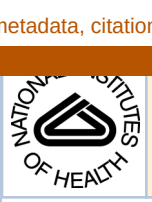

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ATP-P2X$_7$ receptor signaling controls basal and TNFα-stimulated glial cell proliferation

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Abstract

Activation and proliferation of glial cells and their progenitors is a key process of neuroinflammation associated with many neurodegenerative disorders. Under neuropathological conditions where glial cell activation and proliferation is evident, controlling the population of glia might be of therapeutic importance. The proliferative action of the cytokine tumor necrosis factor alpha (TNFα) on microglia has been reported, but the molecular mechanism of TNFα regulation of glial cell proliferation is largely unknown. Using a model of organotypic hippocampal-entorhinal cortex (HEC) slice culture, we investigated the role of ATP-P2X$_7$ receptor signaling in glial proliferation by TNFα. Populations of proliferating cells in HEC culture were labeled with 5-bromo-2′-deoxyuridine (BrdU). Treatment with TNFα induced strong expression of P2X$_7$ receptor mRNA and immunoreactivity in BrdU+ cells while markedly increasing proliferation of BrdU+ cells. In addition, TNFα increased aquaporin 4 (AQP4) expression, an ion channel involved in glial proliferation. The proliferative action of TNFα was attenuated by blocking the P2X$_7$ receptors with the specific antagonists oxATP, BBG and KN62, or by lowering extracellular ATP with ATP hydrolysis apyrase. Basal proliferation of BrdU+ cells was also sensitive to blockade of ATP-P2X$_7$ signaling. Furthermore, TNFα activation of P2X$_7$ receptors appear to regulate AQP4 expression through protein kinase C cascade and down regulation of AQP4 expression can reduce TNFα-stimulated BrdU+ cell proliferation. Taken together, these novel findings demonstrate the importance of ATP-P2X$_7$ signaling in controlling proliferation of glial progenitors under the pathological conditions associated with increased TNFα.

Keywords

neuroinflammation; cytokines; purinergic signaling; microglia; progenitor

INTRODUCTION

The activation and proliferation of glial cells in response to brain insults such as brain trauma, ischemia, and inflammation is an active process that has both beneficial and detrimental effects on brain repair and recovery. Microglia are the predominant immune cell of the CNS and are first to be activated following an insult, rapidly migrating to the injury site and initiating further glial reaction and communication with the immune system (Robel et al., 2011). Similarly, astrocytes are the most abundant cell type in the CNS and react promptly to a variety of CNS pathologies leading to astrogliosis (Eng and Ghirnikar, 1994). Astrogliosis is characterized by an aberrant proliferation of astrocytes as well as an increase in the number of intermediate filaments with accompanying cellular hypertrophy. These

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activated astrocytes and activated microglia then release proinflammatory cytokines/mediators such as tumor necrosis factor alpha (TNFα), interleukin (IL)-1β, and inducible nitric oxide synthase (Griffin, 2006; Dheen et al., 2007), contributing to further brain inflammation and neurodegeneration (Scholz and Woolf, 2007; Inoue and Tsuda, 2009). Thus, studying the mechanism by which glial cells proliferate in response to neurological insults may provide valuable information on modulating glial cells in the compromised brain.

TNFα is a proinflammatory cytokine involved in systemic inflammation and induces neuronal cell death through several mechanisms such as direct apoptotic action (Yu et al., 2006) and inhibition of glutamate uptake leading to increased extracellular glutamate levels and excitotoxicity (Zou and Crews, 2005; Zou and Crews, 2006). TNFα exerts its biological effects through activation of two distinct receptor subtypes, TNFR1 and TNFR2 (Wajant, 2003), and TNFα and its receptors are expressed on progenitor cells of the rat hippocampus (Klassen et al., 2003; Iosif et al., 2006). Recent evidence suggests that TNFα positively or negatively modulates progenitor cell proliferation. Indeed, TNFα may up-regulate the proliferation and differentiation of neural progenitors in the subventricular zone after CNS injury (Wu et al., 2000; Katakowski et al., 2007), which may be mediated via IKK/NF-κB signaling (Widera et al., 2006). On the other hand, TNFα has also been found to suppress neural progenitor proliferation and neurogenesis through TNFR1 signaling (Iosif et al., 2006) and to compromise the survival of hippocampal progenitors *in vitro* (Cacci et al., 2005).

Cellular proliferation, especially the propagation of glia, is tightly controlled by complex microenvironments through cell-cell interactions and specific receptor families. The $P2X_7$ receptor ($P2X_7R$) is a member of the purinergic P2X family of ATP-gated ion channels, and a high level of extracellular ATP is required for the activation of $P2X_7Rs$. This receptor mediates the influx of Na^+ and Ca^{2+} during neuronal activation and the concomitant efflux of K^+ (Gudipaty et al., 2003; Witting et al., 2004). In addition, sustained activation of $P2X_7Rs$ may generate non-selective pores that are permeable to small molecules up to 900 Da in size (Virginio et al., 1999; Di Virgilio et al., 2001). Although expression of the $P2X_7R$ is primarily associated with immune and hematopoietic cells (Surprenant et al., 1996; Di Virgilio et al., 2001), its mRNA or protein has been identified in all brain cell types in the CNS (Ferrari et al., 1999; Choi et al., 2007; Yu et al., 2008). Importantly, the $P2X_7R$ is highly expressed on microglia and activation of these receptors is correlated with release of the proinflammatory cytokines IL-1β (Ferrari et al., 1997; Lister et al., 2007) and TNFα (Hide et al., 2000; Lister et al., 2007). The functional responses of $P2X_7R$ activation by ATP are associated with ongoing cellular damage and chronic brain inflammation. Indeed, recent experimental evidence indicates that stimulation of $P2X_7Rs$ mediate ATP-induced apoptosis through microglial production of superoxide (Parvathenani et al., 2003; Raouf et al., 2007). In addition, expression of the $P2X_7R$ is up-regulated in a transgenic mouse model of Alzheimer's disease (Parvathenani et al., 2003) and amyloid-β-treated rat microglia (McLarnon et al., 2006). The $P2X_7R$ might also play a role in microglial proliferation since down-regulation of the $P2X_7R$ is involved in LPS-induced reduction of microglial proliferation (Bianco et al., 2006). Thus, identification of the role of the $P2X_7R$ in cytokine-induced inflammation will provide further insight into its role in the pathological brain.

