of ANOVA. All tests were two-tailed, and P-values <0.05 were considered significant. All data are indicated as mean ± standard deviation.

Results

Microglia. Iba-1-positive cells, indicating the microglia, were observed in all regions of the brain including the cerebral cortex and hippocampus in the naïve group. In contrast, Iba-1-positive microglia were also detected in both the ipsilateral and contralateral cortices and the hippocampus 3 days after the CCI in the CCI-control group. However, based on the morphological features (27), Iba-1-positive cells were mostly activated (amoeboid) microglia (enlarged cell body and shorter protrusions) in the ipsilateral cortex and ipsilateral hippocampus, and mostly resting (ramified) microglia (small cytoplasm with elongated protrusions) in the contralateral cortex. On the other hand, Iba-1-positive cells were mostly morphologically resting ramified microglia in both the contralateral cortex and the ipsilateral cortex and hippocampus in the CCI-5-BDBD, CCI-AZ11645373, and CCI-5-BDBD + AZ11645373 groups (Figs. 1 and 2). The Iba-1 expression level in the peri-contusional cortex as quantified by western blotting was significantly increased

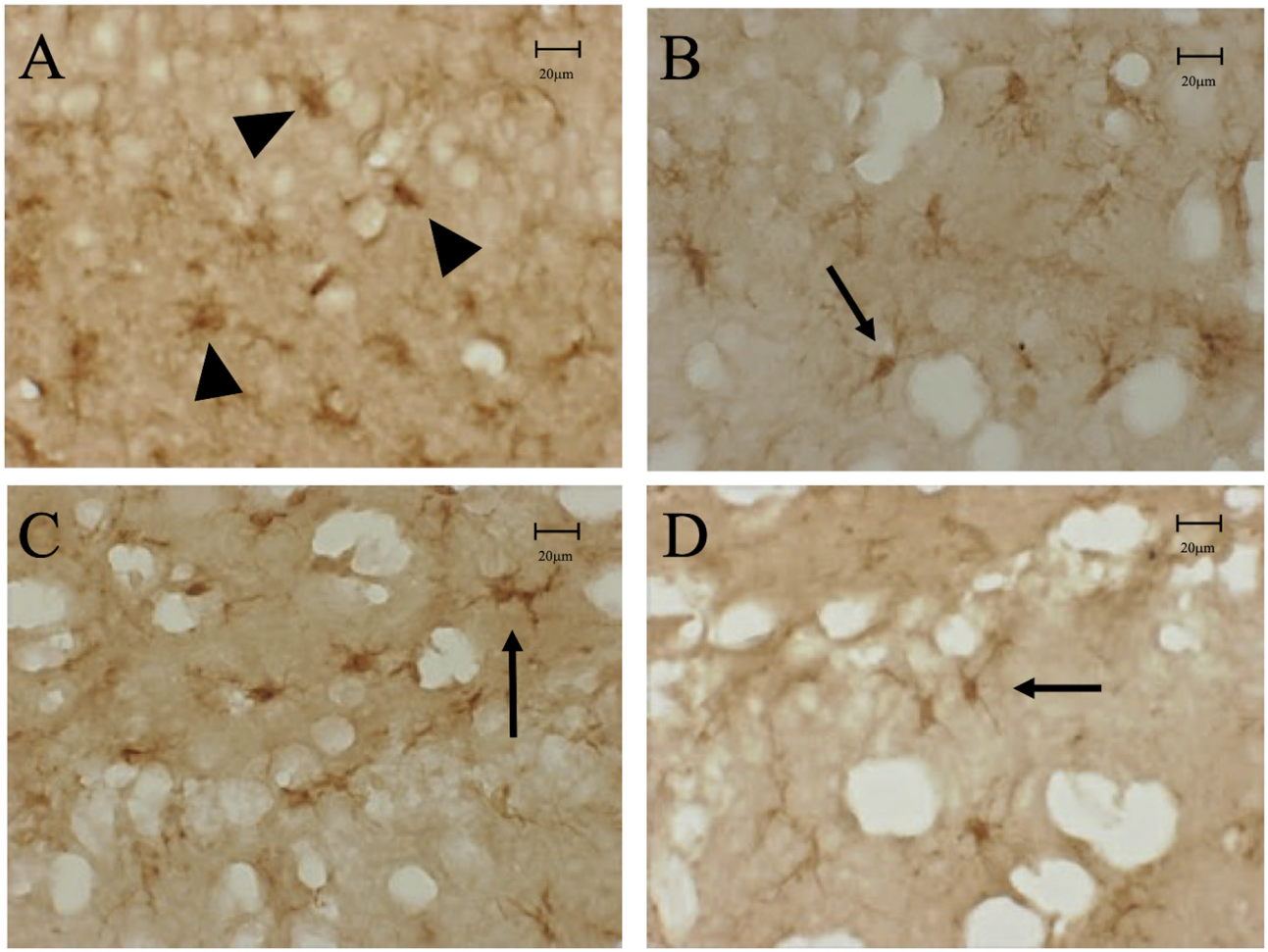

Figure 2. (A) In the ipsilateral CCI hippocampus in the CCI-control group, despite the absence of direct damage caused by CCI, the number of Iba-1-positive microglia had increased with morphology of activated (amoeboid) form, not resting (ramified) form, 3 days after the CCI. In contrast, in the (B) CCI-5-BDBD, (C) CCI-AZ11645373, (D) CCI-5-BDBD + AZ11645373 groups, Iba-1-positive microglia were present in the ipsilateral hippocampus, with a lower number of amoeboid microglia compared with the CCI-control group. Resting microglia are indicated with arrows and activated microglia are indicated with arrowheads. Scale bar=20 μm. Iba-1, ionized calcium-binding adaptor molecule 1; CCI, cortical contusion injury.

