An adenosine A1R-A2aR imbalance regulates low glucose/hypoxia-induced microglial activation, thereby contributing to oligodendrocyte damage through NF-κB and CREB phosphorylation

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Abstract. Microglial activation-mediated inflammatory damage to oligodendrocytes is a key step in the etiology of ischemic white matter lesions. The adenosine A1 receptor (A1R) and adenosine A2a receptor (A2aR) have been reported to regulate the activation of microglia, however, the underlying mechanisms remain elusive. Thus, the present study used a microglia/oligodendrocyte co-culture model exposed to low glucose/hypoxia, and treated with agonists/antagonists of A1R and A2aR to investigate the role of A1R and A2aR. Changes in A1R and A2aR expression and inflammatory cytokine secretion by the microglia, and oligodendrocyte damage, after exposure were examined. Low glucose/hypoxia induced a higher elevation of A1R than A2aR. In addition, activation of A1R inhibited A2aR protein expression and vice versa. The A1R antagonist DPCPX (100 nM) and A2aR agonist CGS 21680 (100 nM) inhibited microglial activation, reduced the production of inflammatory cytokines and attenuated oligodendrocyte damage, along with elevating the levels of phosphorylated nuclear factor (NF)-κB and cyclic adenosine monophosphate response element binding protein (CREB). These data indicate that an A1R-A2aR imbalance is able to modulate low glucose-induced microglial activation and the cellular immune response through altering NF-κB and CREB phosphorylation. This suggests that rebalancing A1R-A2aR is a promising approach for treating white matter injury.

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Introduction

Oligodendrocyte damage-induced demyelination is a typical pathological event in white matter impairment in numerous neurological disorders, including stroke, Alzheimer's disease, intracranial tumors, cerebral hemorrhage and chronic cerebral hypoperfusion (1). Oligodendrocytes, as the primary component of periventricular white matter and the only myelin-producing cells in the central nervous system (CNS), are fragile and vulnerable to ischemic white matter lesions (WMLs) (2). Inflammatory cytokines derived from activated microglia and astrocytes are the main factors responsible for oligodendrocyte injury (3).

Microglia are the predominant resident immune cell in the human brain and white matter (4). Microglia becomes activated under oxygen and glucose deprivation (OGD) or low glucose/hypoxia. Once activated, the morphology and secretory phenotype of the microglia changes; protrusion retraction, polarization and an increase in the soma area occurs (5). A recent study reported that OGD activated microglia, which then had neurotoxic effects on oligodendrocyte progenitor cells by inducing the rapid release of proinflammatory molecules and free radicals (6). Due to sharing analogous pathological mechanisms (7), it may be speculated that microglia will exhibit similar effects under low glucose/hypoxia conditions. Therefore, selective modulation of the activation of microglia may be a strategy for the treatment of white matter injury, such as WMLs.

There is an urgent need for treatments for microglia-induced neuroinflammation of the ischemic brain. Adenosine has been highlighted as a crucial regulatory autocrine and paracrine factor, which is required for microglial-mediated inflammatory activity (8). The extracellular ectonucleotidases cluster of differentiation (CD)39 and CD73 metabolize adenosine triphosphate (ATP) and adenosine diphosphate to adenosine monophosphate (AMP), and then metabolize AMP into

adenosine. Subsequently, adenosine triggers an efflux of K^+ from the cell, followed by a Ca^{2+} influx and activation of phosphatidylcholine-specific phospholipase C and calcium-independent phospholipase A2, which induces an unconventional release of GAPDH and inflammatory cytokines from the microglia (9). However, the role of adenosine receptors (ARs) in the modulation of the secretion of inflammatory cytokines from the microglia during hypoxia is not well understood.

Adenosine is a ubiquitous nucleoside that has an influence on the immune properties of microglia through interactions with four AR subtypes; A1, A2a, A2b and A3 (10). The adenosine A1 receptor (A1R) and adenosine A2a receptor (A2aR) have been reported to form complicated tetrameric heteromers in astrocytes and neurons, suggesting a putative regulatory interaction with A1R and A2aR (11). A1R and A2aR have antagonistic effects on gliosis and the release of glutamate because of different couplings with the guanine nucleotide-binding (G) proteins G_i and G_s, and their elicitation of the release of Ca^{2+} from intracellular stores (12). Furthermore, activation of A2aR reduces the affinity of A1R to agonists during the formation of A1R-A2aR heteromers in mammalian cells, providing a switch mechanism by which low (0.3 μM) and high (3-10 μM) concentrations of adenosine can inhibit and stimulate glutamatergic neurotransmission (13). Little is known about whether an imbalance of A1R to A2aR contributes to the immune cascade in microglia. Our group previously demonstrated that ablation of the A2aR gene promotes microglial activation and deteriorates chronic cerebral hypoperfusion-induced WMLs (14). Given that chronic cerebral ischemia can induce a downregulation of adenosine A1R during white matter damage, the functional antagonistic interactions between A1R and A2aR that modulate the release of inflammatory cytokines from the microglia should be further investigated. In the present study, a co-culture model of microglia/oligodendrocytes undergoing low glucose/hypoxia exposure to mimic chronic cerebral hypoperfusion was utilized to determine whether an A1R-A2aR imbalance regulates the activation of microglia.

Mechanically, activation of microglia is linked to the response of transcription factors, including cyclic adenosine monophosphate (cAMP) response element binding protein (CREB) and nuclear factor (NF)-κB (15). A1R and A2aR affect the level of cAMP through G_i and G_s proteins, activating cAMP-dependent protein kinase A (PKA), and promoting the phosphorylation of CREB and NF-κB, thereby regulating microglial activation and the production of inflammatory cytokines (16,17). Nevertheless, a better understanding of the distinct role of A1R and A2aR in CREB and NF-κB phosphorylation is required. The present study aimed to investigate whether an imbalance of A1R-A2aR regulates low glucose/hypoxia-induced microglial activation, thereby contributing to oligodendrocyte injury through modulating the phosphorylation of NF-κB and CREB.

Materials and methods

Experimental animals. Animals were provided by Animal Center of Third Military Medical University (Chongqing, China). A total of 8 Sprague Dawley rats (3-days-old) were used in the present study. Prior to the experiment, the mice were housed in a cage at a constant temperature (22±2˚C) and humidity (60±5%) with a 12-h light/dark cycle. The rats had free access to food and water. All animal care and experimental procedures were approved by the Institutional Animal Care and Use Committee of Third Military Medical University (approval no. SYXK-PLA-2007035). Efforts were made to minimize animal suffering and to reduce the number of animals used. All surgeries were performed under sodium pentobarbital anesthesia and mice were sacrificed by cervical dislocation under deep anesthesia.