Aquaporin 4 (AQP4) is the most abundant water channel protein in the CNS (Jung et al., 1994) and strongly expressed in astrocytes (Nielsen et al., 1997). Recent studies indicate that AQP4 plays a role in regulating neural stem cell proliferation and neurogenesis (Saadoun et al., 2005; Kong et al., 2008; Kong et al., 2009) as well as proliferation of astrocytes in striatal primary cultures (Kuppers et al., 2008) and cocaine-treated animals (Xie et al., 2009). The expression of AQP4 protein may be regulated by $P2X_7$ activation (Lee et al.,

2008) and the AQP4-dependent Ca^{2+} signaling may be mediated, in part, by autocrine purinergic signaling (Thrane et al. 2011), suggesting an interaction between $P2X_7$ and AQP4. In support, a study using a single intranigral injection of LPS found that AQP4 mRNA and protein are expressed in reactive microglial cells (Tomas-Camardiel et al., 2004). Furthermore, TNFα increases proliferation and AQP4 expression in astrocytes (St Hillaire et al., 2005; Alexander et al., 2008). However, little research has been performed to determine whether AQP4 is involved in glial progenitor proliferation regulated by ATP-$P2X_7$ or TNFα signaling.

The aim of this study was to investigate whether ATP-$P2X_7$R signaling is involved in TNFα-stimulated proliferation of glial cells labeled by the dividing cell marker BrdU. The model of organotypic rat hippocampal-entorhinal cortical (HEC) slice culture was used in the present study. Our results demonstrated that ATP-$P2X_7$ receptor signaling interacts with AQP4 to control the proliferative actions of cytokine TNFα on proliferating glial cells.

MATERIALS AND METHODS

Hippocampal-entorhinal cortical slice culture

All protocols followed in this study were approved by the Institutional Animal Care Use Committee of The University of North Carolina at Chapel Hill and were in accordance with National Institute of Health regulations for the care and use of animals in research. Organotypic hippocampal-entorhinal cortical (HEC) slice cultures were prepared from Sprague-Dawley rat neonates according to the techniques of Stoppini and colleagues (Stoppini et al., 1991) with modifications. Briefly, rat neonates at postnatal day 7 were decapitated, brain removed and hippocampal-entorhinal complex dissected in Gey's buffer. Slices were transversely cut with McIlwain tissue chopper at a thickness of 375 μm and placed onto a 30 mm diameter membrane tissue insert, 8-10 slices/tissue insert. Slices were cultured with medium containing 75% MEM with 25 mM HEPES and Hank's salts + 25% horse serum + 5.5 g/L glucose + 2 mM L-glutamine in a humidified 5% CO_2 incubator at 36.5°C.

5-Bromo-2′-deoxyuridine (BrdU) labeling, immunohistochemistry and quantification

HEC slices at 10 days *in vitro* (DIV) were treated with BrdU (50 μM, Sigma-Aldrich) for 24 hr and then returned to BrdU-free culture medium up to the end of experiments. BrdU was left in the culture medium through the end of experimentation in a time course study of control and TNFα-treated cultures. Various treatments and experimental designs were performed in the BrdU-treated slices in a BrdU-free medium. At the end of each treatment, the slices were removed and fixed with 4% paraformaldehyde + 5% sucrose in 0.1 M PBS overnight at 4°C. BrdU immunohistochemistry follows the method described elsewhere (Nixon and Crews, 2002) with a slight modification. For BrdU-positive cell quantification, the pixel density of BrdU immunoreactivity in the region of hippocampal dentate gyrus was determined by Bioquant imaging software as described previously (Crews et al., 2006).

Drug treatments

All chemicals, except for those specified, were purchased from Sigma-Aldrich. BrdU-treated slices were assigned to the experimental groups treated with TNFα (25 ng/ml, R&D Systems, Minneapolis, MN) in the presence or absence of other pharmacological reagents including ATPase apyrase (25 and 50 U/ml), BzATP (10, 50, 100 and 300 μM), oxATP (10, 50, 100 and 300 μM), brilliant blue G (BBG, 100 μM), H-7 (50 μM), tetraethylammonium (TEA, 50 and 100 μM), suramin (100 μM) and ivermectin (300 μM), NTPDase inhibitor ARL 67156 (10 and 50 U/ml, Tocris, Ellisville, MO), KN62 (50 μM, Chemicon), ERK inhibitor PD 98059 (50 μM, Chemicon) and p38MAPK inhibitor SB 203580 (50 μM,

Chemicon) in BrdU-free medium. At the end of the experiment, all slices were removed for other purposes.

Double immunofluorescence and confocal analysis

Free-floating slices were processed for double immunofluorescence staining. The primary antibodies used were as follows: mouse or rat anti-BrdU for progenitor cells (1:500); neuronal-specific nuclear protein (NeuN) for neurons (1:500, Chemicon), Iba-1 (1:1000, Wako, Richmond, VA) or ED-68 (1:100, Serotec, Raleigh, NC) for microglia, rabbit anti-GFAP (1:1000, Dako, Carpinteria, CA) or mouse anti- GFAP (1:1000, Sigma-Aldrich) for astrocytes, rabbit anti-$P2X_7$ receptor C-terminus (1:100, Sigma-Aldrich or Santa Cruz, Santa Cruz, CA). Alexa Fluor 594 anti-rabbit and Alexa Fluor 488 anti-mouse IgG (1:1000, Invitrogen, Carlsbad, CA) were secondary. A negative control in which the primary antibody was omitted was used to assess nonspecific binding of the secondary antibody. All slices were incubated with primary antibody for 48 hr at 4°C and 2 hr with second antibody at room temperature. Confocal analysis was performed using a LeicaSP2 AOBS Upright Laser Scanning Confocal in the Michael Hooker Microscopy Facility at UNC. The co-localization of BrdU or $P2X_7R$ with neuronal specific (NeuN), microglial specific (Iba-1/ED-68) or astrocytic specific (GFAP) proteins was analyzed with confocal microscopy. For quantification of BrdU+/Iba-1+ cell co-localization, a series of sections at the Z-plane were collected at 1-2 μm thickness, and the number of cells was counted at two adjacent sections (1800-3600 cells in each group).