in the CCI-control group (0.113±0.027) compared to the naïve groups (0.005±0.012) (P<0.001) 3 days after the CCI. In contrast, the Iba-1 expression levels in the peri-contusional cortex of the CCI-5-BDBD, CCI-AZ11645373, and CCI-5-BDBD + AZ11645373 groups were 0.039±0.018, 0.051±0.019, and 0.029±0.020, respectively. These expression levels were significantly lower compared to those in the CCI-control group (P<0.001, P<0.001, and P<0.001, respectively) (Fig. 3A).

Astrocyte. The GFAP expression level, indicating astrocytes, of the peri-contusional cortex was also quantified by western blotting. The GFAP level was not significantly increased in the CCI-control group (0.422±0.225) compared to the naïve group (0.143±0.010). In contrast, the GFAP expression levels of the peri-contusional cortex in the CCI-5-BDBD, CCI-AZ11645373, and CCI-5-BDBD + AZ11645373 groups were 0.949±0.332, 0.895±0.300, and 1.185±0.339, respectively. These expression levels were significantly higher compared to those in the CCI-control group (P=0.029, P=0.042, and P<0.001, respectively) (Fig. 3B).

Expression of inflammatory factors. The mRNA levels of IL-1β, IL-6, and TNFα as inflammatory cytokines were measured by PCR in the peri-contusional cortex, the ipsilateral distal cortex, and the ipsilateral hippocampus 3 days after the CCI. In addition, IL-1β in the peri-contusional cortex was quantified by ELISA 3 days after the CCI. As shown in Fig. 4, mRNA expressions of IL-1β (0.914±0.274), IL-6 (1.670±0.691), and TNFα (1.001±0.030) in the peri-contusional cortex were high in the CCI-control group compared to the naïve group (IL-1β: 0.146±0.012, P=0.011; IL-6: 0.451±0.085, P=0.005; TNFα: 0.414±0.077, P<0.001). In contrast, 5-BDBD treatment decreased expression of IL-6 (0.838±0.194, P=0.010), and 5-BDBD, AZ11645373, and 5-BDBD + AZ11645373 treatment decreased expression of TNFα (0.693±0.118, P=0.004; 0.740±0.079, P=0.013; and 0.603±0.136, P<0.001, respectively). As shown in Fig. 5, expression of IL-1β was significantly increased in the CCI-control group (345.8±42.1 pg/ml) compared with the naïve group (80.4±7.3 pg/ml, P=0.001). Expression of IL-1β was significantly suppressed in the CCI-5-BDBD (178.8±22.4 pg/ml, P=0.002), CCI-AZ11645373 (221.1±30.1 pg/ml, P=0.005), and CCI-5-BDBD + AZ11645373 groups (169.4±54.9 pg/ml, P=0.001).

