Caffeine Treatment Promotes Differentiation and Maturation of Hypoxic Oligodendrocytes via Counterbalancing Adenosine 1 Adenosine Receptor-Induced Calcium Overload

Ting Cao* 
Teng Ma* 
Yang Xu 
Yanping Tian 
Qiyan Cai 
Baichuan Li 
Yanping Tian 
Qiyan Cai 
Hongli Li

* Ting Cao and Teng Ma contributed equally to this article

Corresponding Author: Hongli Li, e-mail: lihongli@tmmu.edu.cn

Source of support: This work was supported by National Natural Science Foundation of China (No. 31471148 by Hongli Li)

Background: We aimed to explore the involvement of adenosine 1 adenosine receptor (A1AR) in hypoxia-induced poor differentiation of oligodendrocytes (OLs), and the underlying mechanism of caffeine treatment in hypoxic injuries.

Material/Methods: Real-time polymerase chain reaction (RT-PCR) was used to assess the alterations of AR expression in cultured hypoxic OLs with or without caffeine treatment. Then, intracellular alterations of Ca\(^{2+}\) concentrations ([Ca\(^{2+}\)]) were detected by confocal Fluo-3 imaging. The subsequent changes of myelin related protein expression were determined by western blot and immunofluorescence.

Results: Three hours after hypoxia, significantly upregulated expression of A1AR was observed, accompanied with significantly decreased expression of oligodendrocyte transcription factor (Olig2). In addition, either hypoxia stimulation or 100 μM adenosine induced apparent elevation of resting [Ca\(^{2+}\)] in cultured OLs. However, pretreatment with DPCPX (A1AR selective antagonist) or caffeine abolished the [Ca\(^{2+}\)] increase, and the subsequent adenosine of high dose induced Ca\(^{2+}\) activity in developing OLs. Furthermore, caffeine or DPCPX improved the expression MBP and CNPase proteins after hypoxia stimulation, which resulted in the morphological maturation of OLs.

Conclusions: Caffeine treatment exerted protective effects on neonatal hypoxia injuries. It prevented Ca\(^{2+}\) overload injury, kept Ca\(^{2+}\) homeostasis in hypoxic developing OLs, and facilitated optimal expression of myelin related proteins by inhibiting A1AR in vitro. This study also provided experimental evidence for clinical application of caffeine in early treatment of neonatal hypoxia, and highlighted the potential significance of A1AR in anti-hypoxic drug discovery.

MeSH Keywords: Adenosine • Caffeine • Calcium • Oligodendroglia • Receptor, Adenosine A1

Abbreviations: CNS – central nervous system; OLs – oligodendrocytes; A1AR – A1 adenosine receptor; IP,R – inositol trisphosphate receptor; Ca\(^{2+}\) – calcium; OGD – oxygen and glucose deprivation; OPCs – oligodendrocyte progenitor cells; Olig2 – oligodendrocyte transcription factor 2; PBS – phosphate-buffered saline; BSA – bovine serum albumin; RT – room temperature; DIC – differential interference contrast

Full-text PDF: https://www.medscimonit.com/abstract/index/idArt/915147
Backgroud

Neonatal hypoxia always causes severe and irreversible damage to the central nervous system (CNS) [1]. Studies have reported that poor differentiation of oligodendrocytes (OLs) and myelination disturbances were the main pathogenesis of hypoxic injury [2]. Thus, the development of OLs is a major concern in hypoxia. However, during distinct developmental stages of OLs lineage, early stage OLs (preOLs or developing OLs) are vulnerable to many factors, including hypoxia [3].

Caffeine, as a common therapeutic drug available in the neonatal intensive care unit, has been utilized for neonatal hypoxia treatment over 30 years [4,5]. Clinical studies have demonstrated that caffeine exerts therapeutic effects in neonatal hypoxic encephalopathy via counterbalancing the hypoxia-induced white matter injury and improving ventilation [6]. Additionally, caffeine has been shown to directly decrease hypoxia-induced ventriculomegaly and myelination disturbances in an animal model of chronic hypoxia [2]. However, until now, reports assessing the protective molecular mechanism of caffeine treatment are scarce.