AQP4 knockdown with siRNA

Rat AQP4 siRNA (Santa Cruz, Cat# sc-156007) was used to knock down AQP4 expression. Preparation of transfection reagents and transfection were performed according to the manufacturer's siRNA transfection protocol. Briefly, after 10 min of equilibration at room temperature, siRNA solution was combined with Lipofectamine 2000 solution to form siRNA liposomes for a further 20 min at room temperature. The transfection mixture was added to serum-free N2 medium at a final concentration of 20 nM siRNA + 8 μl Lipofectamine 2000 in a total volume of 1.5 ml (500 μl on top of slices and 1 ml at bottom of the cultures). Vehicle controls were treated with the same N2 medium containing Lipofectamine only or with negative control siRNA (Ambion). After 24 hr transfection, siRNA-containing medium was replaced with regular serum-free N2-supplemented medium and the slices were cultured for another 48 hr in the presence of TNFα. At the end of experiments, the slices were removed and BrdU+ cell determined.

RNA isolation, reverse transcription and real time quantitative RT-PCR

For each specific experiment, the slices were removed at the end of experiment, rinsed with cold PBS, and followed by total RNA purification using the RNeasy Mini Kit (Qiagen, Valencia, CA). The total amount of RNA was quantified by spectrophotometry at 260 nm. For reverse transcription, 2 μg of RNA was used to synthesize the first strand of cDNA using random primers (Invitrogen) and reverse transcriptase Moloney murine leukemia virus (Invitrogen). After a 1:2 dilution with water, 2 μl of the first strand cDNA solution was used for RT-PCR. The primer sequences for real time RT-PCR were designed by Integrated DNA Technologies (Coralville, Iowa). The following primers were used: for $P2X_7$ receptor, forward 5′-TGTCCCTATCTCTCCACGACTCAC-3′ and reverse 5′ ATTTCCACACTGGCACCAACTCGG-3′; for AQP4, forward 5′-CCATTAACTGGGGCTCAG-3′ and reverse 5′-AACTGCAGGGCCAAAGGATCG-3′ and for β-actin, forward 5′-CTACAATGAGC-TGCGTGTGGC-3′ and reverse 5′-CAGGTCCAGACGCAGGATGGC-3′. SYBER Green Supermix (AB system, UK) was used as a RT-PCR solution. The real time RT-PCR was run with initial activation for 10 min at 95°C and followed by 40 cycles of denaturation (95°C, 40 s), annealing (58°C, 45 s) and

extension (72°C, 40 s). All experiments were run in triplicate. The threshold cycle (C_T) of each target product was determined and normalized to internal standard β-actin. Difference in C_T · values (C_T) of two genes was calculated [difference = $2^{-(C_T \text{ of target genes} - C_T \text{ of } \beta\text{-actin})} = 2^{-C_T}$], and the result was expressed as the percentage or fold difference compared to control.

Statistical Analysis

Statistical significance was analyzed by *ANOVA* and Student's *t* test. Experimental results are presented as a mean ± SEM. Differences were considered to be statistically significant at a $p<0.05$.

RESULTS

Proliferation of BrdU+ cells in HEC slices

To monitor cell proliferation, slices at 10 DIV were treated with BrdU (50 μM) and BrdU immunohistochemistry was used to detect BrdU+ cells. The pixel density of BrdU+ immunoreactive (IR) cells was measured in the hippocampal dentate gyrus with computer-assisted BioQuant Imaging software throughout the study (Figure 1-A). BrdU immunohistochemistry revealed widespread proliferation of BrdU+ cells in HEC slices. However, the distribution of these cells on the surface of the slice was heterogeneous as some regions, such as the dentate gyrus and layer 4 of entorhinal cortex, had a dense population of BrdU+ cells, whereas others had fewer BrdU+ cells (Figure 1-A). The time course study of proliferation found that the BrdU+IR increased progressively over time suggesting that proliferating cells were actively undergoing cell cycle progression under the culture conditions used in the present study (Figure 1-B).

TNFα stimulates robust proliferation of BrdU+/Iba-1+ cells

We investigated whether TNFα modulates proliferation of BrdU+ cells in this model. The BrdU-treated slices were incubated with BrdU-free medium containing TNFα (25 ng/ml) for various time periods and BrdU+IR was determined. As shown in Figure 2 (top panel), TNFα stimulated a rapid and robust increase in BrdU+ cell proliferation. TNFα treatment increased BrdU+IR by 160%, 375%, 283%, and 213% at 8, 24, 48, and 96 hr, respectively.

To identify cellular phenotypes, we used confocal microscopy and double fluorescent immunostaining for BrdU and cell type specific markers (Iba-1 for microglia; NeuN for neurons; GFAP for astrocytes). In a representative experiment with TNFα treatment for 48 hr, BrdU+IR was markedly increased in the TNFα-treated group (Figure 2, bottom panel A-B). Confocal analysis of double fluorescent immunostaining revealed that approximately 50% of BrdU+ cells co-expressed Iba-1 (Figure 2, bottom panel C). BrdU+ cells rarely co-expressed the neuronal marker NeuN or astrocytic marker GFAP (data not shown). Thus, about 50% of proliferating cells are Iba-1+ microglia-like cells, and the remaining 50% of proliferating cells that were not identified by cell markers use. The percentage of BrdU+ cells co-expressing Iba-1 were significantly increased from 48% in control to 90% in TNFα-treated slices at the 48 hr time point (Figure 2, bottom panel D).