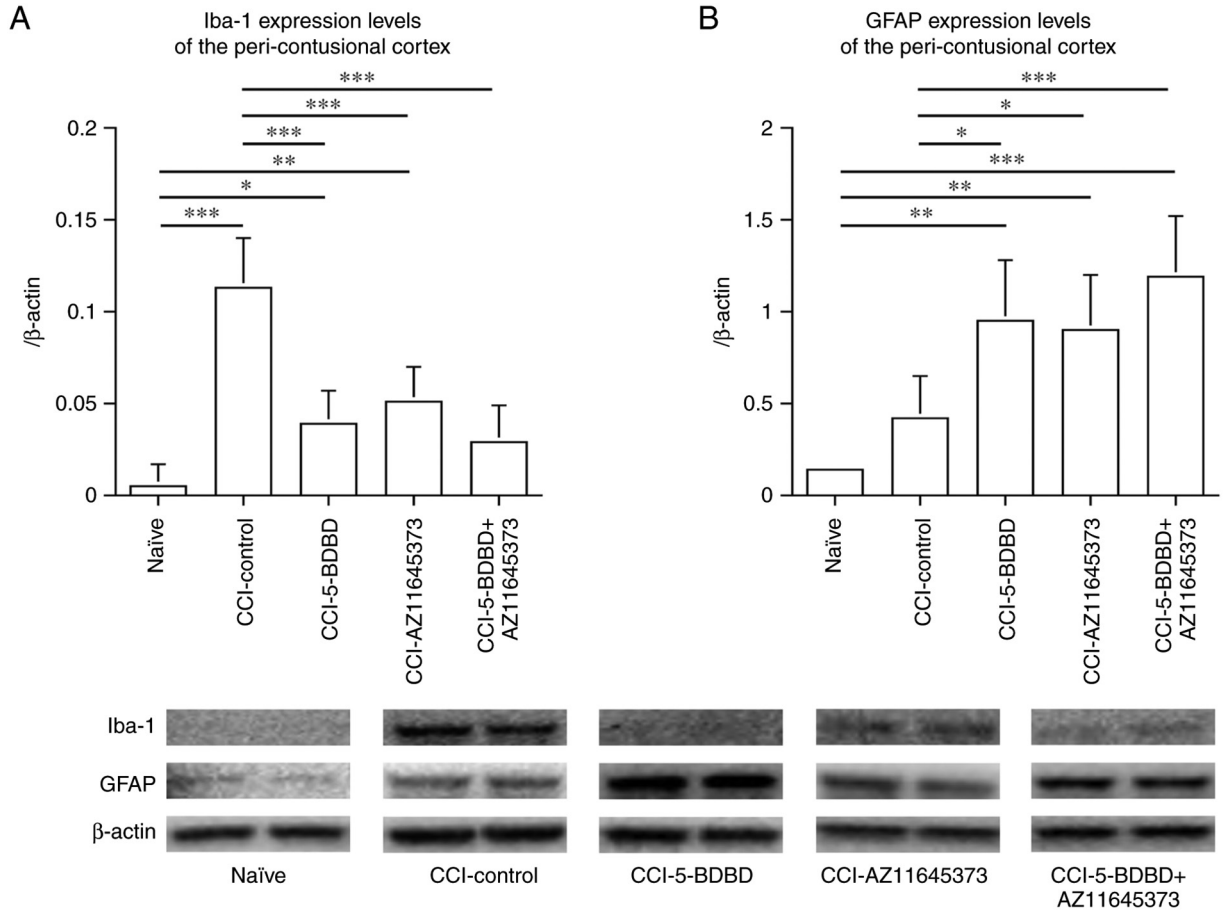

Figure 3. (A) Iba-1 expression levels in the peri-contusional cortex measured by western blotting. The Iba-1 expression levels in the CCI-5-BDBD, CCI-AZ11645373 and CCI-5-BDBD + AZ11645373 groups were significantly lower compared with those in the CCI-control group. (B) GFAP expression levels in the peri-contusional cortex measured by western blotting. The GFAP levels in the CCI-5-BDBD, CCI-AZ11645373 and CCI-5-BDBD + AZ11645373 groups were significantly higher compared with those in the naïve group and CCI-control group. *P<0.05, **P<0.01, ***P<0.001. Iba-1, ionized calcium-binding adaptor molecule 1; CCI, cortical contusion injury; GFAP, glial fibrillary acidic protein.