Caffeine inhibits the function of extracellular adenosine, which is drastically elevated more than 100-fold under hypoxia condition [7,8]. It is also a non-selective agonist of adenosine receptors (ARs), which consist of A1, A2a, A2b, and A3 subtypes. All ARs are G-protein coupled receptors and associated with disease development in CNS, especially adenosine 1 adenosine receptor (A1AR) [9]. Cheng et al. reported the chronic ischemia-induced downregulation of A1AR contributed to white matter damage [10]. Activation of A1AR lead to white matter loss and ventriculomegaly [11], indicating that A1AR function was related to white matter damage. However, the role of A1AR in myelin forming cells, OLs, under hypoxic condition was still unclear.

Additionally, A1AR activation also mobilizes intracellular calcium signals (Ca++) via inositol triphosphate receptor (IP,R) [12]. In physiological conditions, sleep deprivation induces the release of adenosine in cholinergic basal forebrain to stimulate A1AR, resulting in IP,R activation and alterations of transcription factors [13]. In pathological conditions, like hypoxia, abnormal elevation of adenosine might induce over-activation of A1AR, and lead to dysregulation of IP,R and Ca++ signals. But whether this was the underlying mechanism of A1AR induced hypoxic injuries, requires experimental explorations.

This study was performed on cultured OLs in vitro. Oxygen and glucose deprivation (OGD) was utilized to simulate hypoxic conditions. We aimed to provide evidence for the involvement of A1AR in hypoxia-induced poor differentiation of OLs, and the underlying mechanism of caffeine treatment in hypoxic injuries.

Material and Methods

Animals and drugs

All procedures were conducted in accordance with the University Committee on Animal Care and Use. Sprague-Dawley (SD) neonatal rats (1 to 3 day postnatal, P1–P3, n=10) were purchased from the Animal Facility Center of the Army Medical University, P.R. China, and housed in separate cages in a controlled environment.

For adenosine receptor ligands, adenosine and caffeine (Sigma-Aldrich, St. Louis, MO, USA) were utilized; the selective antagonist DPCPX (8-cyclopentyl-1,3-dipropylxanthine) (Abcam, Cambridge, UK) was tested as well.

Culture of purified OPCs (OL progenitor cells)

The rat OL progenitor cells (OPCs) were propagated as previously described [14]. In brief, cell suspensions were collected from the cerebral cortex of postnatal 1 to 3 days old SD rats and then cultured in OPCs proliferation medium, containing: DMEM/F12 (Hyclone); 1% N2 supplement; 5 mg/mL N-acetyl-L-cysteine (Amresco, Solon, OH, USA); 1% fetal bovine serum (FBS); 5 mg/mL insulin, 15 to 30 minutes at room temperature and centrifuged at 1000 rpm for 5 minutes. Then, isolated OPCs were cultured with OPCs-proliferation medium in dishes or cover slips coated with poly-D-lysine. For OPCs differentiation experiments, OPCs were incubated with OPCs-proliferation medium in dishes or cover slips coated with poly-D-lysine. For OPCs differentiation experiments, OPCs were incubated with OPCs differentiation medium containing: DMEM/F12; 1% N2 supplement; 5 mg/mL N-acetyl-L-cysteine (Amresco, Solon, OH, USA); 1% fetal bovine serum (FBS); 5 mg/mL insulin. At the distinct time point of differentiation stage, cells were collected and fixed for experiments.

Hyposic model of cultured oligodendrocytes

For oxygen and glucose deprivation (OGD) in OLs medium, cultures were maintained in DMEM with 10% FBS in incubator at 37°C in a humidified atmosphere with 5% CO2. Then, external O2 and glucose were replaced with N2 and sucrose (10 mM), respectively. Finally, 20 μM iodoacetate was used to block glycolysis. After 30 minutes in OGD conditions, OLs were returned to fresh media. The hypoxic damage was detected by immunocytochemistry and western-blot of CNPase (a maker of immature OLs), MBP (a maker of mature OLs), and GFAP (a marker expressed in immature oligodendrocytes and astrocytes) as well as Ca++ alteration at different post stimuluses (1 day to 6 days).