ATP-P2X$_7$ receptor signaling controls the basal proliferation of BrdU+ cells

We performed experiments to directly test the hypothesis that endogenous ATP could be released by cells in response to damage or other physiological responses that may drive the basal proliferation of BrdU+ cells by activating P2X$_7$Rs. The first series of experiments examined the growth promoting role of endogenous ATP-P2X$_7$ receptor signaling by manipulating the extracellular ATP level with ATPase apyrase or the NTPDase inhibitor, ARL 67156. The results revealed that apyrase significantly reduced BrdU+ cell proliferation

at the concentrations 10 and 50 U/ml, and ARL 67156 increased BrdU+ cell proliferation at the concentrations 10 and 50 U/ml (Figure 3A). The second set of experiments tested the effects of the $P2X_7R$ agonist BzATP (10, 50, 100, and 300 μM) and the antagonist oxATP (10, 50, 100, and 300 μM) on basal proliferation of BrdU+ cells. Treatment of the slices with BzATP for 48 hr resulted in a dose-dependent increase in BrdU+ cell proliferation whereas treatment with oxATP reduced BrdU+ cell proliferation. Other $P2X_7R$, antagonists, such as KN62 (50 μM) and Brilliant Blue B (BBG, 100 μM) as well as $P2X_4R$ antagonists suramin (100 μM) and agonist ivermectin (50 μM) were also tested. Both KN62 and BBG significantly inhibited basal proliferation of BrdU+ cells, but suramin and ivermectin has no effect on proliferation (Figure 3C). Further characterization of the cellular expression of $P2X_7Rs$ with double immunofluorescence staining and confocal analysis revealed that $P2X_7Rs$ were widely expressed on proliferating cells labeled by BrdU (Figure 4, panel A). Consistent with other reports (Xiang and Burnstock, 2005; Choi et al., 2007), $P2X_7Rs$ were expressed on microglia, neurons, and astrocytes in the cultured slices (Figure 4, panel B-D). These results indicate that endogenous ATP could directly stimulate $P2X_7Rs$ on proliferating cells labeled by BrdU and drive BrdU+ cell proliferation under the culture conditions used in the present study.

TNFα induces $P2X_7$ receptor expression

We hypothesized that the proliferative actions of TNFα may be mediated through ATP-$P2X_7$ receptor signaling in this model. Double immunofluorescence staining revealed that $P2X_7R$+IR was strongly co-expressed on BrdU+ cells in slices treated with TNFα (Figure 5A). RT-PCR analysis found that $P2X_7R$ mRNA levels were elevated 10-fold at 24 hr and 8-fold at 48 hr of treatment with TNFα (Figure 5B). Examination of the effects of TNFα on NTPDase mRNA expression with several isoforms of NTPDases revealed that TNFα treatment did not significantly alter NTPDase mRNA levels (data not shown).

Proliferative effect of TNFα is sensitive to ATPase and blockade of $P2X_7$ receptors

We further investigated the role of ATP-$P2X_7$ receptor signaling on the proliferative actions of TNFα on BrdU+ cell proliferation. All $P2X_7R$ antagonists assessed effective blocked TNFα-stimulated BrdU+ cell proliferation. Treatment with TNFα for 48 hr increased BrdU +IR by 235% relative to control, and the presence of $P2X_7R$ antagonists reduced TNFα-stimulated BrdU+IR cells by 43%, 56% and 73% with KN62 (50 μM), BBG (100 μM), and oxATP (100 μM), respectively (Figure 6). Among the $P2X_7R$ antagonists tested, oxATP was the most effective in blocking TNFα-stimulated BrdU+ cell proliferation. The $P2X_4R$ antagonist suramin (100 μM) did not alter TNFα-stimulated BrdU+ cell proliferation (Figure 6). These results support a specific role of $P2X_7R$ in regulating TNFα-stimulated proliferation of cells labeled by BrdU in this model.

Since levels of extracellular ATP could limit activation of $P2X_7R$, we could not exclude the possibility that TNFα may increase ATP release resulting in enhanced activation of $P2X_7R$. Administration of the ATPase apyrase, which lowers extracellular ATP levels, reduced TNFα-stimulated proliferation of BrdU+ cells by 52% and 82% at concentrations of 25 U/ml and 50 U/ml, respectively (Figure 6). Taken together, these results demonstrate that ATP-$P2X_7$ signaling mediates the proliferative action of TNFα on BrdU+ cells, in which BrdU+/Iba-1+ cells account for the increases in this model.

Involvement of AQP4 and downstream signaling pathways in proliferative action of TNFα

We examined the possibility that TNFα activation of $P2X_7Rs$ might regulate AQP4 expression and subsequently influence the proliferative actions of TNFα. We first measured AQP4 mRNA expression in slice cultures treated with TNFα in the absence or presence of the $P2X_7R$ antagonist oxATP (100 μM) or protein kinase C (PKC) inhibitor, H-7 (50 μM).

Treatment with TNFα induced strong expression of AQP4 mRNA that was completely blocked by both oxATP and H-7 (Figure 7A). These data suggest that TNFα induction of AQP4 gene expression is associated with activation of $P2X_7Rs$ and downstream PKC cascade signaling. Consistent with the AQP4 mRNA data, BrdU+IR analysis from the same experiment revealed that the proliferative actions of TNFα were completely abolished by both oxATP and H-7 (Figure 7B). To directly test the role of AQP4 in the proliferative actions of TNFα, the post-BrdU treated slices were pretreated with AQP4 inhibitor TEA (50 and 100 μM) or knockdown AQP4 by using siRNA during TNFα treatment. As shown in Figure 7B, the AQP4 inhibitor TEA dose-dependently reduced TNFα-induced BrdU+ cell proliferation by 41% and 63%, respectively. Similar results were also obtained by knocking down AQP4 with siRNA. Furthermore, the role of $P2X_7R$ downstream signaling cascade including ERK and p38MAPK in the proliferative action of TNFα was also evaluated in the present study. Both the ERK inhibitor, PD 98059 (50 μM), and the p38MAPK inhibitor, SB 203580 (50 μM), significantly blocked TNFα-stimulated proliferation of BrdU+ cells by 38% and 43%, respectively (Figure 8). Taken together, these results suggest interactive role between activation of $P2X_7Rs$ and AQP4 in regulation of proliferative actions of TNFα in this model.