As shown in Fig. 4, mRNA expression of IL-1β (0.877±0.361) and IL-6 (1.178±0.157) in the ipsilateral distal cortex were high in the CCI-control group compared to the naïve group (IL-1β: 0.074±0.085, P=0.001; IL-6: 0.315±0.110, P<0.001). However, mRNA expression of TNFα (1.296±0.449) in the CCI-control group showed no significant difference compared to the naïve group (0.665±0.141). In contrast, 5-BDBD, AZ11645373, and 5-BDBD + AZ11645373 treatment reduced expression of IL-1β (0.400±0.203, P=0.012; 0.314±0.094, P=0.003; and 0.389±0.089, P =0.013, respectively) compared to the CCI-control group. 5-BDBD and 5-BDBD + AZ11645373 treatment reduced expression of IL-6 (0.569±0.128, P=0.023 and 0.486±0.134, P=0.005, respectively) compared to the CCI-control group.

As shown in Fig. 4, the changes in inflammatory cytokines 3 days after CCI were similar in the ipsilateral hippocampus and the ipsilateral distal cortex. mRNA expressions of IL-1β (1.151±0.192) and IL-6 (1.007±0.079) were high in the CCI-control group compared to the naïve group (IL-1β: 0.095±0.014, P<0.001 and IL-6: 0.433±0.138, P=0.001). mRNA expression levels of TNFα (0.836±0.294) in the CCI-control group showed no significant difference compared to the naïve group (0.836±0.294). In contrast, 5-BDBD, AZ11645373, and 5-BDBD + AZ11645373 treatment reduced expression of IL-1β (0.355±0.163, P<0.001; 0.449±0.159, P<0.001; and 0.473±0.093, P<0.001, respectively) and IL-6 (0.591±0.168, P=0.002; 0.459±0.165, P<0.001; and 0.419±0.025, P<0.001, respectively) compared to the CCI-control group.

Behavioral test. Sensorimotor dysfunction using the corner turn test and the cylinder test, sensorimotor coordination of the four limbs using the grid walking test, and spatial memory function using the plus maze test were assessed in the present study. Detailed data is not shown here, but no significant differences were found between the CCI-control group and the CCI-5-BDBD + AZ11645373 group in the tests other than the plus maze test.

In the plus maze test, the criterion of 4/5 alternation 14 days after CCI in the CCI-control group and the CCI-5-BDBD + AZ11645373 group was 55.18±17.79 and 73.59±8.28, respectively (P=0.077). The criterion of the 4/5 alternation 28 days after CCI in the CCI-control group and the CCI-5-BDBD+AZ11645373 group was 53.53±14.18 and 74.89±12.44, respectively (P=0.047). No significant differences were found in the 4/5 alternation criterion 14 days after CCI, but significant increase occurred 28 days after CCI (Fig. 6).

