P2X7 receptor modulation of the viability of radial glial clone L2.3 cells during hypoxic-ischemic brain injury

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Received December 7, 2011; Accepted February 22, 2012

DOI: 10.3892/mmr.2012.816

Abstract. The purinergic P2X7 receptor (P2X7R) can be activated by ATP and plays significant and complex roles in neuropathology. However, research is limited concerning the role of P2X7R in radial glia following hypoxia-ischemia (HI). In this study, radial glial clone L2.3 cells were cultured and subjected to oxygen-glucose deprivation (OGD) to generate an HI model *in vitro*. We found that HI decreased P2X7R expression in the L2.3 cells. Activation of P2X7R in L2.3 cells by 3'-O-(4-benzoylbenzoyl) adenosine 5'-triphosphate (BzATP) led to cell death in a dose- and time-dependent manner, while a P2X7R antagonist, oxidized ATP (oATP), alleviated the injury induced by BzATP or HI. We also found that P2X7R modulated the phosphorylation of glycogen synthase kinase-3β (GSK-3β). The present findings suggest that L2.3 cells express P2X7R, and this receptor may be involved in HI injury of radial glia by mediating phosphorylation of GSK-3β.

Introduction

As we know hypoxia-ischemia (HI) can cause cell death and neurological damage to the central nervous system (CNS). Studies propose that radial glia function as neural progenitors during CNS development (1,2). However, research is sparse concerning the injury of radial glia as a result of HI.

Adenosine 5'-triphosphate (ATP) can induce a variety of cellular functions through purinoceptors. As an ATP-gated ion channel, P2X7 receptor (P2X7R) is involved in modulating immune responses, cell proliferation, cell death and cytoskeletal rearrangement (3). Recent studies suggest a link between P2X7R and neurological disorders, including HI (4,5).

Glycogen synthase kinase-3β (GSK-3β), a serine/threonine kinase, is believed to be essential in the regulation of several cellular signaling pathways and transcription factors (6,7). Recent studies have shown that GSK-3β may be important in the pathology of HI injury (8,9).

In this study, we used radial glial clone L2.3 cells to investigate the effects of HI injury and P2X7R in radial glia, and evaluate the relationship between P2X7R and GSK-3β.

Materials and methods

Cell culture. Radial glial clone L2.3 cells were incubated as neurospheres in culture medium at 37˚C in a humidified atmosphere of 95% air and 5% CO_2 as previously described (10).

Oxygen-glucose deprivation (OGD). In vitro HI was simulated by OGD. Briefly, the cells were washed three times with glucose-free medium (125 mM NaCl, 2.8 mM KCl, 1.5 mM $MgCl_2$, 0.05 mM $MgSO_4$, 2 mM $CaCl_2$, 0.83 mM NaH_2PO_4, 24 mM $NaHCO_3$, 2 mM HEPES) prior to oxygen removal and placed in an anaerobic chamber, perfused with 95% N_2 and 5% CO_2 at 37˚C.

Western blot analysis. For western blot analysis total cell extracts were prepared. The protein aliquot (40 μg) was subjected to sodium dodecyl sulphate (SDS) gel electrophoresis and transferred to a PVDF membrane (Millipore). After blocking with 5% non-fat milk, the membranes were incubated with primary antibodies as follows: anti-P2X7R (1:1,000, Epitomics), anti-GSK-3 (1:1,000, Cell Signaling Technology), anti-phospho-GSK-3β (Ser9) (1:1,000, Cell Signaling Technology) and anti-GAPDH (1:500, Santa Cruz Biotechnology, Inc.). The membranes were washed and incubated with horseradish peroxidase-coupled secondary antibodies (1:2,000, Bio-Rad) for 2 h. The membrane was washed and specific bands were detected with Immobilon Western Chemiluminescent HRP Substrate (Millipore) and bands were quantified using the Gel-Pro image analyzer (Omega).