DISCUSSION

The present study investigated the growth-promoting role of ATP-$P2X_7$ receptor signaling in BrdU+ proliferating cells in a model of organotypic brain slice culture. This series of experiments is the first to demonstrate that: (1) $P2X_7$ receptors are widely expressed on BrdU-labeled proliferating cells; (2) the proinflammatory cytokine TNFα exerts a strong proliferative action on BrdU+ cells in parallel with enhanced expression of $P2X_7Rs$; (3) basal and TNFα- stimulated proliferation of BrdU+ cells is sensitive to the blockade of ATP-$P2X_7$ receptor signaling with ATPase apyrase or $P2X_7R$ antagonists; and (4) AQP4 expression may be regulated by activation of $P2X_7Rs$ and play a role in regulating the proliferative actions of TNFα. These novel findings demonstrate for the first time that ATP-$P2X_7$ receptor signaling plays a critical role in controlling proliferation of glial progenitors.

The growth-promoting role of $P2X_7R$ signaling has been reported in several models. An earlier study demonstrated that transfection of $P2X_7Rs$ into human lymphoblastoid cells that lacked $P2X_7Rs$ stimulates cell proliferation in response to endogenously released ATP (Baricordi et al., 1999). ATP-$P2X_7R$ signaling plays an important role in controlling activation and proliferation of microglial cells. Indeed, blockade of $P2X_7Rs$ with antagonists, treatment with the ATP hydrolase apyrase, and down-regulation of $P2X_7Rs$ with siRNA significantly decrease microglial cell proliferation in cultured N9 cell line (Bianco et al., 2006). Activation and proliferation of microglia via exposure to LPS can be attenuated by administration of $P2X_7R$ antagonists (Bianco et al., 2006; Choi et al., 2007). Recently, Monif and colleagues (Monif et al., 2009) reported that overexpression of $P2X_7Rs$ in the absence of a pathological insult is sufficient to drive the activation and proliferation of microglial cells in rat primary hippocampal cultures. Furthermore, pore conductance rather than cation channels mediate the trophic effects of increased P2X7R expression. Consistent with these reports, our findings demonstrate the strong growth promoting role of ATP-$P2X_7$ receptor signaling in proliferating cells labeled by BrdU in an organotypic brain slice culture model used to identify and study proliferation and neurogenesis (van Praag et al., 2002; Noraberg et al., 2005; Namba et al., 2007). TNFα-induced increases in proliferation of BrdU + cells are completely abolished by ATP hydrolase apyrase and blockade of $P2X_7Rs$ with several antagonists, including oxATP, KN62, and BBG. Conversely, administration of the potent $P2X_7R$ agonist BzATP enhanced basal proliferation of BrdU+ cells. Our results demonstrate that oxATP appears to be the most effective in blocking TNFα proliferative action as a concentration of 300 μM abolished more than 90% BrdU+ cell proliferation.

KN62, an inhibitor of calcium/calmodulin-dependent protein kinase II, has been described as a potent antagonist at the native $P2X_7$-like receptor of human lymphocytes (Gargett and Wiley, 1997), but is inactive at rat $P2X_7$Rs (Humphreys et al., 1998). Several recent studies have further confirmed that KN62 up to 10 μM is ineffective in blocking BzATP-stimulated responses at the rat $P2X_7$R, but can block mouse $P2X_7$Rs (Hibell et al., 2001; Michel et al., 2008; Donnelly-Roberts et al., 2009). Our results show that KN62 effectively reduces TNFα-stimulated BrdU+ cell proliferation in rat brain slice culture. It is not clear whether this is the result of specific actions of KN62 at the concentration used on $P2X_7$Rs. Thus, we could not exclude the possibility that KN62 blocked the proliferative actions of TNFα through inhibition of calcium/calmodulin-dependent protein kinase II. Further experiments such as testing the direct effect of KN62 on BzATP-stimulated BrdU+ cell proliferation may be needed to clarify whether KN62 is effective in blocking $P2X_7$Rs in this model.

Our results found that the $P2X_4$R antagonist suramin at concentration of 100 μM is ineffective in blocking basal and TNFα-stimulated BrdU+ cell proliferation. Suramin has been found to be a non-selective antagonist at native P2X and P2Y receptors (Ralevic and Burnstock, 1998; Lambrecht, 2000) and also has potency at the $P2X_7$R (Hibell et al., 2001). Considering the fact that suramin is a wide range P2 antagonist, further experiments such as a dose-dependent study as well as BzATP stimulation may be needed to clarify whether suramin can effectively block BrdU cell proliferation in this model.

TNFα is known to promote glial cell proliferation (Arnett et al., 2001; Mander et al., 2006), while inhibiting neural progenitor cell proliferation and neurogenesis (Monje et al., 2003; McCoy and Tansey, 2008). Although the mechanisms by which TNFα increases microglial cell proliferation remain unclear, some studies have reported that oxidative stress and MAPK are involved (Mander et al., 2006). Direct evidence linking ATP-$P2X_7$ receptor signaling to the proliferative action of TNFα is also lacking. Our results reveal for the first time that TNFα treatment strongly upregulates gene and protein expression of $P2X_7$Rs in proliferating cells labeled by BrdU in a brain slice culture as 90% of BrdU+ cells co-expressed the microglial marker Iba-1 after TNFα stimulation. The proliferative effects of TNFα on microglia are effectively blocked by administration of either ATP hydrolase apyrase or $P2X_7$R antagonists. These findings clearly indicate that ATP-$P2X_7$ receptor signaling plays a critical role in mediating the proliferative actions of TNFα in this model. Combined with the nature of slice culture modeling, these findings are quite relevant to neuropathological conditions such as neuroinflammation, brain trauma, and ischemic damage where there is increased release of cytokines such as TNFα and IL-1β as well as increased endogenous ATP from damaged cells (Tansey and Goldberg, 2010; Rojo et al., 2008). Microenvironments under these conditions are favorable for the proliferation of microglia and astrocytes, which act as a danger/damage sensor and release cytokines and ATP when activated. This self-propagation cycle could greatly exacerbate brain damage and neuronal cell death. Thus, ATP hydrolase apyrase and $P2X_7$R antagonists are effective in inhibiting basal or TNFα-induced proliferation of BrdU-labeled cells and may have significant implication for new therapeutic options that target ATP-$P2X_7$ receptor signaling in order to regulate proliferation of glial cells in response to neuroinflammatory insults.