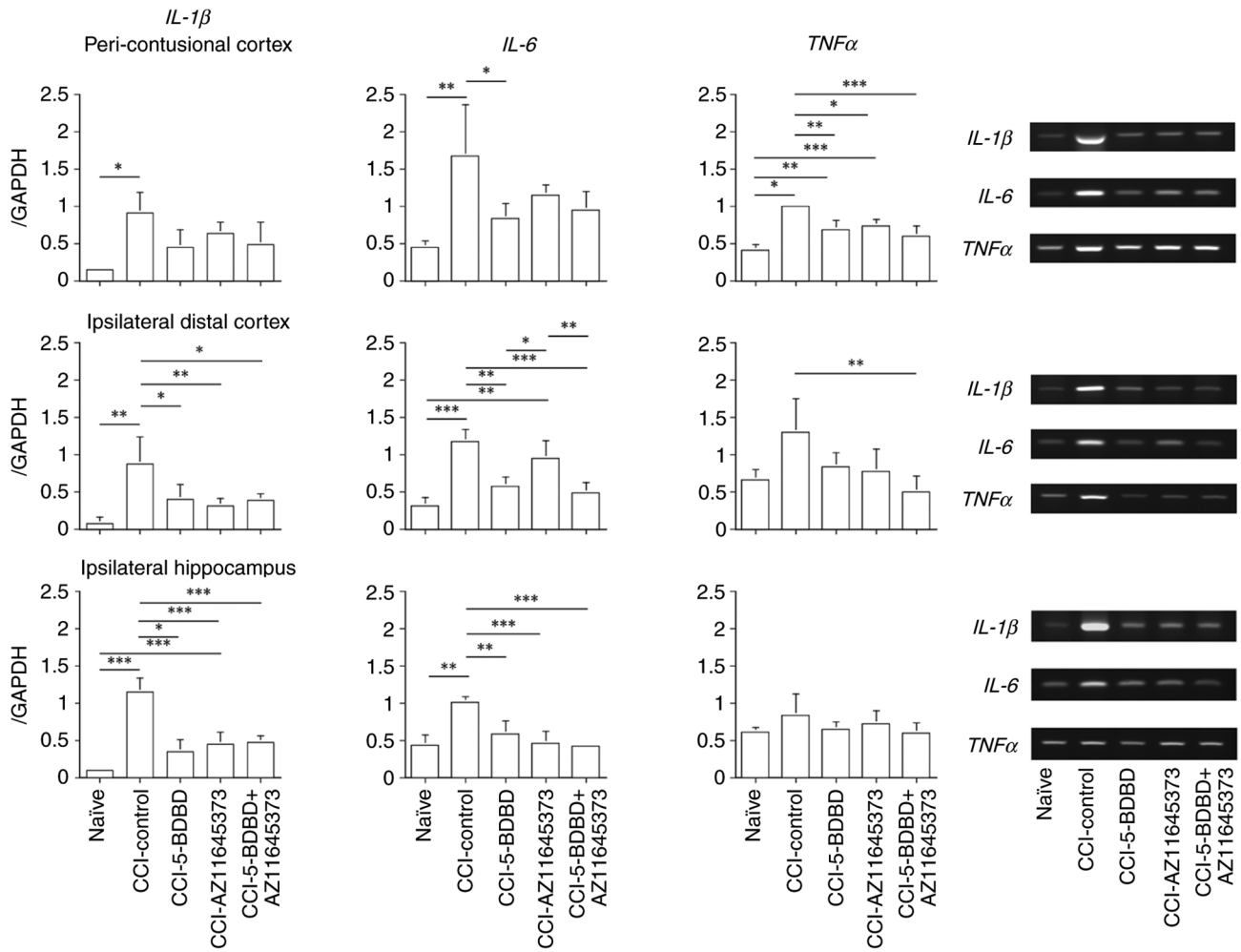

Figure 4. Expression of the cytokines measured by PCR in the peri-contusional cortex 3 days after the CCI. Expression levels of IL-1β, IL-6 and TNFα were increased by the CCI. Furthermore, treatment of 5-BDBD, AZ11645373 or 5-BDBD + AZ11645373 decreased expression of TNFα. Expression of cytokines measured by PCR in the ipsilateral distal cortex 3 days after the CCI. Expression levels of IL-1β and IL-6 were increased by the CCI, but not of TNFα. Furthermore, treatment of 5-BDBD, AZ11645373 or 5-BDBD + AZ11645373 decreased expression of IL-1β, and treatment of 5-BDBD or 5-BDBD + AZ11645373 decreased expression of IL-6. Expression of cytokines measured by PCR in the ipsilateral hippocampus 3 days after the CCI. Expression levels of IL-1β and IL-6 were increased by the CCI, but not of TNFα. Furthermore, treatment of 5-BDBD, AZ11645373 or 5-BDBD + AZ11645373 decreased expression levels of IL-1β and IL-6. *P<0.05, **P<0.01, ***P<0.001. CCI, cortical contusion injury; IL, interleukin; PCR, polymerase chain reaction; TNFα, tumor necrosis factor α; GAPDH, glyceraldehyde-3-phosphate dehydrogenase.

Discussion

Increased extracellular ATP level immediately after the insult has been demonstrated in several experimental models including TBI (9,28-30). The source of the rapid ATP increase is considered to be intracellular ATP leakage from damaged cells and subsequent cellular release from activated brain cells. The released ATP binds to the purinergic receptors on microglia and activates these cells. Activated microglia initiates inflammatory responses such as migration to the damaged area and phagocyte debris (2,31), engulfing or stripping of synapses (3,32), and release of inflammatory cytokines (33-35). Strong inflammatory response is well known to occur following TBI (11,36), but excess inflammatory responses including the release of enormous amounts of inflammatory cytokines can be harmful to the brain (7,37) (Fig. 7). Stimulation of P2Y6 receptors (P2 receptor is an ATP-gated ion channel receptor) is mandatory for microglia phagocytosis (2). Other previous studies have demonstrated that treatment with P2Y1 receptor antagonist decreases extracellular ATP levels via restriction of cellular ATP signal propagation in various experimental models (13,38-40). In our previous study, in situ administration of a competitive P2Y1 receptor antagonist MRS2179 significantly reduced the extracellular ATP level both before and after CCI in a rat model (9). Other studies reported that administration of MRS2179 in a TBI model reduced inflammatory responses (15).