Drugs. The following drugs were used in the present study (Table I): A1R agonist, 2-chloro-N6-cyclopentyladenosine (CPA); A1R antagonist, cyclopentyl-1,3-dipropylxanthine (DPCPX); A2AR agonist, 2-p-(carboxyethyl) phenethylamino-5'-N-ethylcarboxamideadenosine hydrochloride (CGS 21680); and A2AR antagonist, 2-(2-furanyl)-7-(2-phenylethyl)-7H-pyrazolo[4,3-e] [1,2,4]triazolo[1,5-c]pyrimidin-5-amine (SCH 58261). Saline, 5 mM dimethylsulfoxide and 10 mM ethanol were used for the vehicle. The drugs were purchased from eBioscience (Thermo Fisher Scientific, Inc., Waltham, MA, USA).

Microglia culture. The microglial cell line BV2 (Cell Resource Center, Institute of Basic Medical Sciences, Chinese Academy of Medical Sciences/Peking Union Medical College, Beijing, China) was used. The cells were cultured in Dulbecco's modified Eagle medium (low glucose; Invitrogen; Thermo Fisher Scientific, Inc.), 5% fetal bovine serum (FBS; HyClone; GE Healthcare Life Sciences, Logan, UT, USA), 4 mM glutamine (Invitrogen; Thermo Fisher Scientific, Inc.), 100,000 U/l penicillin G and 100 mg/l streptomycin (Mediatech, Inc., Herndon, VA, USA), and were maintained at 37˚C with 5% CO_2.

Oligodendrocyte culture. Primary oligodendrocyte cultures were isolated and maintained as described by Seki *et al* (18). Briefly, the subventricular zone was removed from 3-day-old Sprague Dawley rats (n=8) using a dissecting microscope. The tissues were mechanically dissociated into single cells on 100-mm-pore nylon mesh cell strainers (BD Biosciences, Franklin Lakes, NJ, USA) and collected in PBS. Subsequently, the cells were filtered through 40-mm-pore nylon mesh cell strainers (BD Biosciences) and centrifuged at 800 x g for 5 min at 4˚C. The cell pellet was re-suspended in cold Neurobasal Medium supplemented with 2% B27, 1% L-glutamine, 1% penicillin/streptomycin/amphotericin B (all Thermo Fisher Scientific, Inc.), 20 ng/ml epidermal growth factor and 10 ng/ml basic fibroblast growth factor (both Sigma-Aldrich, Merck KGaA, Darmstadt, Germany). The cell suspension was plated into poly-L-lysine coated 12-well plates at a density of $1.5x10^5$ cells/well. Cells were maintained at 37˚C in an atmosphere of 5% CO_2. At day 7, triiodothyronine (T3; 30 μg/ml) and thyroxine (T4; 40 μg/ml) (both Sigma-Aldrich; Merck KGaA) were added to the culture media. A total of 14 days after the addition of T3 and T4, the differentiated oligodendrocytes were subjected to further experiments.

Co-culture model of microglia and oligodendrocytes. As previously described (18), BV2 microglial cells were plated on tissue culture inserts for 12-well plates (Greiner

Table I. A1R and A2aR agonists and antagonists used and their effects.

Drug	Dosage (nM)	Effect
CPA	1,000	Activated A1R
DPCPX	100	Inhibits'A1R
CGS	100	Activates A2aR
SCH	100	Inhibits A2aR

A1R, adenosine A1 receptor; A2aR, adenosine A2a receptor.

Bio-One GmbH, Frickenhausen, Germany) at a density of $5x10^5$ cells/well. The microglial cells were incubated for 12 h in the presence of CPA (1 μM), DPCPX (100 nM) CGS (100 nM) or SCH (100 nM). In triplicate, using 5-μm pore Transwell filters (Corning Incorporated, Corning, NY, USA), each BV2 culture insert was placed on the primary oligodendrocytes ($1.5x10^5$ cells/cm^2) in the 12-well plates. Both layers of cells were submerged in Neurobasal Medium with the aforementioned supplements. A total of 24 h after a 37˚C incubation, the upper cells in the filter inserts were removed from the 12-well plates. Thus, only oligodendrocytes were involved in the subsequent lactate dehydrogenase (LDH) and Cell Counting Kit (CCK)-8 assays. Morphological changes in the primary microglia and oligodendrocytes were observed under a phase-contrast microscope (magnification, x200). All procedures were performed in triplicate independently in this experiment.

Low glucose/hypoxia stimulation. In conventional experiments, cells are cultured under normoxic conditions (5% CO_2, 20% O_2 and 3.0 g/l glucose). For the low glucose/hypoxia-mimicking assessments in the present study, the cells were cultured at 37˚C in low glucose medium, in which glucose was partly replaced by 10% FBS under hypoxic conditions (5% CO_2, 1.5% O_2 and 1.4 g/l glucose). To maintain cell viability, high glucose and oxygen recovery after exposure to low glucose/hypoxia is required. Thus, the cells suffered from low glucose/hypoxia for 0, 2, 4, 6, 8, 10 and 12 h, followed by 24 h of high glucose and oxygen recovery treatment (5% CO_2, 30% O_2 and 4.5 g/l glucose).

NO production assessment. NO production from microglia was used as an indicator of microglial activation. Thus, the accumulation of NO^{2-}, a stable end product of NO production, was assayed using the Griess reaction as previously reported (19). Mouse BV2 cells were plated on 96-well tissue culture plates (Greiner Bio-One GmbH) at a density of $1x10^5$ cells/200 μl medium. The cells were pre-incubated under low glucose/hypoxia conditions for 12 h followed by 24 h of high glucose and oxygen recovery incubation. Subsequently, the cell-free supernatants were assayed for NO accumulation using a Griess assay kit (Dojindo Molecular Technologies, Inc., Kumamoto, Japan) and read at 550 nm using a microplate reader (Multiscan MS; Thermo Labsystems, Helsinki, Finland).

ELISA. The secretion of inflammatory cytokines, including interleukin (IL)-6 (cat. no. BMS603HS), interferon (IFN)-β (cat. no. BMS606), IL-1β (cat. no. KMC0012) and tumor necrosis factor (TNF)-α (cat. no. BMS607HS), was detected using ELISA kits (Invitrogen; Thermo Fisher Scientific, Inc.). BV2 cells were pre-incubated in low glucose/hypoxia plus CPA (1 μM), DPCPX (100 nM), CGS (100 nM) or SCH (100 nM) for 8 h, and the conditioned media was collected for detection. All ELISA procedures were performed according to the manufacturer's protocol. Optical densities were determined by the measurement of indicator color shifts at 450 nm on a microplate reader (Multiscan MS).

LDH assay. Oligodendrocyte cell damage was determined by the colorimetric measurement of LDH, the increased production of which is an indicator of damage. Cells were collected 24 h after co-culture. The LDH level was measured by a spectrophotometric enzyme assay using an LDH Assay kit (Wako Pure Chemical Industries, Ltd., Osaka, Japan). The assay was performed according to the manufacturer's protocol. In brief, LDH converts pyruvate into lactate that reduces the developer to a colored product with absorbance at 450 nm measured by a microplate reader (Multiscan MS).