Another significant finding in this study is that AQP4 expression is influenced by activation of $P2X_7$Rs and is involved in the proliferative actions of TNFα. AQP4 is the predominant water channel proteins involved in maintaining water and ion homeostasis in physiological and pathological conditions in the CNS. Both AQP4 and $P2X_7$Rs are highly expressed on astrocytes in the CNS (Cavazzin et al., 2006; La Porta et al., 2006). Recent work from other laboratories indicate that AQP4 plays a role in regulating neural cell proliferation and neurogenesis (Saadoun et al., 2005; Kong etal., 2008; Kuppers et al., 2008; Kong et al., 2009) as well as proliferation of astrocytes in striatal primary cultures (Kuppers et al., 2008)

and cocaine-treated animals (Xie et al., 2009). On the other hand, TNFα can increase AQP4 expression in astrocytes (St Hillaire et al., 2005; Alexander et al., 2008). However, there is little information available regarding the interaction of AQP4 and $P2X_7$Rs in the regulation of cell proliferation. One study by Lee and colleagues (Lee et al., 2008) reported that activation of $P2X_7$Rs in astrocytes is associated with down-regulation of AQP4 in response to brain injury, and pretreatment with the $P2X_7$R antagonist oxATP abolished this down-regulation. Under these conditions, down-regulation of AQP4 was a protective mechanism, which may inhibit water influx to the cells and attenuate the acute cytotoxic brain edema after brain injury. In contrast to this report, our data clearly indicate that the cytokine TNFα increases both $P2X_7$R and AQP4 gene expression that is accompanied by strong proliferative action on BrdU+ cells. By blocking $P2X_7$R activation with the antagonist oxATP, TNFα induction of AQP4 mRNA expression was abolished, suggesting a role of $P2X_7$Rs in the regulation of AQP4 expression by TNFα. With regard to the regulation of $P2X_7$R or AQP4 expression, the literature has confirmed that TNFα up-regulates their expression (St Hillaire et al., 2005; Alexander et al., 2008). In this regard, our results are consistent with these reports. For the first time, we provide direct evidence of an interaction of the $P2X_7$R and AQP4 in the proliferative actions of TNFα. Indeed, TNFα- stimulated proliferation of BrdU+ cells can be effectively blocked by down-regulating AQP4 expression or function with an AQP4 inhibitor or siRNA knockdown. It is not clear whether reduction induced by inhibition of AQP4 is directly mediated through blocking AQP4 in those BrdU+/Iba-1+ microglia or influenced by blocking AQP4 in the surrounding cells. A recent study has indicated that AQP4 mRNA and protein can be expressed in reactive microglial cells stimulated by LPS (Tomas-Camardiel et al., 2004). This would support our data that TNFα-stimulated BrdU+/Iba-1+ microglia may express AQP4 in our model. Future studies are needed to examine if TNFα-stimulated BrdU+/Iba-1+ microglia are co-expressing AQP4 in this model.

Activation of $P2X_7$Rs activate PKC that is associated with AQP4 expression (Kleindienst et al., 2006). The PKC inhibitor H-7 used in the present study effectively blocked TNFα induction of AQP4 mRNA expression as well as proliferation of BrdU+ cells. Activation of PKC by TPA is reported to decrease AQP4 mRNA expression as well as water permeability (Kleindienst et al., 2006). Although we did not examine the effects of TNFα on PKC, blockade of TNFα induction of AQP4 mRNA expression as well as BrdU+ cell proliferation by PKC inhibitor H-7 would suggest the involvement of PKC in the proliferative actions of TNFα. These effects could occur either through activation of $P2X_7$Rs and subsequent activation of downstream signaling cascade including PKC, or direct activation of TNFα on PKC as reported by many studies in different cell types (Zhou et al., 2006; Peng et al., 2011). However, it has been reported that TNFα-induced increases in AQP4 expression may be mediated by p38MAPK (St Hillaire et al. 2005) since we also note that the p38MAPK inhibitor significantly blocked the proliferative actions of TNFα in this model. Together, our data provide the evidence that AQP4 might be involved in TNFα-stimulated proliferation of BrdU+ cells though activation of $P2X_7$ receptors and a downstream signaling cascade that involves PKC and p38MAPK as well as ERK, collectively resulting in alteration of AQP4 function. This blocking AQP4 with inhibitor or knockdown AQP4 with siRNA may be a useful tool in preventing gliosis in certain neuropathological conditions.

In conclusion, our results indicate that basal proliferation of BrdU+ cells is sensitive to blockade of ATP-$P2X_7$ receptor signaling suggesting that endogenous ATP might be released from slices during culture and drive the basal proliferation (Figure 9). Administration of the proinflammatory cytokine TNFα exaggerates cell proliferation through enhanced actions on $P2X_7$R signaling (Figure 9). The proliferative action of TNFα can be effectively blocked by lowering extracellular ATP with apyrase or by blocking $P2X_7$Rs with antagonists as well as by blocking AQP4 expression and downstream signaling

cascades (Figure 9). These findings are particularly relevant to the neuropathological conditions such as brain trauma, ischemia and neuroinflammation as well as several neurodegenerative disorders, which involve increased ATP release and $P2X_7R$ expression as well as cytokine release. The results of the studies herein warrant further experiments to test pharmacological inhibition or stimulation of ATP-$P2X_7$ receptor signaling as a therapeutic strategy in these pathological conditions. Glial cells may respond to their microenvironments by actively proliferating and releasing various factors, which may be detrimental or beneficial to brain repair or recovery. Therefggore, targeting the ATP-$P2X_7$ receptor signaling cascade may effectively control populations of proliferating glial cells and protect neuronal cells. Two studies have recently demonstrated that *in vivo* administration of the $P2X_7R$ antagonist BBG effectively blocked neuronal apoptosis triggered by $P2X_7R$ stimulation in mouse models of Huntington's disease (Diaz-Hernandez et al., 2009) and that administration of BBG in a spinal cord injury model reduced abnormal morphology, improved motor recover, and reduced the local activation of astrocytes and microglia (Peng et al., 2009). Our findings may add another dimension to further support the effectiveness of this strategy.