In the present study, we hypothesized that the blockade of P2X4 and/or P2X7 receptors after TBI may potentiate an anti-inflammatory effect through suppression of microglial activation, and may provide therapeutic benefits. Several astrocytic functions are essential to maintain homeostasis in the injured brain, so we hypothesized that blocking the activation of microglia without influencing the activation of astrocytes might be the ideal approach to treat damage in the brain. The ATP leakage from damaged cells (primary injury) caused by TBI cannot be prevented, even with MRS2179 administration. Therefore, the present study focused on

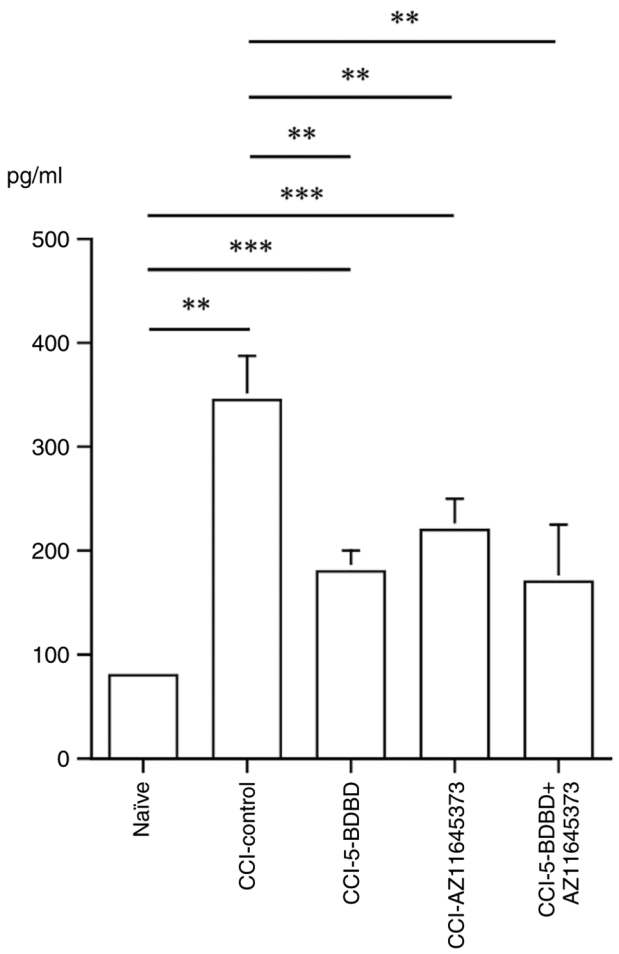

Figure 5. IL-1β concentration in the peri-contusional cortex 3 days after the CCI measured by ELISA. IL-1β levels in the CCI-control group were significantly suppressed in the CCI-5-BDBD, CCI-AZ11645373, and CCI-5-BDBD + AZ11645373 groups. **P<0.01, ***P<0.001. CCI, cortical contusion injury; IL, interleukin; ELISA, enzyme-linked immunosorbent assay.

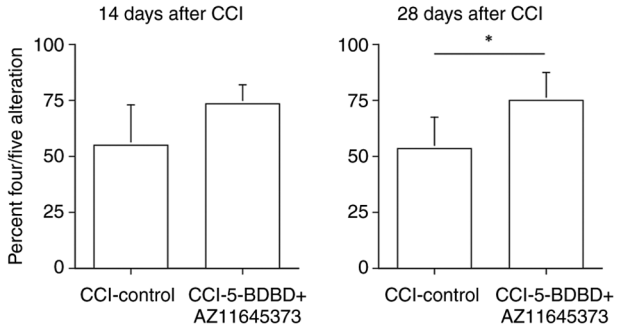

Figure 6. Observed 4/5 alternations in the plus maze test. There was no significant difference in the 4/5 alternation criteria 14 days after CCI, but significant increase occurred 28 days after CCI, indicating improving the spatial memory function. *P<0.05. CCI, cortical contusion injury.

blocking the receptors on microglia that receive ATP signals by 5-BDBD and/or AZ11645373, an antagonist of P2X4 and P2X7 receptors. In the CCI-control group, very high numbers of activated microglia were histologically observed in the ipsilateral cortex and hippocampus compared to the contralateral cortex and hippocampus. In contrast, in the CCI-5-BDBD, CCI-AZ11645373, and CCI-5-BDBD + AZ11645373 groups, smaller numbers of activated microglia were observed in both the ipsilateral cortex and hippocampus. Western blotting with Iba-1 antibody confirmed that administration of CCI-5-BDBD and/or CCI-AZ11645373 significantly suppressed the expression of microglia 3 days after CCI. Therefore, blockade of P2X4 and P2X7 receptors was effective to regulate the activation of microglia in the present injury model. Unlike treating systemic inflammation, anti-inflammation therapy for brain lesions is more complicated, but we showed that the present method is a viable option to control the activity of microglia.