For adenosine treatment, 100 μM adenosine [15] was used to mimic hypoxic injury. OLs were exposed to adenosine solution
20 minutes before returning to fresh medium with or without 0.1 mM caffeine, 100 nM DPCPX (selective A1AR antagonist). After adenosine stimulation, cells were cultured for 3 to 6 days for various experiments. For observation of Ca\textsuperscript{2+} dynamic alteration, we tested Ca\textsuperscript{2+} response of OLs to adenosine. Solution in concentration ranged from 100 μM and 300 μM. Adenosine 300 μM was utilized to induce temporal and drastic Ca\textsuperscript{2+} response in cultured OLs.

**Reverse transcription polymerase chain reaction (RT-PCR)**

Total RNA was extracted and purified from cultured cells, according to the experiment protocol of TRIzol Reagent (Life Technologies, USA). Briefly, the reverse transcription of total RNA into single strand cDNA was performed using random primer (Takara) and Super Script III (Life Technologies, USA). The sequences of specific primers were listed as follows: A1AR, 5'-TGGTCATTGGCCTTGTCCT-3' and 5'-GATCTGAGCTGGGATGTA-3'; A2aAR, 5'-CTGCTCTTCTCCCTGGTT-3' and 5'-GCTGGAGTTCCTTTCTTCT-3'; A2bAR, 5'-GTCATGGAACAGACGACAGA-3' and 5'-ATGGACAGTGAGAGAAAGACA-3'; A3AR, 5'-ACCTTCTATTTCATCTTCCCTA-3' and 5'-TTCTTCTTGTAGTGAAGCCTTA-3'; Olig2, 5'-TCAAGTCATCTTCCTCCAGCAC-3' and 5'-GGTGGAGTTCCCGTCTTTCT-3'; A1R, 5'-TCAAGTCATCTTCCTCCAGCAC-3' and 5'-GGTGGAGTTCCCGTCTTTCT-3'; CNPase, 5'-GATCTTGACTCGGAGGTATCGA-3'; Olig2, 5'-CTTCTTTGAGTGGTAACCGTTCTA-3'; A3aR, 5'-ACCTTCTATTTCATCTTCCCTA-3' and 5'-TTCTTCTTGTAGTGAAGCCTTA-3'; CNPase, 5'-TGCTCATTGCCTTGGTCTCT-3'; MBP, 5'-GATCTTGACTCGGAGGTATCGA-3'; Olig2, 5'-CTTCTTTGAGTGGTAACCGTTCTA-3'; A3aR, 5'-ACCTTCTATTTCATCTTCCCTA-3' and 5'-TTCTTCTTGTAGTGAAGCCTTA-3'; and Olig2, 5'-CTTCTTTGAGTGGTAACCGTTCTA-3'.

**Immunofluorescence staining**

For immunofluorescence staining, cells were first treated in a mixture of 3% H\textsubscript{2}O\textsubscript{2}, methanol and phosphate-buffered saline (PBS) at the ratio of 1: 1 to suppress endogenous peroxidase activity followed by 2 PBS washes. Then, sections were blocked with 5% bovine serum albumin (BSA) and incubated overnight at room temperature using primary antibodies: anti-A1R (1: 200, Boster, China); Olig2 (1: 200, Abcam, USA); MBP (1: 200, Santa Cruz, CA, USA); A1R (1: 200, Boster, China); CNPase (1: 200, Santa Cruz, CA, USA); Olig2 (1: 200, Abcam, USA); anti-IP2 (1: 500) and anti-R2 (1: 500) and anti-β-actin (1: 1000, Boster, China). After BSA blocking for 1 hour, primary antibodies were used overnight at 4°C, including: anti-CNpase (1:1000, Santa Cruz, CA, USA), anti-MBP (1:1000, Santa Cruz, CA, USA), anti-GFAP (1:1000, Santa Cruz, CA, USA), anti-IP2 (1:500) and anti-β-actin (1:1000, Boster, China). Then, HRP-conjugated secondary antibodies were used (1:1000, Boster, China) and ECL plus detection kit (ECL plus, GE Healthcare) were conducted to reveal the immunoreactive bands. Image-Pro Plus (Media Cybernetics, MD, USA) was used for statistical analysis of western blot products (normalized to β-actin).