CCK-8 assay. To assess cell proliferation, oligodendrocytes that had undergone low glucose/hypoxia plus CPA (1 μM), DPCPX (100 nM) CGS (100 nM) or SCH (100 nM) treatment for 12 h were seeded into 96-well cell culture plates (Corning Incorporated) at a concentration of $2x10^4$ cells/well in a volume of 100 μl and cultured overnight at 37˚C. CCK-8 reagents (Dojindo Molecular Technologies, Inc.) were added to each well at 0, 2, 4, 6, 8, 10 and 12 h. The plates were then incubated for another 2 h at 37˚C in the dark. The absorbency of the wells was measured at 450 nm using the Immuno-mini NJ-2300 microplate reader (InterMed, Tokyo, Japan).

Reverse transcription-quantitative polymerase chain reaction (RT-qPCR) analysis. RT-qPCR analyses of inflammatory cytokines were performed as previously reported (20). A Transcriptor First Strand cDNA Synthesis kit (Roche Applied Science, Penzberg, Germany) was used according to the manufacturer's protocol. Briefly, microglial cultures were collected to extract total RNA using TRIzol reagent (Invitrogen; Thermo Fisher Scientific, Inc.), which was reverse transcribed with a combination of anchored-oligo(dT) and random primers that were included in the kit. Gene expression analysis was performed for four inflammatory cytokines (IL-6, IFN-β, IL-1β and TNF-α) and the primers (5'-3') were as follows: IL-1β forward (F), CAACAACAAGTGATATTCTCCATG and reverse (R), GATCCACACTCTCCAGCTGCA; TNF-α F, GCGGTGCCTATGTCTCAG and R, GCCATTTGGGAACTTCTCATC; IFN-β F, CCCTATGGAGATGACGGAGA and R, CTGTCTGCTGGTGGAGTTCA; IL-6 F, ATGAACTCCTTCTCCACAAGC and R, CTACATTTGCCGAAGAGCCCTCAGGCTGGACTG; and β-actin F, AGAGGGAAATCGTGCGTGAC and R, CAATAGTGATGACCTGGCCGT. qPCR analysis was performed in a final volume of 10 μl using 5 ng cDNA/well and 5 μl LightCycler® 480 Probes Master (Roche Applied Science). The reagents and samples were pipetted by an epMotion 5070

Liquid Handling Robot (Eppendorf, Hamburg, Germany). The thermocycling conditions were as follows: Enzyme activation at 95˚C for 10 min; 45 cycles of amplification at 95˚C for 10 sec, 60˚C for 30 sec and signal detection at 72˚C for 1 sec; and cooling at 40˚C for 30 sec. The expression levels were quantified using the $2^{-\Delta\Delta Cq}$ method (21). For data normalization, the β-actin control was used. Interactive dot diagrams were used to represent the scale of the differences, and indicate the specificity and sensitivity values of the analyzed markers.

Western blotting. Western blotting was performed according to previously described method (22). Primary microglia were rinsed with ice-cold PBS and lysed in 8 M urea, 2% SDS, 100 mM DTT and 375 mM Tris (pH 6.8) by heating at 37˚C for 2 h. The proteins were resolved by 5-10% SDS-PAGE. A total of 30 μg protein was loaded in each lane. Following this, the gels were transferred to polyvinylidene difluoride membranes using a semidry transfer system. Subsequently, the membranes were immunoblotted overnight at 4˚C with the following primary antibodies: Mouse anti-A2a (1:1,500; cat. no. ab79714; Abcam, Cambridge, MA, USA), rabbit anti-A1 (1:1,000; cat. no. ab82477; Abcam), rabbit anti-NF-κB p65 (1:2,000; cat. no. 04-1008; EMD Millipore, Billerica, MA, USA), rabbit anti-phosphorylated (p)-NF-κB p65 (1:1,000; cat. no. ab222494; Abcam), mouse anti-CREB antibody (1:1,000; cat. no. MAB5432; Millipore), mouse anti-phosphorylated CREB (1:1,000; cat. no. 05-667; Millipore), mouse anti-phosphorylated protein kinase C (p-PKC; 1:1,000; cat. no. ab75837; Abcam) and mouse anti protein kinase C (PKC; 1:1,000; cat. no. 05-983; Millipore). The membranes were then washed five times in 0.1% TBST and incubated with the secondary antibody [horseradish peroxidase-conjugated goat anti-mouse/rabbit immunoglobulin (Ig)G antibody; 1:1,000; cat. no. A0208 and A0216; Beyotime Institute of Biotechnology, Haimen, China] for 2 h at room temperature. The immunoreactive bands were developed using a chemiluminescent detection kit, visualized by ChemiDoc Imaging system (Bio-Rad Laboratories, Inc., Hercules, CA, USA) and quantified using ImageJ software (version 1.50i; National Institutes of Health, Bethesda, MD, USA). The membranes were then stripped and re-probed with a rabbit anti-α-tubulin polyclonal primary antibody at 4˚C overnight (1:1,000; cat. no. 11224-1-AP; Wuhan Sanying Biotechnology, Wuhan, China), followed by incubation with a horseradish peroxidase-conjugated goat anti-rabbit IgG antibody (1:1,000; cat. no. A0216; Beyotime Institute of Biotechnology) for 2 h at room temperature.

Statistical analysis. Statistical analyses were performed using GraphPad Prism (version 5.0; GraphPad Software, Inc., La Jolla, CA, USA). All experiments were repeated at least three times. All data were presented as the mean ± standard error of the mean. Comparisons between groups were performed using one-way analysis of variance followed by a Bonferroni post hoc test where appropriate. $P<0.05$ was considered to indicate a statistically significant difference.

Results

Imbalanced elevation and the antagonism between A1R and A2aR after low glucose/hypoxia in microglia. To mimic general ischemic injury, cells were cultured in low glucose and hypoxic conditions. Then, the expression of A1R and A2aR was measured in microglia exposed to low glucose/hypoxia for up to 10 h. This revealed that low glucose/hypoxia induced an upregulation of A1R and A2aR at 4, 6, 8 and 10 h after exposure (Fig. 1A and B). Furthermore, the expression of A1R enhanced faster than that of A2aR at 8 and 10 h after low glucose/hypoxia, suggesting an imbalance in A1R vs. A2aR expression. Next, agonists and antagonists of A1R and A2aR were applied to treat microglia that had been exposed to low glucose/hypoxia for 8 h (Fig. 1C and D). Interestingly, activation of A1R by CPA reduced the expression of A2aR, whereas inactivation of A1R by DPCPX did not notably increase A2aR expression. Conversely, activation of A2aR by CGS significantly reduced the expression of A1R, while the inhibition of A2aR with SCH did not significantly increase A1R expression. These data indicate an imbalance of A1R-A2aR expression after low glucose/hypoxia. However, this phenomenon requires verification under different metabolic tissue-specific conditions *in vivo* in the future.