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Key words: hypoxia-ischemia, radial glial cells, P2X7 receptor, glycogen synthase kinase-3β

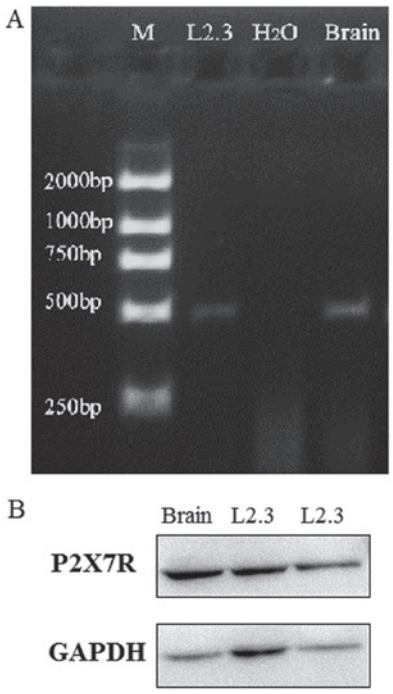

Figure 1. Cultured L2.3 cells express P2X7R. (A) RT-PCR showing P2X7R mRNA expression in L2.3 cell cultures. The expected product size was 468 bp. H_2O and brain tissue were used as the negative and positive control, respectively. (B) Western blot analysis showing P2X7R protein expression in L2.3 cell cultures.

Reverse transcription-PCR (RT-PCR). Total-RNA was extracted from the cultured L2.3 cells by TRIzol reagent (Invitrogen). RNA was reverse-transcribed using the reverse transcription kit (Takara) following the manufacturer's protocol. PCR amplification of P2X7R-subunit-specific fragments was performed with the following primer pairs as previous reported: sense primer, 5'-AAT GAG TCC CTG TTC CCT GGC TAC-3' and antisense primer, 5'-CAG TTC CAA GAA GTC CGT CTGG-3' (11).

Cell viability assay. Cell viability was assessed by the Cell Counting Kit-8 (CCK-8, Dojindo). L2.3 cells dispensed as 100 μl of cell suspension were seeded in 96-well plates (50,000 cells/well). After the different treatments, 10 μl of CCK-8 solution was added to each well following the manufacturer's protocol. After incubation at 37˚C for 3 h in a humidified CO_2 incubator, absorbance at 450 nm was measured with a micro-plate reader (Bio-Rad). The values were used to calculate cell viability by setting the normoxic control to 100%.

Statistical analysis. Data are expressed as the mean ± standard deviation (SD). One-way ANOVA was performed for multiple comparisons. A value of P<0.05 was considered to indicate statistical significance.

Results

Expression of P2X7R in clone L2.3 cells. In order to determine whether the L2.3 cells expressed the P2X7R, RT-PCR and western blot analysis was utilized. We found that P2X7R was stably expressed in the L2.3 cells (Fig. 1).

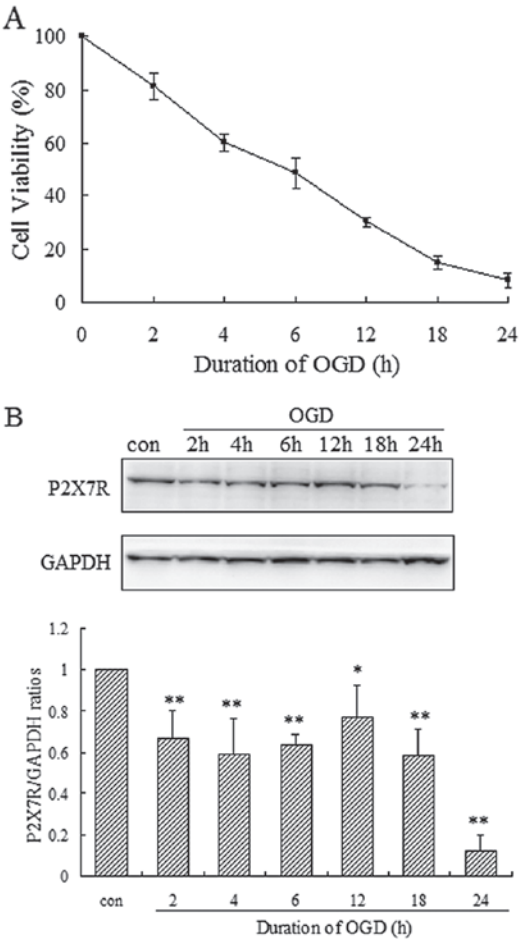

Figure 2. Effects of OGD insult on the expression of P2X7R. (A) Time course of cell viability of L2.3 cells subjected to OGD insult. Cell viability was determined by CCK-8 assay as described in Materials and methods. (B) OGD insult reduced P2X7R protein expression. The bar chart represents the relative ratios of signal intensities for P2X7R versus GAPDH. The ratio for normoxic control was set to 1. Data represent the mean ± SD of three independent experiments. *P<0.05 and **P<0.01, compared with the normoxic controls.