**Western blot analysis**

After experimental intervene, total proteins were isolated from cultured OLs using the RIPA lysis buffer with 1% PMSF solution (Beyotime, China). Protein amounts were quantified by the Coomassie brilliant blue G250 method. Equal amounts of protein in different groups were denatured and loaded separately on SDS-PAGE gels. After electrophoresis, the target bands were transferred onto polyvinylidifluoride (PVDF) membranes. After BSA blocking for 1 hour, primary antibodies were used overnight at 4°C, including: anti-CNpase (1:1000, Santa Cruz, CA, USA), anti-MBP (1:1000, Santa Cruz, CA, USA), anti-GFAP (1:1000, Santa Cruz, CA, USA), anti-IP2 (1:500) and anti-β-actin (1:1000, Boster, China). Then, HRP-conjugated secondary antibodies were used (1:1000, Boster, China) and ECL plus detection kit (ECL plus, GE Healthcare) were conducted to reveal the immunoreactive bands. Image-Pro Plus (Media Cybernetics, MD, USA) was used for statistical analysis of western blot products (normalized to β-actin).

**Quantitative image analysis**

For each experiment, 10 randomly chosen fields at 20× magnification were recorded for cell counting. Image-Pro Plus software (n=10) was used to quantify the immunofluorescence intensity of immunostaining, Fluo-3 staining and cell counting. The relative optical density of PCR or western blot results were quantified by Image-Pro Plus image analysis system (normalized to β-actin).

**Statistical analysis**

Statistical analysis was performed through SPSS18.0 for Windows (SPSS Inc, Chicago, IL, USA) in one- or two-way analysis of variance (ANOVA) followed by Tukey's post-hoc test. Comparisons of 2 experimental groups were carried out using statistical analysis.
Student’s t-test. Data was presented as mean ± standard deviation (SD) and P<0.05 was considered statistically significant.

Results

To explore whether the expression of ARs were altered in hypoxic OLs and whether caffeine functions as the antagonist of ARs, mRNA expressions of ARs were detected by RT-PCR in normal and hypoxia conditions, with or without 0.1 mM caffeine treatment (Figure 1). After hypoxia, in cultured developing OLs, a rapid elevation of A1AR mRNA was found from 1 to 12 hours, peaking at 3 hours in the OGD group (P<0.05; Figure 1A, 1B). However, treatment with 0.1 mM caffeine early abolished the elevation of A1AR observed in the OGD group. In contrast, A2aAR, A2bAR, and A3AR did not display significant alterations in the early stage of hypoxia as well as in caffeine treatment (Figures 1D–1F). Besides, in normal condition, OLs expressed sufficient oligodendrocyte transcription factor 2 (Olig2) (Figure 1A). PCR results revealed that the expression of Olig2 mRNA was notably decreased in the OGD group from 1 hour to 12 hours (P<0.05; Figure 1C). After caffeine treatment, OLs displayed similar expression of Olig2 mRNA with control (CTL) group. It indicates hypoxia interfered the differentiation of OLs, which was the hallmark of injury and caffeine treatment reversed this hypoxic injury.

In addition, immunofluorescence was used to reveal the expression of A1AR and Olig2 in cultured OLs of 1 day (Figure 1G). In the CTL group, A1AR was found distributed in the cytoplasm and the fluorescence intensity was weak on cell protuberance. In the OGD group, the fluorescence intensity of A1AR in the cytoplasm and cell protuberance were apparently increased (P<0.05, Figure 1G, 1H). After caffeine treatment, the fluorescence intensity of A1AR was decreased and similar to the CTL group. Additionally, the expression of Olig2 was significantly decreased in the OGD group while this effect was also recovered by caffeine treatment (P<0.05, Figure 1G, 1I).