Effects of A1R and A2aR on the activation of microglia. Compared to normal resting microglia, cells undergoing low glucose/hypoxia displayed a unique activation-associated morphology, including a larger and round soma, retracted projections and intercellular adhesion (Fig. 2A). To detect the effects of low glucose/hypoxia on the activation of microglia, the level of NO, an indicator of activated microglia, was measured in cultures exposed to low glucose/hypoxia for 2, 4, 6, 8, 10 and 12 h. Low glucose/hypoxia induced an increased release of NO within 12 h, peaking at 8 h (Fig. 2B). Furthermore, following an 8 h exposure to low glucose/hypoxia with agonists or antagonists of A1R and A2aR, the NO concentration in the microglia cultures was assayed (Fig. 2C). Inactivation of A1R by DPCPX and activation of A2aR by CGS significantly reduced the NO level in microglial cultures under low glucose/hypoxia conditions. Notably, inhibition of A2aR with SCH significantly increased the NO level. The A1R agonist CPA had no significant effect on NO levels. These findings suggest that A1R and A2aR serve distinct roles in the activation of microglia under low glucose/hypoxia conditions.

Effects of A1R and A2aR on the production of IL-6, IFN-β, IL-1β and TNF-α by microglia. To determine whether activated microglia secrete proinflammatory cytokines, the protein and mRNAs level of IL-6, IFN-β, IL-1β and TNF-α were measured in cultures after low glucose/hypoxia treatment, and exposure to agonists or antagonists of A1R and A2aR, for 8 h (Table II and Fig. 3). The inhibition of A1R and activation of A2aR reduced the concentration of IL-6, IL-1β and IFN-β in cultures (Fig. 3A, B and D). By contrast, activation of A1R or suppression of A2aR increased the production of IL-6, IL-1β and TNF-α (Fig. 3A-C). Notably, the expression of IFN-β mRNA was promoted by the A2aR antagonist SCH and reduced by its agonist CGS (Fig. 3H). In addition, the A1R agonist CPA significantly enhanced the mRNA expression of IL-6, IL-1β and TNF-α (Fig. 3E-G). These results indicate that the activation of A1R and/or inactivation of A2aR cause the secretion of microglia-derived proinflammatory cytokines under low glucose/hypoxia conditions.

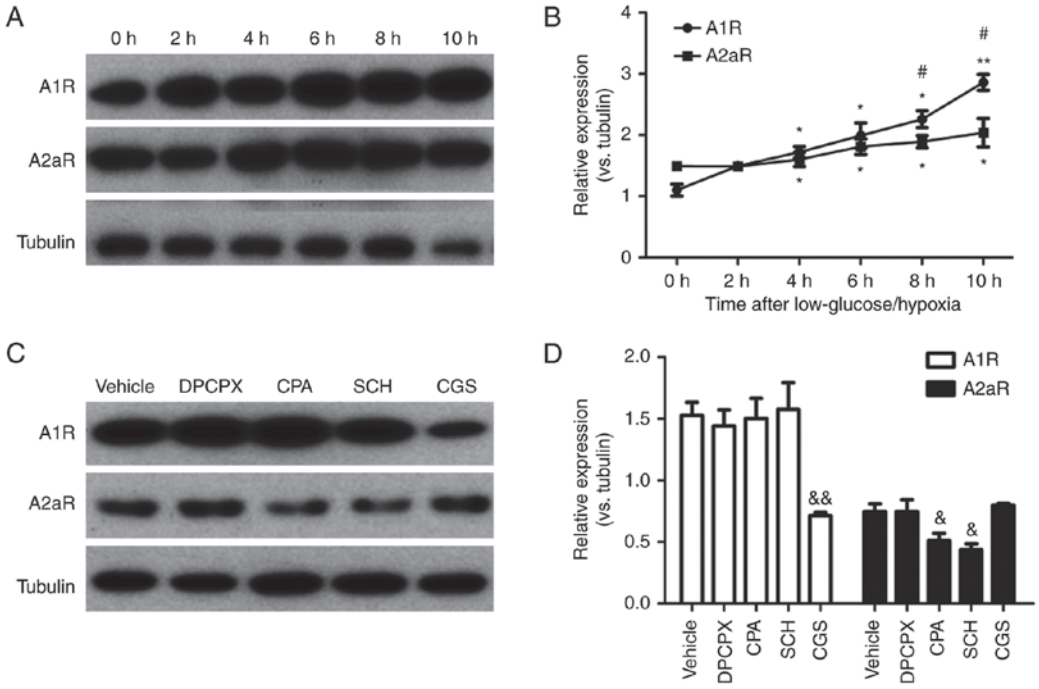

Figure 1. Imbalanced elevation and the antagonism between A1R and A2aR after low glucose/hypoxia in microglia. (A) Western blotting and (B) quantification of the expression of A1R and A2aR after exposure to low glucose/hypoxia for 0, 2, 4, 6, 8 and 10 h. *P≤0.05, **P≤0.01 vs. the 0 h group; $^{\#}$P≤0.05 vs. same time point in the A2aR group. (C) Western blotting and (D) quantification of the expression of A1R and A2aR after exposure to A1R and A2aR agonists and antagonists (n=6/group). $^{\&}$P≤0.05, $^{\&\&}$P≤0.01 vs. the vehicle group. A1R, adenosine A1 receptor; A2aR, adenosine A2a receptor.