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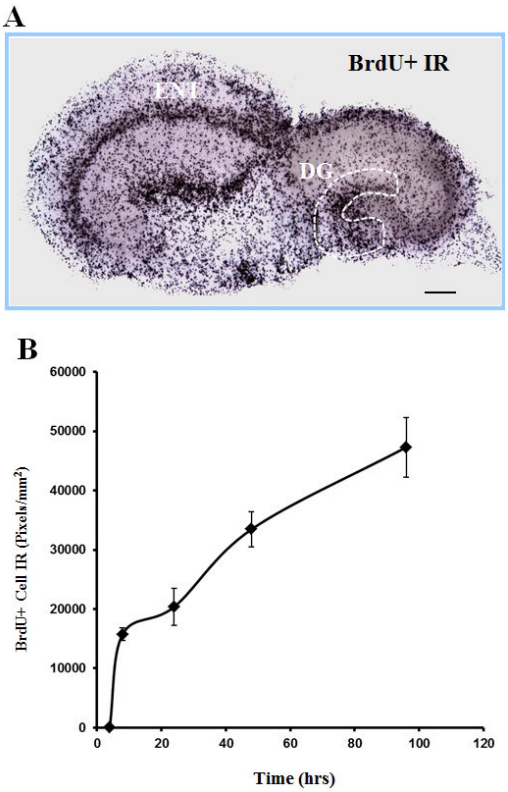

Figure 1. Proliferation of progenitor cells labeled by BrdU in HEC slice

HEC slices at 10 DIV were treated with BrdU (50 μM) for 24 hr and fixed at different time points. BrdU immunohistochemistry was used to visualize BrdU+ cells. The whole-mounted HEC slice (A) shows the distribution of BrdU+ cells in slice after 4 day post-BrdU (total 14 DIV), with dense BrdU+ cells in dentate gyrus (DG) of the hippocampus and layer IV of entorhinal cortex (ENT) (Scale bar = 500 μm). With computer-assisted imaging software, BrdU+ cell immunoreactivity (IR) in the DG defined region defined in the box was measured and quantified throughout study. Depicted in the graph (B) is the mean ± S.E.M. of BrdU+IR measured from slices (n = 6) in a time course study, indicating that BrdU-labeled cells are constantly dividing under the culture conditions used.

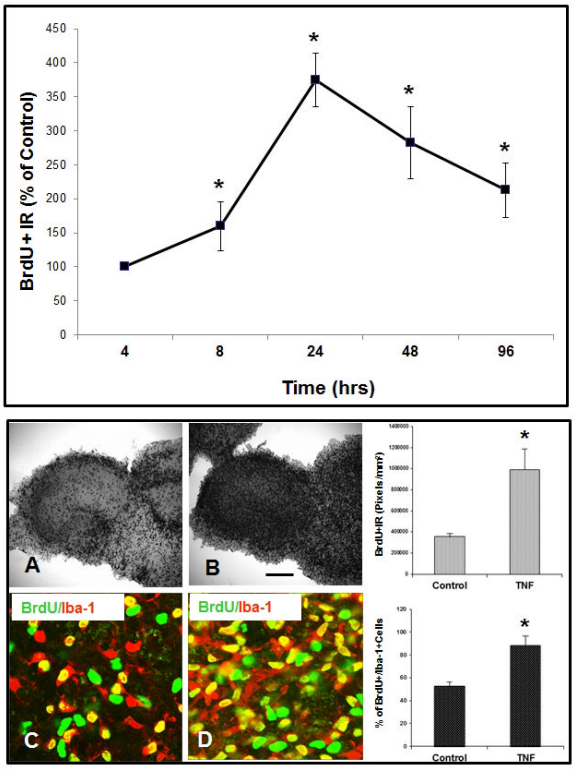

Figure 2. TNFα-stimulated BrdU+ cell proliferation

Top panel: Time course study of BrdU proliferation showing that TNFα (25 ng/ml) stimulates a robust increase in BrdU+ cell populations. Data are presented by percentage change relative to control * $p<0.001$ compared to Control, n = 6-8 slices). Bottom panel: Representative images of BrdU immunohiochemistry are shown in Control (A) and TNFα (B) at 48 hr time point (Scale bar = 500 μm). The confocal z-section images shown are the double immunofluorescent staining of BrdU (green) and microglial marker Iba-1 (red) (original magnification 40x). The bar graph to the upper right depicts BrdU+ IR at 48 hr time point measured from control- and TNFα-treated groups (n = 6-8). Bar graph on the bottom right represents the percentage of BrdU+ cells co-expressing Iba-1.

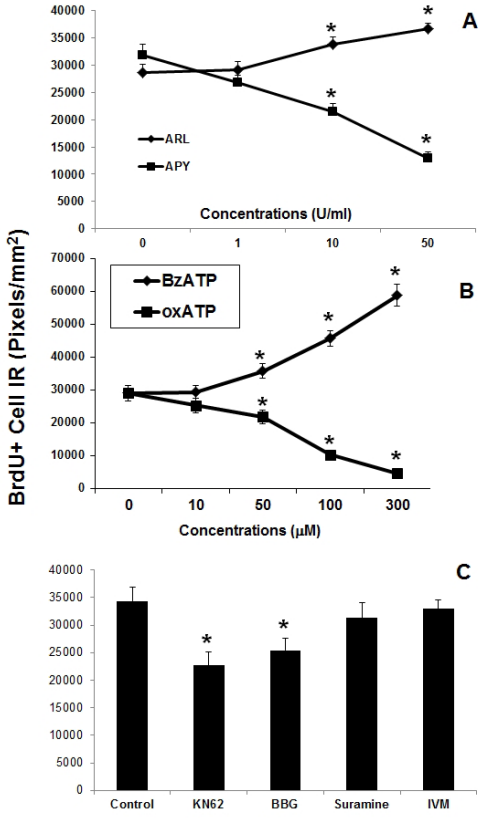

Figure 3. ATP-P2X$_7$ receptor signaling regulates basal proliferation of BrdU+ cells