OGD induces downregulation of P2X7R expression in clone L2.3 cells. To evaluate the role of P2X7R during HI, we used the OGD model and detected P2X7R expression by western blot analysis. As shown in Fig. 2A, viability of the L2.3 cells decreased progressively with the time of exposure to OGD. We also found that OGD caused a decrease in P2X7R expression when compared with the control group (P<0.05) (Fig. 2B).

P2X7R is involved in modulation of the viability of clone L2.3 cells. As OGD reduced P2X7R protein expression in the L2.3 cells, we hypothesized that P2X7R may be involved in modulating the viability of clone L2.3 cells. We used the P2X7R selective agonist, 3'-O-(4-benzoylbenzoyl) adenosine 5'-triphosphate (BzATP, Sigma), to activate P2X7R with a concentration range from 25 to 400 μM. As a result, we found that the viability of the L2.3 cells decreased with increasing BzATP concentration. Dose-response effects of BzATP on L2.3 cell viability is shown in Fig. 3A. The viability of L2.3 cells was also found to decrease progressively with the time of incubation with 100 μM BzATP (Fig. 3B). Involvement of

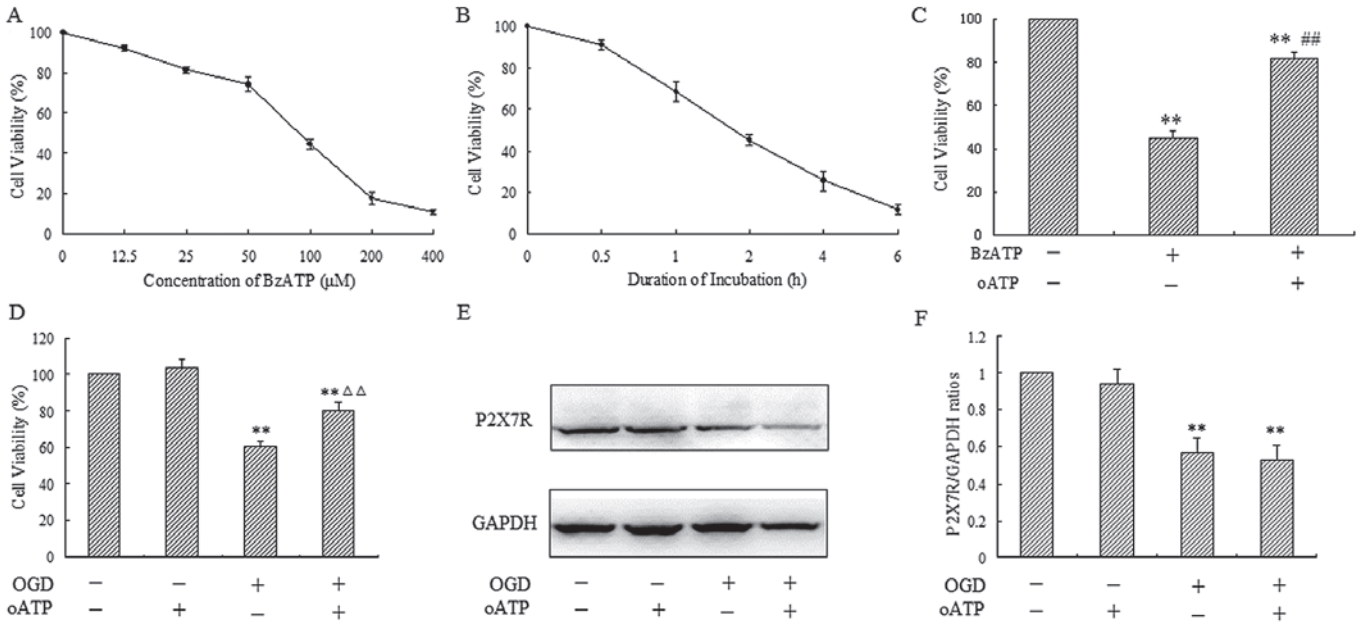

Figure 3. Effects of P2X7R activation on L2.3 cells. (A) The P2X7R agonist BzATP was toxic to L2.3 cells in a dose-dependent manner. L2.3 cells were exposed to a range of BzATP concentrations for a 2-h treatment. (B) The P2X7R agonist BzATP was toxic to L2.3 cells in a time-dependent manner. L2.3 cells were exposed to 100 μM BzATP for 0.5, 1, 2, 4, 6 h, respectively. (C) The P2X7R antagonist oATP attenuated the toxicity of BzATP. oATP (200 μM) was applied to L2.3 cells for 30 min before and during the 2-h 100 μM BzATP application. (D) P2X7R inhibition increased the viability of L2.3 cells following OGD. L2.3 cells were incubated under normoxic conditions in the presence or absence of oATP (200 μM) for 30 min before a 4-h OGD insult. (E) oATP had no effects on OGD-induced downregulation of P2X7R in L2.3. (F) The bar chart represents the relative ratios of signal intensities for P2X7R versus GAPDH. The ratio for normoxic control was set to 1. Data represent the mean ± SD from three independent experiments. **P<0.01, compared with the control; $^{\#\#}$P<0.01, compare with the BzATP treated group; $^{\triangle\triangle}$P<0.01, compared with the OGD group.

P2X7R was further confirmed by using the P2X7R antagonist oxidized ATP (oATP; Sigma). After pretreatment with 200 μM oATP for 30 min, 100 μM BzATP was added to the culture medium and incubated for 2 h. The results showed that oATP alleviated the BzATP-induced cell injury (Fig. 3C).