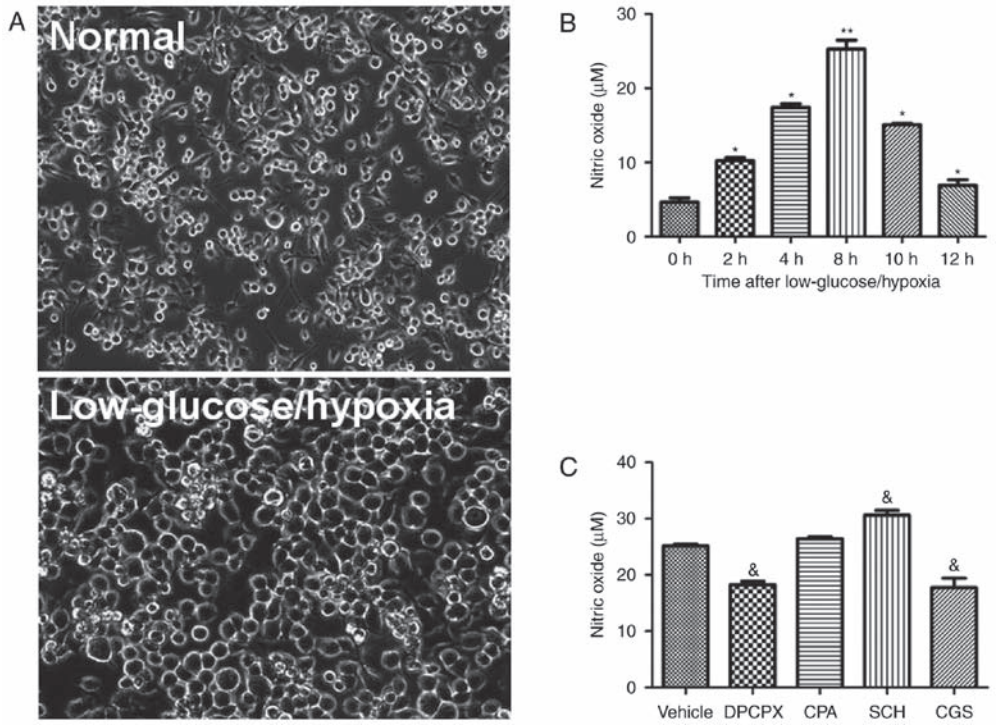

Figure 2. Effects of A1R and A2aR on the activation of microglia. (A) Phase-contrast microscopy images of microglia cell morphology under normal and low glucose/hypoxia conditions (magnification, x200). (B) Concentrations of NO, an indicator of microglial activation, at 0, 2, 4, 6, 8, 10 and 12 h after exposure to low glucose/hypoxia. (C) NO levels following treatment with A1R and A2aR agonists and antagonists (n=6/group). *P≤0.05, **P≤0.01 vs. the 0 h group; $^{\&}$P≤0.05 vs. the vehicle group. A1R, adenosine A1 receptor; A2aR, adenosine A2a receptor; NO, nitric oxide.

Effects of A1R and A2aR on oligodendrocyte damage. To further determine the effects of activated microglia on oligodendrocyte growth, a co-culture model of microglia and oligodendrocytes was used. The cytotoxicity induced by microglia-derived inflammatory cytokines was investigated by an LDH release assay and cell viability was measured using a

Table II. Effects of the A1R and A2aR agonists and antagonists on proinflammatory cytokine levels.

	Drug			
Cytokine	DPCPX (A1R inhibitor)	CPA (A1R activator)	SCH (A2aR inhibitor)	CGS (A2aR activator)
IL-6 protein	Downregulation[a]	Upregulation[b]	Upregulation[a]	Downregulation[a]
IL-6 mRNA	-	Upregulation[a]	Upregulation[a]	Downregulation[a]
IL-1β protein	Downregulation[a]	Upregulation[a]	Upregulation[a]	Downregulation[a]
IL-1β mRNA	-	Upregulation[b]	-	-
TNF-α protein	-	Upregulation[a]	Upregulation[a]	Downregulation[a]
TNF-α mRNA	-	Upregulation[a]	-	Downregulation[a]
IFN-β protein	-	Upregulation[b]	Upregulation[a]	Downregulation[a]
IFN-β mRNA	Downregulation[a]	-	Upregulation[a]	-

A1R, adenosine A1 receptor; A2aR, adenosine A2a receptor; IL, interleukin; IFN, interferon; TNF, tumor necrosis factor. [a]P≤0.05, [b]P≤0.01 vs. the vehicle group.

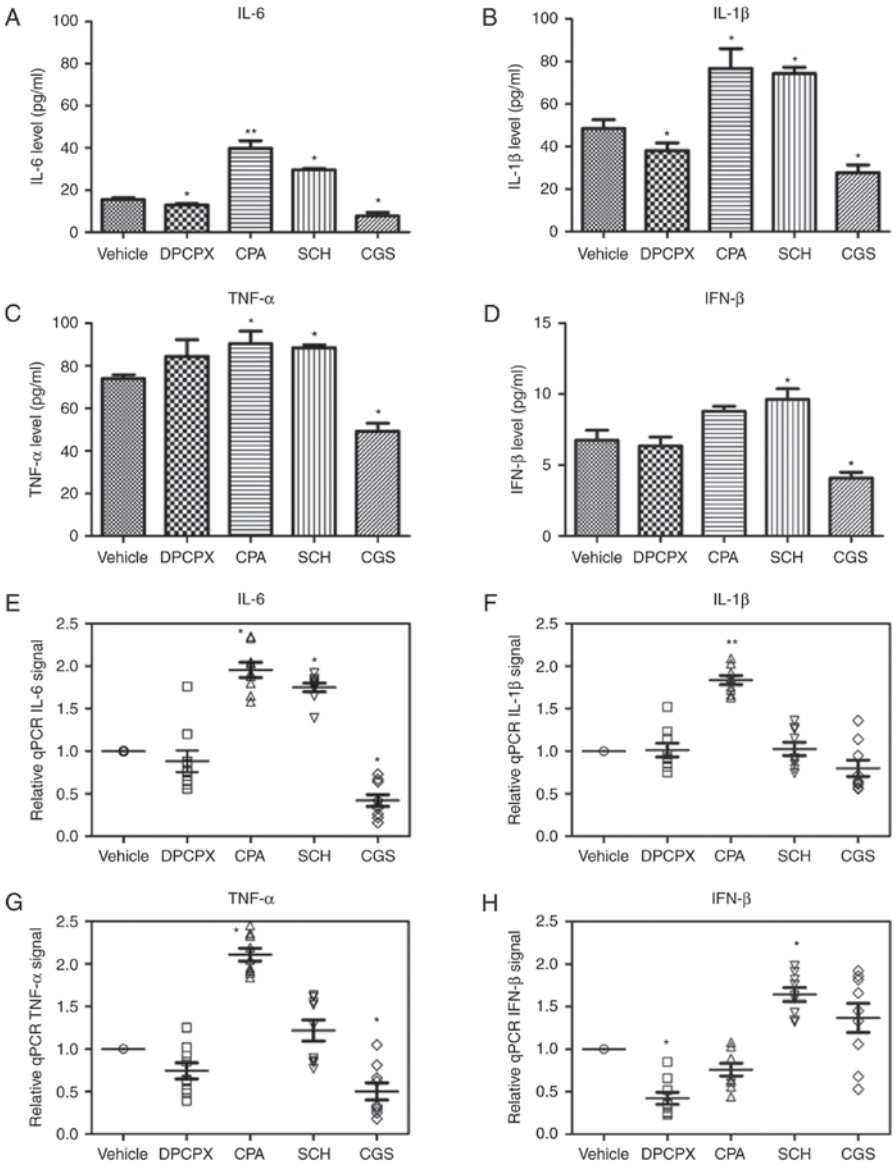

Figure 3. Effects of A1R and A2aR on the production of IL-6, IFN-β, IL-1β and TNF-α by microglia. The concentration of (A) IL-6, (B) IL-1β, (C) TNF-α and (D) IFN-β after exposure to A1R and A2aR agonists and antagonists (n=9/group) was detected by ELISAs. The mRNA levels of (E) IL-6, (F) IL-1β, (G) TNF-α and (H) IFN-β after exposure to A1R and A2aR agonists and antagonists (n=9/group) were determined by reverse-transcription quantitative polymerase chain reaction analysis. *P≤0.05, **P≤0.01 vs. the vehicle group. A1R, adenosine A1 receptor; A2aR, adenosine A2a receptor; IL, interleukin; IFN, interferon; TNF, tumor necrosis factor.