Cytokines are involved in both improvement and exacerbation depending on the timing after injury (41). However, the influence of inflammatory cytokines after TBI is not fully understood. IL-1β or TNFα receptor blockers suppressed neural cell death and improved motor coordination function in a rat TBI model (35). In addition, administration of IL-1β neutralizing antibodies improved cognitive function (42) but only inhibition of cytokine secretion could not provide anti-inflammatory effects (43). In this study, administration of 5-BDBD and/or AZ11645373 reduced the IL-1β level and improved behavioral outcome. We note that cytokine secretion can be controlled by inhibiting purinergic signaling which represents another potential therapeutic approach for investigation in the future. Further types of cytokines might be simultaneously suppressed by this approach. However, we found obvious differences in cytokine secretion levels between 5-BDBD and AZ11645373. The P2X4 receptor seems to be more involved in cytokine secretion and no synergistic effect was seen with the combination of 5-BDBD and AZ11645373. Our study confirmed suppression of cytokine release by the administration of 5-BDBD and/or AZ11645373, and our results have established a highly significant new perspective on treatment methods mediated through gliotransmission control.

Gliosis is well known to occur after TBI (42). Increased expression levels of GFAP in the acute phase are considered to reflect an increase in reactive astrocytes which are believed to partly remain as gliosis in the chronic phase (21). Reactive astrocytes may also protect against oxidative stress (44) and the blood-brain barrier (45), and reduce glutamate toxicity (46), brain edema in stroke (22), and the inflammatory response in various conditions (47-49). However, gliosis also potentiates adverse effects especially in the chronic phase such as inhibiting nerve regeneration (40) and can act as an epileptic focus (50). In the present study, GFAP expression increased after CCI, reflecting the increase of reactive astrocytes in the acute phase, as previously reported (51). However, interestingly, treatment with 5-BDBD and/or AZ11645373 further increased GFAP expression. These findings suggest that 5-BDBD and/or AZ11645373 suppressed microglial activation, but not reactive astrocytes. No other studies show similar results in a TBI model, but this trend was similar to a study using an ischemia model in a P2X7 receptor knockout mice (52). Astrocyte response to P2 receptor antagonists is important and we plan a detailed study (including GFAP staining) in the near future.

The corner turn test, the cylinder test, and the grid walking test, which evaluate unilateral sensorimotor dysfunction and sensorimotor coordination of the four limbs, did not show any significant changes after administration of 5-BDBD and/or AZ11645373, whereas the plus maze test, which reflects spatial memory function and involves the hippocampal function,