To investigate whether caffeine reversed hypoxia-induced disturbance of OLs differentiation, we observed the morphological differentiation of OLs after OPCs were cultured for 3 days (Figure 2A, 2B). During the maturation process, immature OLs passed through a series of morphological changes. Compared

| A | B | C | D | E | F |
|---|---|---|---|---|---|
| A1AR | Relative optical density of A1AR mRNA (% to actin) | Relative optical density of Olig2 mRNA (% to actin) | Relative optical density of A2aAR mRNA (% to actin) | Relative optical density of A2bAR mRNA (% to actin) | Relative optical density of A3AR mRNA (% to actin) |
| A | B | C | D | E | F |
| CTL 0h 0h 0h 0h 0h | +Caffeine 0h +Caffeine 0h +Caffeine 0h +Caffeine 0h +Caffeine 0h | +Caffeine 0h +Caffeine 0h +Caffeine 0h +Caffeine 0h +Caffeine 0h | +Caffeine 0h +Caffeine 0h +Caffeine 0h +Caffeine 0h +Caffeine 0h | +Caffeine 0h +Caffeine 0h +Caffeine 0h +Caffeine 0h +Caffeine 0h | +Caffeine 0h +Caffeine 0h +Caffeine 0h +Caffeine 0h +Caffeine 0h |
| OGD 1h OGD 3h OGD 6h OGD 12h | Relative optical density of A1AR mRNA (% to actin) | Relative optical density of Olig2 mRNA (% to actin) | Relative optical density of A2aAR mRNA (% to actin) | Relative optical density of A2bAR mRNA (% to actin) | Relative optical density of A3AR mRNA (% to actin) |
| +Caffeine 1h +Caffeine 3h +Caffeine 6h +Caffeine 12h | +Caffeine 1h +Caffeine 3h +Caffeine 6h +Caffeine 12h | +Caffeine 1h +Caffeine 3h +Caffeine 6h +Caffeine 12h | +Caffeine 1h +Caffeine 3h +Caffeine 6h +Caffeine 12h | +Caffeine 1h +Caffeine 3h +Caffeine 6h +Caffeine 12h | +Caffeine 1h +Caffeine 3h +Caffeine 6h +Caffeine 12h |

Indexed in: [Current Contents/Clinical Medicine] [SCI Expanded] [ISI Alerting System] [ISI Journals Master List] [Index Medicus/MEDLINE] [EMBASE/Excerpta Medica] [Chemical Abstracts/CAS]

This work is licensed under Creative Common Attribution-NonCommercial-NoDerivatives 4.0 International (CC BY-NC-ND 4.0)
with immature OLs, mature OLs formed more primary and secondary cell processes to present a net-like structure. In the CTL group, more than 60% of OLs displayed mature morphological features and arborization was observed. However, in the OGD group, only 18.4% displayed mature features (P<0.01). OLs were swelling and cell processes were ruptured. Then, we used 100 μM adenosine to simulate hypoxic injury and the mature percentage of OLs was decreased to 24.1%, which was similar to the OGD group (P<0.01). In contrast, when treated with 0.1 mM caffeine or 100 nM DPCPX (A1AR selective antagonist), in the OGD group, differentiation disturbance was morphologically recovered and the percentage of mature OLs both reached approximately 57%.

To explore the mechanism of hypoxic induced poor differentiation of OLs and caffeine effects, confocal Ca²⁺ imaging was utilized to detect intracellular resting [Ca²⁺] via quantifying the fluorescence intensity of Fluo-3 labelling OLs (Figure 2C, 2D). Fluorescence signals were normalized to average values measured before drug application. In the CTL group, the fluorescence intensity of Fluo-3 pseudo color was stable and not intense. In the OGD group, the fluorescence intensity of Fluo-3 was significantly increased compared to the CTL group (P<0.05) and similar elevation was recorded after 100 μM adenosine treatment. In contrast, 0.1 mM caffeine or 100 nM DPCPX treatment prevented elevation of the fluorescence signal in the OGD group. This indicated that 0.1 mM caffeine or 100 nM DPCPX treatment prevented abnormal elevation of intracellular resting [Ca²⁺] in developing OLs in hypoxia.