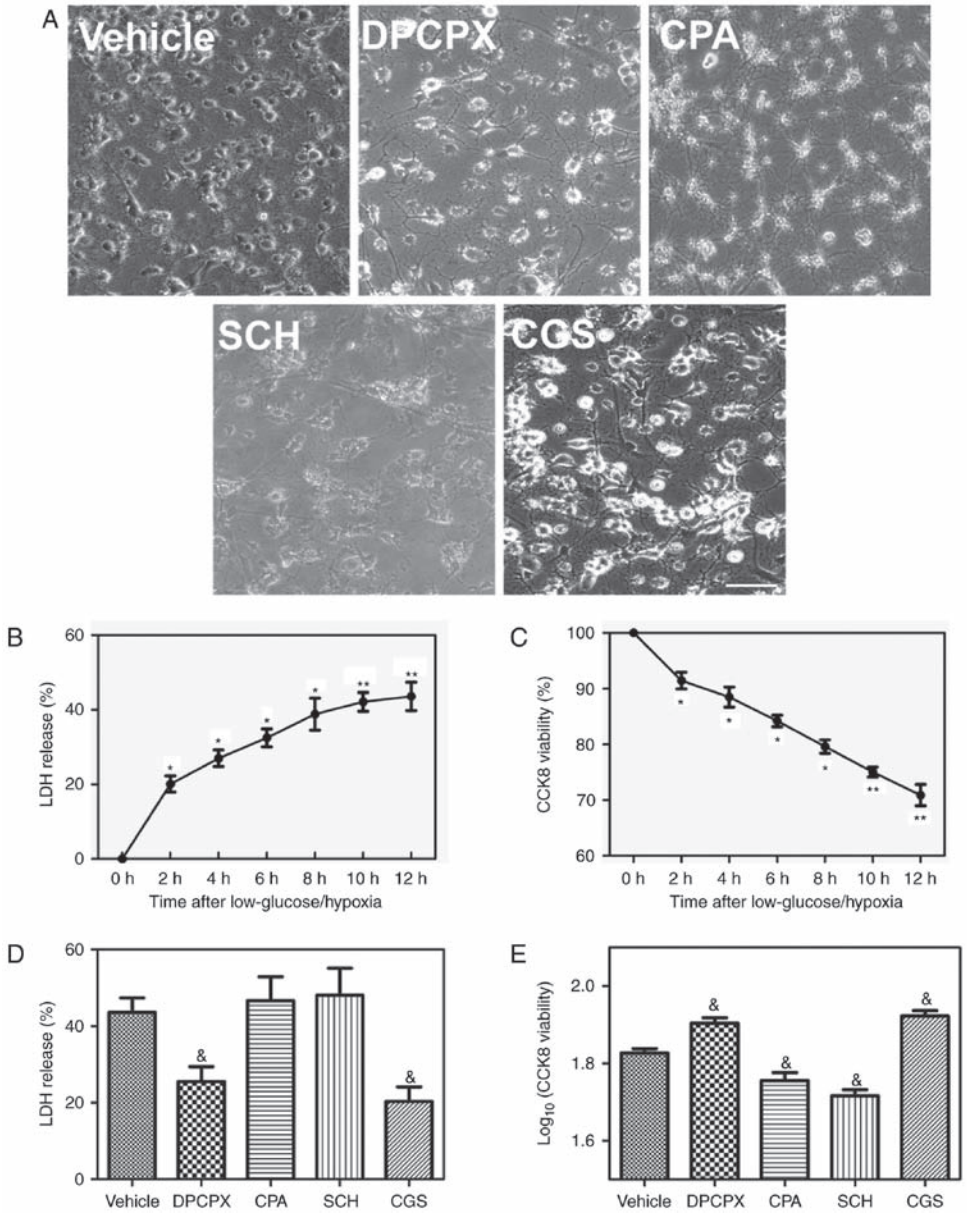

Figure 4. Effects of A1R and A2aR on oligodendrocyte damage. (A) Phase-contrast microscopy images of oligodendrocyte cell morphology (magnification, x200) after exposure to A1R and A2aR agonists and antagonists. Changes in the (B) LDH released and (C) CCK-8 assay-measured viability of oligodendrocytes at 0, 2, 4, 6, 8, 10 and 12 h after exposure to low glucose/hypoxia. (D) LDH release (E) viability of oligodendrocytes after exposure to A1R and A2aR agonists and antagonists (n=3/group). *P≤0.05, **P≤0.01 vs. the 0 h group; $^{\&}$P≤0.05 vs. the vehicle group. A1R, adenosine A1 receptor; A2aR, adenosine A2a receptor; LDH, lactate dehydrogenase; CCK-8, Cell Counting Kit-8.

CCK-8 assay. Reduced cell density and processes were observed in the low glucose/hypoxia stimulated groups (Fig. 4A). Low glucose/hypoxia caused a significant increase in LDH release and significantly decreased the viability of oligodendrocytes within 12 h compared with the vehicle group (Fig. 4B and C). Inactivation of A1R by DPCPX and activation of A2aR by CGS significantly reduced LDH release and significantly increased viability within 12 h compared with the vehicle group (Fig. 4D and E). Although activation of A1R by CPA and inactivation A2aR by SCH did not further enhance LDH release, they significantly reduced cell viability compared with the vehicle group (Fig. 4D and E). These data suggest that A1R and A2aR serve distinct roles in oligodendrocyte impairment. The inactivation of A1R and activation of A2aR may be an effective way of reducing oligodendrocyte damage after low glucose/hypoxia.

NF-κB and CREB are involved in the effects of the interaction between A1R and A2aR. NF-κB and CREB are critical transcriptional regulators of inflammation. Thus, whether an A1R-A2aR imbalance could affect the expression of NF-κB and CREB in microglia was investigated (Fig. 5). Compared with the vehicle group, inactivation of A1R by DPCPX and activation of A2aR by CGS significantly reduced the phosphorylation of NF-κB p65 (Fig. 5B). Paradoxically, activation of A1R also significantly reduced the expression of p-NF-κB (Fig. 5B). Inactivation of A2aR by SCH significantly enhanced the expression of NF-κB but not p-NF-κB (Fig. 5B and C).