To further identify the role of P2X7R during OGD, we blocked P2X7R with oATP (200 μM), which was added to the culture medium and incubated for 30 min before the 4-h OGD. We found that cell death induced by OGD was rescued by approximately 20% after treatment with oATP, when compared with the OGD group. This indicates that PX7R may participate in OGD injury (Fig. 3D). However, the oATP pretreatment did not affect the reduced expression of P2X7R induced following OGD insult (Fig. 3E).

P2X7R activation mediates GSK-3β phosphorylation. It is well known that GSK-3β plays a vital role in regulating critical cellular functions. Previous studies indicate that GSK-3β may be a therapeutic target for HI injury and provide evidence that P2X7R may modulate GSK-3β phosphorylation (8,9,12). In this study, we investigated the relationship between P2X7R and GSK-3β in L2.3 cells. We detected a higher level of phospho-GSK-3β (Ser9) in the BzATP-treated group, and oATP abrogated this increase (Fig. 4A). In additional, we further examined the possible role of P2X7R activation in mediating GSK-3β phosphorylation in OGD. As shown in Fig. 4B, OGD reduced the phospho-GSK-3β (Ser9) level. Contrary to what would be expected, the level of phospho-GSK-3β (Ser9) decreased by OGD was upregulated when the cells were preincubated with 200 μM oATP (Fig. 4C).

Discussion

The present study provides evidence that radial glial clone L2.3 cells express P2X7R at both the mRNA and protein levels and that OGD decreases P2X7R expression. We also found that antagonist oATP inhibits P2X7R activation-induced cell death and alleviates OGD-induced damage to L2.3 cells. In addition, we found that P2X7R modulates GSK-3β phosphorylation in L2.3 cells.

When ATP was identified as a neurotransmitter in the CNS, the concept of purinergic neurotransmission was formed in 1972 (13). Currently, seven human P2X receptor subunits (P2X1-7) have been cloned. P2X7R acts as a cation channel or a nonselective pore that engages in the release of cytokines, the induction of cell death and a signaling complex. It is suggested that the intracellular 150-amino-acid-long C-terminal tail of the P2X7R is linked to its unique functions as well as cellular localization (14). It has been identified that P2X7R is widely distributed throughout the CNS, including neurons, microglia, astrocytes, oligodendrocytes and oligodendrocyte precursor cells (15-17). In this study, we confirmed that P2X7R is present in cultured radial glia.

P2X7R has been considered as a cytotoxic receptor, since it activates the apoptotic caspase enzyme system and induces the formation of large nonselective pores, eventually leading to cell death (14). This is consistent with what we observed, that is, BzATP induced L2.3 cell death in a dose- and time-dependent manner.