To further our understanding of hypoxia-induced resting [Ca²⁺] elevation, we detected the protein expression of potential [Ca²⁺] release channels, IP₃Rs. Among 3 subtypes of IP₃Rs, only IP₃R2 has been reported in OLs [16]. Our western blot results showed that IP₃R2 was expressed in the CTL group. However, hypoxia induced significant increase in the expression of IP₃R2 protein (P<0.01; Figure 2E, 2F). Treatment of 0.1 mM caffeine notably decreased its expression.
To understand the mechanism of caffeine treatment in regulating Ca\textsuperscript{2+} response, confocal Ca\textsuperscript{2+} imaging was used to detect dynamic intracellular [Ca\textsuperscript{2+}] activity upon ATP or adenosine stimulation (Figure 3). In OPC culture of 3 days, as a positive control, 300 μM ATP triggered overt [Ca\textsuperscript{2+}] transient wave in developing OLs, which peaked sharply and descended quickly (Figure 3A). In contrast, 300 μM adenosine induced apparent [Ca\textsuperscript{2+}] response. The Ca\textsuperscript{2+} transient wave underwent sustained elevation and remained at plateau phase after the descent of ATP-induced [Ca\textsuperscript{2+}] uptake (Figure 3B). When pharmacologically blocked, the activation of A1AR in developing OLs by 0.5 mM caffeine or 300 nM DPCPX pretreatment, Ca\textsuperscript{2+} transient wave did not undergo intensive elevation and [Ca\textsuperscript{2+}] wave remained at relatively lower and stable levels after adenosine stimulation (Figure 3C, 3D). The area under the curve (AUC) and peak value of Ca\textsuperscript{2+} transient waves were calculated (Figure 3E, 3F). Significant differences were found with or without caffeine treatment. Caffeine treatment not only inhibited adenosine-induced Ca\textsuperscript{2+} release at the peak (P<0.01), but decreased intracellular [Ca\textsuperscript{2+}] release after adenosine stimulation (P<0.01). This phenotype was similar with DPCPX treatment.

To verify the effect of caffeine in regulating the differentiation and myelination of hypoxic OLs, firstly we detected quantified CNPase and GFAP protein expression levels by western blot (Figure 4A). Our data showed that hypoxia induced a significant reduction of CNPase proteins (P<0.05, Figure 4B) while inducing an elevation of GFAP proteins (P<0.05; Figure 4C). The alteration was also observed after treatment of OLs with 100 μM adenosine (P<0.05). In contrast, treatment with 0.1 mM caffeine or 100 nM DPCPX reversed the changed expression of CNPase and GFAP proteins in the OGD group.

Three days after hypoxia, CNPase proteins were expressed in OLs and cells presented nearly mature features. However, the fluorescence intensity of CNPase protein was overtly decreased...
and OLs displayed immature features in the OGD group. Caffeine treatment was observed to counterbalance the hypoxic effects on OLs in the expression of CNPase and also in morphological differentiation (Figure 4D, 4E). Besides, in 6 days, OLs in the CTL group and the caffeine group presented high fluorescence intensity of MBP protein and arborization was abundant and dense. The OGD group displayed immature features of OLs with swollen cytoplasm and fewer cell processes. In addition, the fluorescence intensity of MBP protein in the OGD group was weak than in the CTL group, while caffeine treatment recovered the expression of MBP protein (Figure 4D, 4F).

**Discussion**

A1AR is widely expressed in CNS. It is also found in OLs, astrocytes [17], and microglia cells. Turner et al. reported a correlation between A1AR activation and hypoxia-induced white matter loss [11]. Our results also revealed the importance of A1AR in hypoxia. In the early stage of hypoxia (<12 hours), A1AR underwent a rapid upregulation and peaked at 3 hours in developing OLs. This response was associated with stark changes of adenosine concentration, which increased by more than 100-fold within minutes after acute hypoxia. High concentrations of adenosine were also proven to induce hypoxia-like injury in cultured OLs in our experiments via interfering with morphological differentiation. In addition, we did not observe apparent relationships between the other 3 adenosine receptors and cellular hypoxic response. These findings provide evidence that A1AR is involved in hypoxic injury in developing OLs. However, A1AR has been reported to protect the brain in embryo [18,19], which was different from our results. This indicates that A1AR activation might lead to different effects before versus after birth [18].