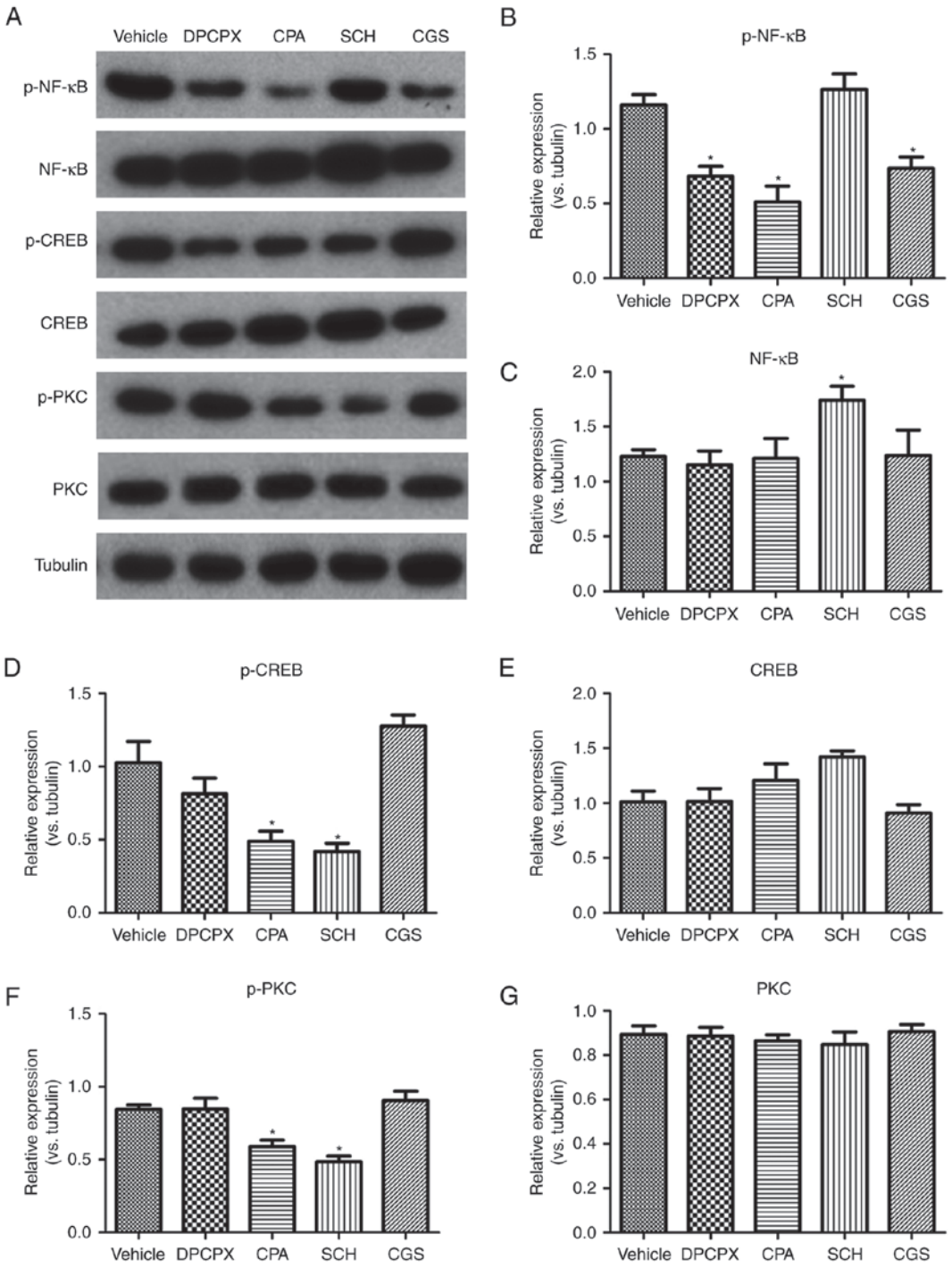

Figure 5. A1R-A2aR imbalance affects NF-κB p65 and CREB expression in microglia. (A) Western blotting and quantification of (B) p-NF-κB p65, (C) NF-κB, (D) p-CREB, (E) CREB, (F) p-PKC and (G) PKC in microglia exposed to low glucose/hypoxia plus A1R and A2aR agonists and antagonists (n=3/group). *P≤0.05 vs. the vehicle group. A1R, adenosine A1 receptor; A2aR, adenosine A2a receptor; NF, nuclear factor; CREB, cyclic adenosine monophosphate response element binding protein; PKC, protein kinase C.

Activation of A1R by CPA and inactivation of A2aR by SCH significantly reduced the protein levels of p-PKC and p-CREB (Fig. 5D and F). However, the expression of PKC and CREB was not significantly changed by any of the drugs (Fig. 5E and G).

Together, as illustrated in the schematic in Fig. 6, an imbalanced elevation of A1R and A2aR can result in the activation of microglia under low glucose/hypoxia conditions. Low glucose/hypoxia induces the phosphorylation of NF-κB p65 and CREB, promoting the release of inflammatory cytokines and causing oligodendrocyte damage. Therefore, rebalancing A1R-A2aR via the inactivation of A1R and activation of A2aR may inhibit this microglia-mediated immune cascade and prevent the damage of oligodendrocytes under low glucose/hypoxia conditions.