Our study showed that oATP partly alleviates the OGD-induced death of L2.3 cells. It is well established that HI

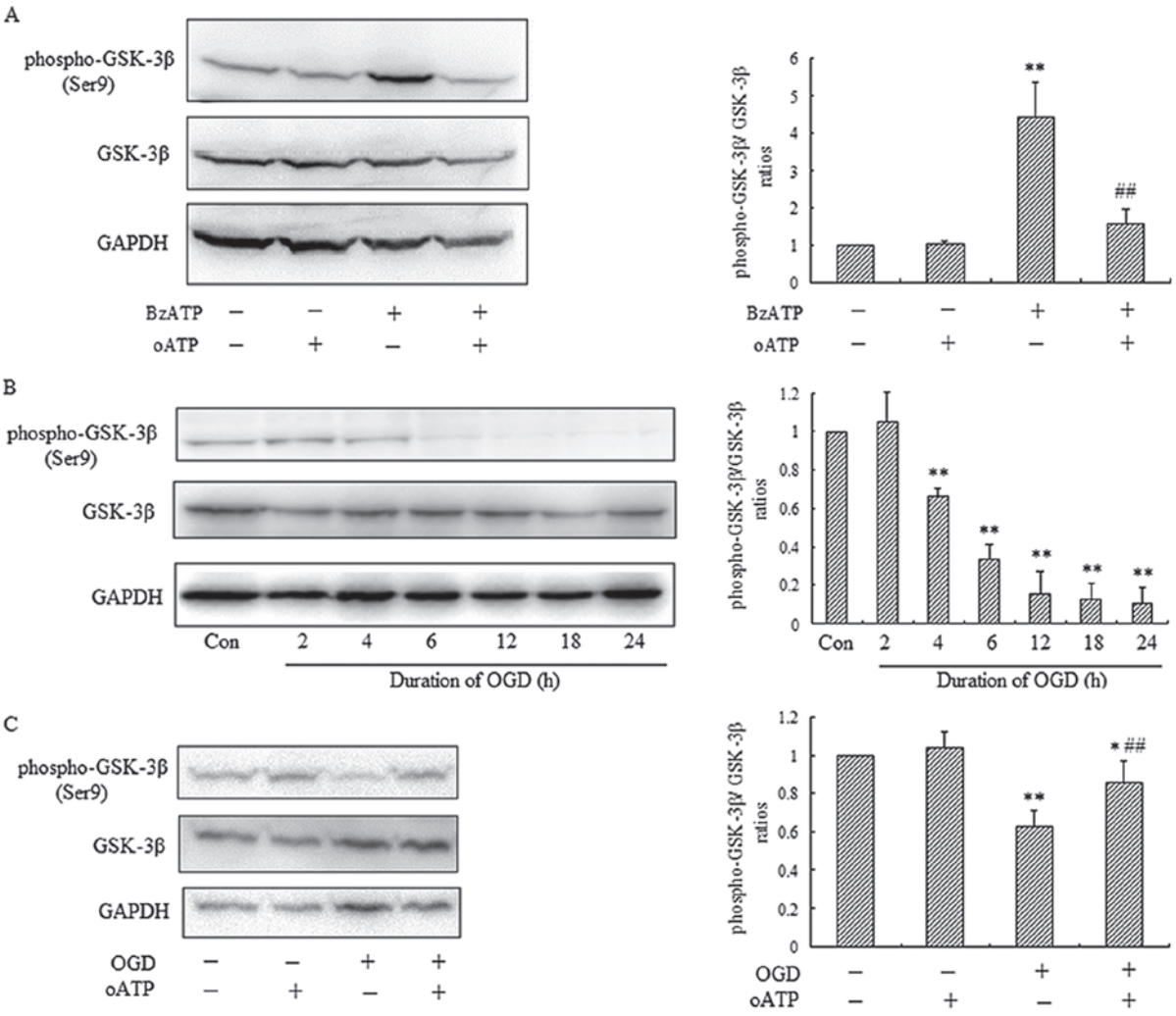

Figure 4. P2X7R activation mediates GSK-3β phosphorylation. (A) A 2-h treatment of 100 μM BzATP increased the level of phospho-GSK-3β (Ser9) in the L2.3 cells, which was inhibited by preincubation with 200 μM oATP. (B) OGD insult caused the downregulation of phospho-GSK-3β (Ser9) in the L2.3 cells. (C) Preincubation with 200 μM oATP for 30 min before OGD upregulated phospho-GSK-3β (Ser9) in the L2.3 cells. The bar charts represent the relative ratios of signal intensities for phospho-GSK-3β vs. GSK-3β. The ratio for normoxic control was set to 1. Data represent the mean ± SD from three independent experiments. *P<0.05 and **P<0.01, compared with the controls; $^{\#\#}$P<0.01, compared with the OGD group.