For years, A1AR has been regarded as an anti-inflammatory in the CNS [20], indicating its protective role in hypoxia. However, this effect has not been considered to be critically important in hypoxic and ischemic models [9]. Our results also strongly indicated that A1AR activation was associated with the developmental disorder of OLs and white matter loss. As caffeine has been used to treat hypoxia-induced white matter loss for over 30 years, we pretreated OLs with 0.1 mM caffeine after hypoxia. Caffeine treatment reversed this potential injury. In conclusion, caffeine exerts antagonistic effects on A1AR and prevents its...
Figure 3. Caffeine decrease adenosine-induced intracellular Ca\(^{2+}\) response in developing OLs via inhibiting A1AR. (A) Ca\(^{2+}\) transient wave of 300 μM ATP in cultured OLs. ATP induced overt [Ca\(^{2+}\)] response in developing OLs, which reached peak value sharply and then descended quickly as a positive control. (B) Ca\(^{2+}\) transient wave of 300 μM adenosine in cultured OLs. Adenosine induced sustained elevation of [Ca\(^{2+}\)] and remained at plateau phase. (C, D) After 0.5 mM caffeine or 300 nM DPCPX treatment, Ca\(^{2+}\) transient wave did not undergo intensive elevation and [Ca\(^{2+}\)] wave remained at relatively lower and stable level after adenosine stimulation. (E, F) AUC and peak value of Ca\(^{2+}\) wave. Significant differences were found between adenosine and caffeine, DPCPX treatment. Basal line of Ca\(^{2+}\) transient waves were normalized to the averaged measured in cultures in standard solution. Values represent mean ± standard deviation. (n=10 for each group) ** P<0.01 versus adenosine group. OLs – oligodendrocytes; A1AR – adenosine 1 adenosine receptor; DPCPX – A1AR selective antagonist; AUC – area under the curve.
overexpression in hypoxia. Caffeine also protects the expression of differentiation related factors of OLs in hypoxia.

Our results also provided evidence that hypoxia and adenosine treatment induced significant elevation in resting intracellular $[\text{Ca}^{2+}]$. When we treated cells with caffeine or the A1AR antagonist DPCPX, resting $[\text{Ca}^{2+}]$ decreased to normal levels in hypoxia. This indicated that caffeine was able to regulate intracellular $[\text{Ca}^{2+}]$ and keep $\text{Ca}^{2+}$ homeostasis in developing OLs during hypoxia by antagonizing A1AR. Furthermore, adenosine stimulation of OLs resulted in long-lasting elevation of $[\text{Ca}^{2+}]$, indicating that adenosine induces sustained elevation of intracellular $[\text{Ca}^{2+}]$. However, the adenosine-induced $[\text{Ca}^{2+}]$ uptake could be inhibited by treatment with caffeine or DPCPX. In conclusion, caffeine treatment inhibited adenosine induced Ca2+ activity and maintained Ca2+ homeostasis. Our findings unveiled the underlying mechanism of OLs injury in hypoxia by observing $[\text{Ca}^{2+}]$: as adenosine increased rapidly and violently...
in hypoxia within minutes, A1AR was stimulated by high adenosine concentrations, which resulted in excessive Ca\(^{2+}\) release. Moreover, this excessive Ca\(^{2+}\) release can directly lead to Ca\(^{2+}\) overload in hypoxia \([21,22]\), and is considered with initiate cell injury. However, previous studies have also reported that A1AR reduces [Ca\(^{2+}\)] by inhibiting voltage-gated Ca\(^{2+}\) channels in excitable cells, including motor neurons \([23]\), and retinal ganglion cells \([24]\). This effect is considered an important way to inhibit the release of excitatory neurotransmitters, such as glutamate \([25]\), in hypoxia. In contrast, our results demonstrated that A1AR contributed to Ca\(^{2+}\) overload in developing OLs. During the whole developmental process of OL lineage, OLs exhibit obvious plasticity to Ca\(^{2+}\) signal at different stages of development and are sensitive to the change of [Ca\(^{2+}\)] \([26]\).