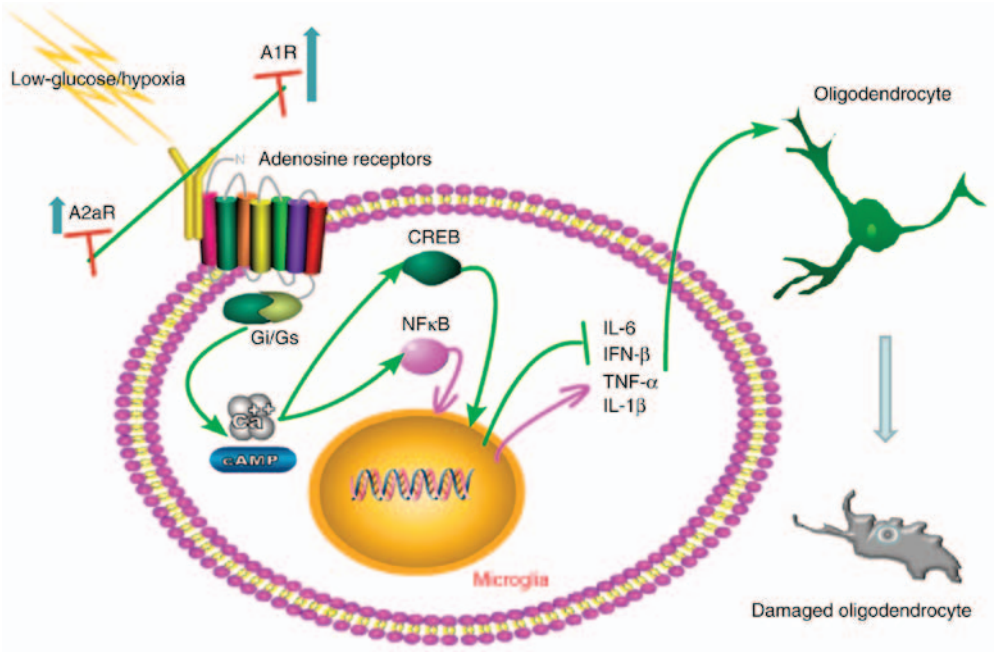

Figure 6. Schematic of the mechanisms by which A1R-A2aR imbalance activates microglia and damages oligodendrocytes under low glucose/hypoxia conditions. Low glucose/hypoxia stimulation causes an imbalance in elevation of A1R and A2a, with A1R levels increasing faster than A2aR levels. This imbalance triggers activation of the microglia and the following immune cascade. Mechanisms proposed to be involved include the inflammatory modulation of adenosine receptors coupled to G_i/G_s proteins. Overexpressed A1R activates cAMP or Ca^{2+}, promoting the phosphorylation of NF-κB p65 and CREB. Activation of these transcription factors regulates the release of inflammatory cytokines from the microglia, causing oligodendrocyte damage. A1R, adenosine A1 receptor; A2aR, adenosine A2a receptor; NF, nuclear factor; cAMP, cyclic adenosine monophosphate; CREB, cAMP response element binding protein; IL, interleukin; IFN, interferon; TNF, tumor necrosis fact.

Discussion

The present study investigated the role of A1R-A2aR imbalance in low glucose/hypoxia-induced microglial activation. The results indicated that an imbalance of A1R-A2aR serves an important role in the onset of microglial activation and inflammation after exposure to a low glucose/hypoxia in a NF-κB- and CREB-dependent manner. In addition, an A1R antagonist and A2aR agonist were applied to rebalance A1R-A2aR, which suppressed microglial activation and exhibited anti-inflammatory activity. Taking into account the results of our previous study, which identified that A2aR in bone marrow-derived dendritic cells is an important modulator of chronic cerebral hypoperfusion-induced WMLs (14), an A1R-A2aR imbalance may have important consequences for neuroinflammation and serve a role in the pathology of numerous CNS diseases.

The release of ATP in the brain serves an irreplaceable role in recruiting and formatting microglia to mount neuroinflammatory responses after hypoxia (15). This response involves the activation of different ATP purinergic 2 receptors, as well as ARs (16). However, the distinct role and interaction of these ARs remains unknown. The present study demonstrated that the activation of A2aR inhibits A1R in microglia, and vice versa. This antagonistic association between A1R and A2aR has been identified in previous studies. For example, A2R inhibits neutrophil adhesion to the endothelial layer, thereby blocking inflammatory initiation, while A1R enhances this process (17). Activation of presynaptic A1R suppresses excitatory transmission by reducing the probability of release, whereas A2aR exerts a facilitating effect on synaptic transmission by inhibiting A1R-mediated suppression (18). The neuromodulatory role of adenosine relies on a balanced activation of inhibitory A1R and facilitating A2aR (19). The current study revealed that the activation of A2aR with CGS attenuated inflammatory activity and improved oligodendrocyte viability. However, certain studies have reported that inhibition of A2aR alleviates the long-term burden of brain disorders in different neurodegenerative and psychiatric conditions, including ischemia, epilepsy, Parkinson's disease and Alzheimer's disease (19,22,23). The distinct role of A2aR in different pathological scenarios requires further study. By contrast, A1R acts as a regulator that effectively controls neurodegeneration if activated in the temporal vicinity of brain damage (11,24). In the present study, the upregulation of A1R was presumably responsible for the activation of microglia. This *in vitro* finding is in agreement with the results of a previous *in vivo* study of $A1R^{-/-}$ mice with neonatal brain hypoxic ischemia (25). Intriguingly, pharmacological preconditioning with an A1R agonist has been demonstrated to suppress the cellular immune response through an A2aR-dependent mechanism (26). These results suggest that targeting A1R and A2aR is a promising approach for researching and treating neuroinflammation.

The distinct effects of A1R and A2aR allude to different mechanisms of control for microglial activation. This mechanism may be similar to the process observed in neurons, in which the interaction between A1R and A2aR modulates neurotransmitter transporters via coupling to G_i/G_s proteins (27,28). A1R and A2aR may closely interact in such a way that the A1R is often inhibitory and couples to G_i/G_o proteins, while A2aR is usually coupled to G_s proteins, enhancing cAMP accumulation and PKA activity (29). Furthermore, the present study revealed

that the A1R antagonist DPCPX reduced the phosphorylation of NF-κB p65, and the production of IL-1β and TNF-α. Conversely, NF-κB has been reported to regulate A2aR gene transcription through a mechanism involving IL-1β and TNF-α (29). A2aR is able to enhance CREB phosphorylation via raising cAMP levels. Furthermore, p-CREB competitively binds with CREB binding protein, a ligand of NF-κB p65, and inhibits NF-κB transcription (30). In addition, the NO/cyclic guanosine monophosphate/protein kinase G/ATP-sensitive K^+ channel and the p38 mitogen-activated protein kinase signaling pathways can modulate CREB and NF-kB expression, insinuating a more complex signaling interaction between A1R and A2aR (31-34). These data indicate that NF-κB and CREB can be exploited as important nodes of the signaling network for the dissection of the interaction between A1R and A2aR.

The present study had several limitations. Firstly, a co-culture model of microglia and oligodendrocytes was used to investigate the effects of microglial-derived inflammatory cytokines on oligodendrocyte damage. However, an *in vivo* study should be performed in the future. Secondly, despite an association between A1R-A2aR imbalance and microglial activation after low glucose/hypoxia being demonstrated in the current study, the underlying mechanisms require elucidation. Thirdly, the mechanisms of the production of inflammatory cytokines from microglia are sophisticated and individualized; thus, additional signaling pathways apart from NF-κB and CREB should be explored. Lastly, A1R and A2aR have been reported to form complicated tetrameric heteromers in astrocytes and neurons using bioluminescence resonance energy transfer (BRET) and fluorescence resonance energy transfer (FRET) methods (13,35). Thus, whether A1R-A2aR heteromerization also occurs in microglia requires more direct evidence by means of BRET or FRET in the future.

In conclusion, the present study demonstrated that there is an imbalanced elevation of A1R-A2aR in microglia after exposure to low glucose/hypoxia, which initiates the release of inflammatory cytokines via modulation of NF-κB and CREB phosphorylation, thus contributing to oligodendrocyte damage. These results implicate an imbalance of A1R-A2aR in white matter impairment-induced demyelinating diseases. Suppression of A1R and activation of A2aR may be beneficial for rebalancing A1R-A2aR, thereby providing novel therapeutic strategies for the treatment of excessive nerve inflammation.

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Competing interests

The authors declare that there are no competing interests.

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