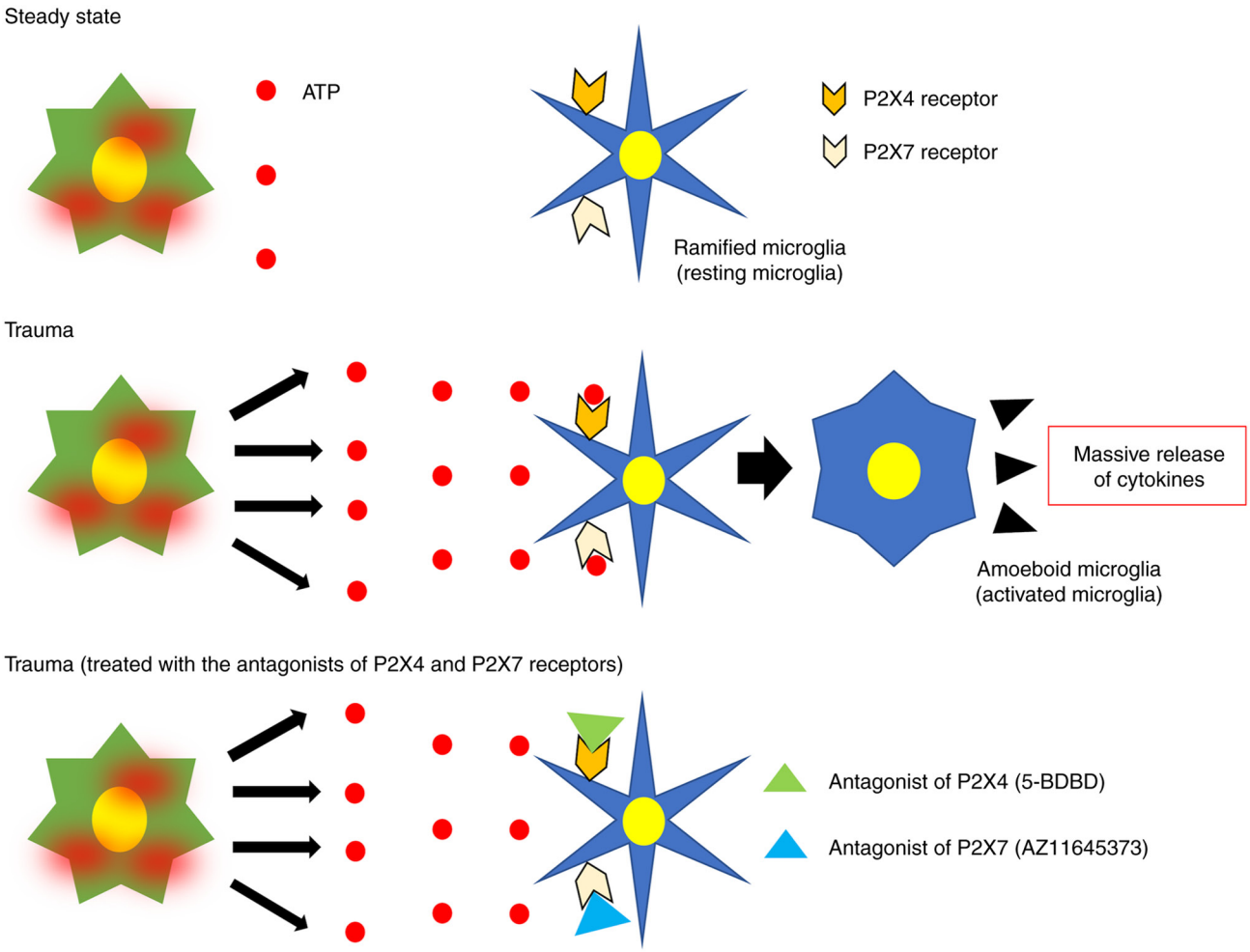

Figure 7. Release of a large amount of ATP, gliotransmitter, into the extracellular space by TBI activates the microglia through the ATP receptor, namely P2X and P2Y receptors. Activated microglia retract their processes, enlarge their cell bodies and release excessive amounts of cytokines, which can cause excessive inflammatory response inducing secondary brain injury. 5-BDBD, an antagonist of the P2X7 receptor, and AZ11645373, an antagonist of the P2X4 receptor, bind to these receptors and block ATP binding, which can reduce microglial activation and thus suppress excessive cytokine release. ATP, adenosine triphosphate; TBI, traumatic brain injury.

showed significant improvement. Since the brain contusion induced in this study was not intense enough to cause direct (primary) damage on both sides of the hippocampus and the contralateral cortex, the improved performance in the plus maze test was considered to indicate the effect of blocking P2X4 and/or P2X7 receptors on the hippocampus despite the absence of observed primary damage, which implies that the ameliorative effect reduced secondary damage to the hippocampus. Cytokine expressions contributed to both improved and worsened outcomes in a rat trauma model (35,42,43,53). Therefore, we considered that secondary brain injury could be effectively treated by suppressing microglial activation and subsequent secretion of cytokines.

In conclusion, secondary brain injury after cerebral contusion is therapeutically important, and has been investigated by various studies. However, no effective treatment has been established. We confirmed that blocking the P2X4 and P2X7 receptors, which are ATP receptors central in gliotransmission, suppressed microglial activation and subsequent cytokine expression after brain injury. These findings indicate the potential as an effective treatment for reducing secondary brain injury.

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Availability of data and materials

All data generated or analyzed during this study are included in this published article.

Authors' contributions

MK and NM designed all the experiments. MK, NM, and TK performed the experiments. AY, KS, TM, and HO helped with data analysis and draft writing. MK, NM, and AY reviewed

the manuscript. All authors read and approved the final manuscript. MK, NM and AY confirm the authenticity of all the raw data.

Ethics approval and consent to participate

This study was approved by the animal care and use committee at Nihon University School of Medicine (approval no. AP16M046-1). All experiments were conducted according to the animal experimental protocol manuals at Nihon University School of Medicine. Efforts were made to avoid pain and distress to the animals.

Patient consent for publication

Not applicable.

Competing interests

The authors declare that they have no competing interests.

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