[Ca\(^{2+}\)] overload in hypoxic developing OLs directly leads to apoptosis, mitochondrial damage, production of reactive oxygen species, and activation of Ca\(^{2+}\)-dependent proteases \([27]\).

In addition, A1AR activation, in a concentration-dependent manner, mobilizes intracellular [Ca\(^{2+}\)] via the IP\(_3\)Rs located on the endoplasmic reticulum membrane \([13,28]\). This mechanism can explain our results and the findings of enhanced [Ca\(^{2+}\)] in isolated OLs via A1AR activation \([29]\).

In cultured developing OLs, 100 μM adenosine was able to decrease the protein expression of CNPase and show a compensatory increase in the expression of GFAP, resulting in morphologically poor differentiation of OLs. When cells were exposed to hypoxia conditions, similar effects were observed. Interestingly, 0.1 mM caffeine or 100 nM DPCPX reversed these effects. It becomes increasingly clear that caffeine inhibits A1AR activation to upregulate the expression of myelin-related proteins and promote the morphological differentiation of OLs. Our findings were in agreement with previous works by Turner and collaborators \([11,30]\), who generated a gene knockout model of A1AR and observed increased expression of MBP proteins and myelination in hypoxic brain. Together, A1AR plays a crucial role in the differentiation of developing OLs, caffeine treatment experts significant protective effects in developing OLs by antagonizing A1AR to promote cell differentiation.

### Conclusions

Our results demonstrated that caffeine treatment was able to prevent Ca\(^{2+}\) overload and keep Ca\(^{2+}\) homeostasis in developing OLs. In addition, in hypoxia conditions, this mechanism antagonizes A1AR and IP\(_3\)R2 activation and results in the expression of myelin-related proteins to initiate maturation and myelination. In terms of potential clinical use, this mechanism might be able to reverse hypoxia-induced neonatal periventricular leukomalacia. In addition, A1AR displayed a potential significant role in anti-hypoxic drug discovery.

### Conflict of interest

None.
22. Annunziato L, Boscia F, Pignataro G: Ionic transporter activity in astrocytes, microglia, and oligodendrocytes during brain ischemia. J Cereb Blood Flow Metab, 2013; 33(7): 969–82
23. Mynlieff M, Beam KG: Adenosine acting at an A1 receptor decreases N-type calcium current in mouse motoneurons. J Neurosci, 1994; 14(6): 3628–34
24. Sun X, Barnes S, Baldridge WH: Adenosine inhibits calcium channel currents via A1 receptors on salamander retinal ganglion cells in a mini-slice preparation. J Neurochem, 2002; 81(3): 550–56
25. Wang SJ: Caffeine facilitation of glutamate release from rat cerebral cortex nerve terminals (synaptosomes) through activation protein kinase C pathway: An interaction with presynaptic adenosine A1 receptors. Synapse, 2007; 61(6): 401–11
26. Soliven B: Calcium signalling in cells of oligodendroglial lineage. Microsc Res Techniq, 2001; 52(6): 672–79
27. Dewar D, Underhill SM, Goldberg MP: Oligodendrocytes and ischemic brain injury. J Cereb Blood Flow Metab, 2003; 23(3): 263–74
28. Kang SS, Han KS, Ku BM et al: Caffeine-mediated inhibition of calcium release channel inositol 1, 4, 5-trisphosphate receptor subtype 3 blocks glioblastoma invasion and extends survival. Cancer Res, 2010; 70(3): 1173–83
29. Peakman MC, Hill SJ: Adenosine A1 receptor-mediated changes in basal and histamine-stimulated levels of intracellular calcium in primary rat astrocytes. Brit J Pharmacol, 1995; 115(5): 801–10
30. Turner CP, Seli M, Ment L et al: A1 adenosine receptors mediate hypoxia-induced ventriculomegaly. Proc Natl Acad Sci USA, 2003; 100(20): 11718–22