results in abundant ATP outflow compatible upon P2 receptor activation. The activation leads to enhancement of Ca^{2+} inward currents, as well as altering synaptic activity and cellular plasticity (18). Actually, HI injury is mediated by a complex pathophysiologic interaction of different mechanisms, such as excitatory amino acid receptor activation, calcium overload, nitric oxide, oxidative stress, and apoptotic caspase activation (19,20). As P2X7R is involved in the mechanisms of HI injury, inhibition of P2X7R activation may rescue L2.3 cells partially from OGD-induced injury.

In addition, we observed that OGD insults decreased the P2X7R expression in L2.3 cells. Similarly, Wang *et al* (17) observed that hypoxia-ischemia downregulated the expression of P2X7R in rat oligodendrocyte precursor cells. However, several previous studies reported inconsistent results. Milius *et al* found an increase in P2X7R immunoreactivity in cortical neurons in response to OGD (16). Furthermore, an increase in P2X7R was also detected in the cortex surrounding the zone of penumbra and infarction (5). Nevertheless, another study found that whole brain P2X7R expression was unchanged following brain injury induced by anoxia (21). These inconsistent results concerning changes in P2X7R expression may have resulted from different area specificity, maturational status and the manner with which injury was induced.

We observed that BzATP stimulates the GSK-3β phosphorylation in L2.3 cells. Ortega *et al* also found that BzATP activates P2X7R and inhibits GSK-3 phosphorylation in cerebellar granule neurons (12). GSK-3β is a multifunctional serine/threonine kinase, playing a vital role in signal transduction to regulate various cellular functions, such as metabolism, formation of the cytoskeleton, gene expression, cell growth and apoptosis. It has been reported that inhibition of GSK-3β can relieve ischemia injury (8,9). Several mechanisms have been identified that contribute to the regulation of GSK-3β, and the most widely known and well-studied is GSK3β phosphorylation on Ser9 residues (6). It is well documented that the inhibitory serine residues of GSK-3β, the Ser9 residues, can be phosphorylated by the PI3-K/Akt pathway (7) and P2X7R has been described to couple with Akt phosphorylation (22). Recent studies have also revealed that ATP-induced Ser9-GSK3β

phosphorylation may be inhibited by the downregulation of protein kinase C (PKC) (23), indicating that PKC may be a key step in P2X7R-stimulated GSK-3β phosphorylation.

Here we observed that OGD insult induced a decline in phospho-GSK-3β. However, when oATP was used to inhibit P2X7R activity in OGD, GSK-3β phosphorylation increased to a normoxic control level. This was contrary to what was expected in that P2X7R activation by BzATP in normoxic condition increased the phosphorylation level of GSK-3β. One possible explanation is that the effects of BzATP on GSK-3β phosphorylation might be different from the effects of ATP released in OGD. It is reported that BzATP and ATP activate downstream signals via P2X7R through different signaling pathways (24). ATP also activates other P2X receptors (3) and plays more complex roles than BzATP. In addition, OGD causes more extensive irreversible damage than does BzATP, and other crosstalk mechanisms may regulate GSK-3β phosphorylation. Therefore, further investigation is needed to further elucidate the linkage and mechanisms between P2X7R and GSK-3β phosphorylation.

In conclusion, we found that P2X7R was expressed in radial glial clone L2.3 cells and played a critical role both in normoxic conditions and in hypoxic/ischemic injury. P2X7R blockade increased the L2.3 cell survival during OGD insult via upregulation of phospho-GSK-3β. These findings suggest that the activities of P2X7R may be beneficial for the treatment of HI. More in-depth studies may reveal the role of P2X7R in physiological and pathological conditions, particularly in signaling pathways downstream of P2X7R and their crosstalk. Formulation of a more complete image of the involvement of P2X7R in radial glia may offer a new therapeutic strategy to rescue HI brain injury.

Acknowledgements

This study was supported by grants from the National Natural Science Foundation of China (nos. 30772343 and 30973215), the Program for Changjiang Scholars and the Innovative Research Team in University (no. IRT0935).

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