A: After BrdU treatment, slices were incubated with ATPase apyrase (APY: 1, 10, and 50 U/ml) or NTPDase inhibitor ARL 67156 (ARL: 1, 10, and 50 U/ml) for 48 hr and then BrdU +IR determined. Lowering extracellular ATP level by APY inhibits while increasing extracellular ATP level by ARL augments basal proliferation of BrdU+ cells under normal culture conditions used. B: After BrdU treatment, slices were with P2X$_7$R agonist BzATP (10, 50, 100, and 300 μM) or antagonist oxATP (10, 50, 100, and 300 μM) for 96 hr and then BrdU+IR was determined as described in the methods. Activation of P2X$_7$R with agonist BzATP or blockade of P2X$_7$ receptors with antagonist oxATP dramatically increases or decreases proliferation of cells labeled by BrdU in a dose dependent manner respectively (* p<0.001 comparing to control). C: After BrdU treatment, slices were cultured with the presence of other P2X$_7$R antagonists KN62 (50 μM) and BBG (100 μM) or P2X$_4$R antagonist suramin (100 μM) or agonist invemectin (IVM: 50 μM). The experiments were repeated at least three times with similar designs and results.

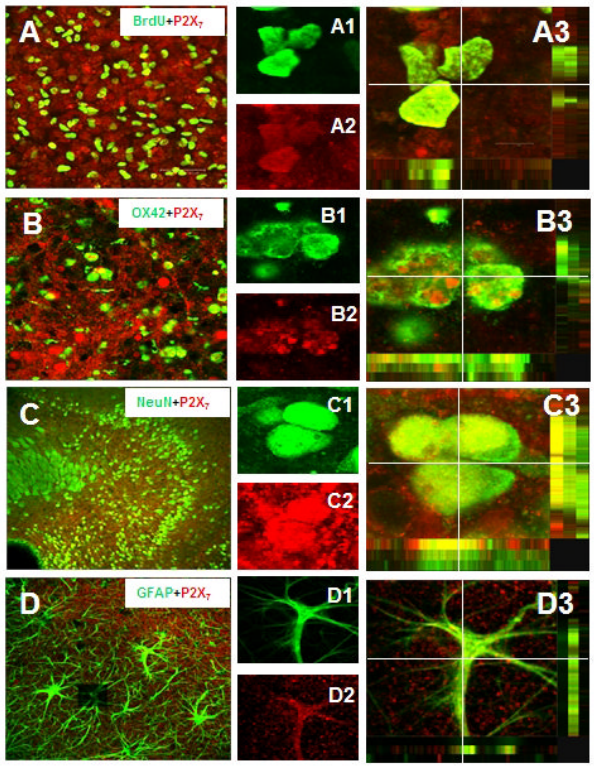

Figure 4. Cell type expression of $P2X_7$ receptors in HEC slices

Panel A: The double immunofluorescent staining of anti-BrdU (green) and anti- $P2X_7$ (red) shows that the majority of BrdU+ cells co-expressing $P2X_7R$ (A-20x; A1-2-80x; A3-120x). Panel B: The double immunofluorescent staining of anti-OX42 (green) and anti- $P2X_7$ (red) shows co-localization of $P2X_7Rs$ in OX42+ microglial cells (B-20x; B1-2-80x; A3-320x). Panel C: The double immunofluorescent staining of anti-NeuN (green) and anti- $P2X_7$ (red) shows strong expression of $P2X_7R$ on granule neurons of the hippocampus (C-20x; C1-2-120x; C3-320x). Panel D: The double immunofluorescent staining of anti-GFAP (green) and anti- $P2X_7$ (red) shows expression of $P2X_7R$ in GFAP+ astrocytes (D-20x; D1-2-80x; D3-160x).

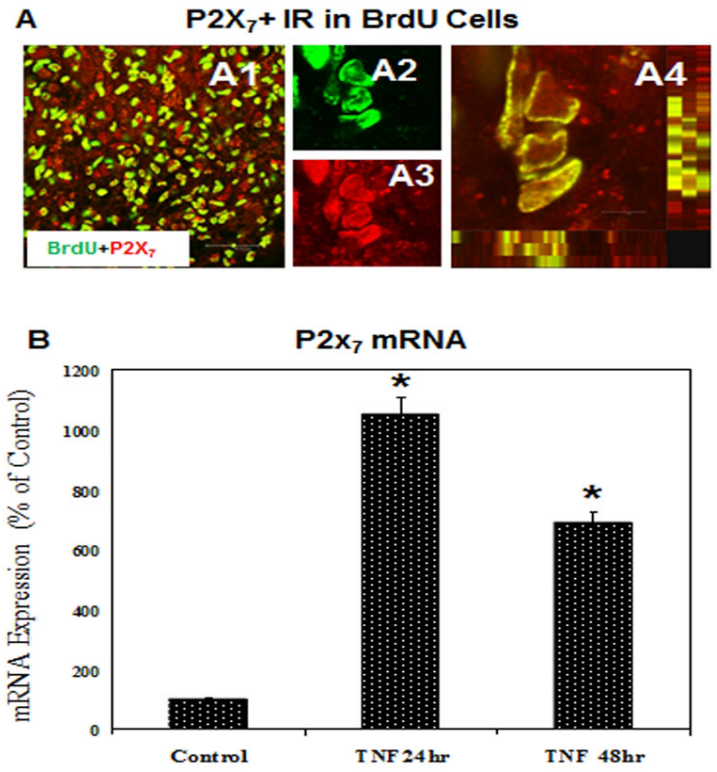

Figure 5. TNFα induction of $P2X_7$ receptors

Panel A: Representative photographs of the double immunofluorescent staining with anti-BrdU (green) and anti-$P2X_7R$ (red) show strong expression of $P2X_7Rs$ in BrdU+ cells in TNFα-treated slices (A1-20x; A2-3-80x; A4-240x). Panel B: Bar graph indicates TNFα-induced expression of $P2X_7R$ mRNA (* $p<0.0001$, n = 3). Panel C: Shown in bar graph are mean ± SEM of mRNA levels of NTPDase family (n = 3).

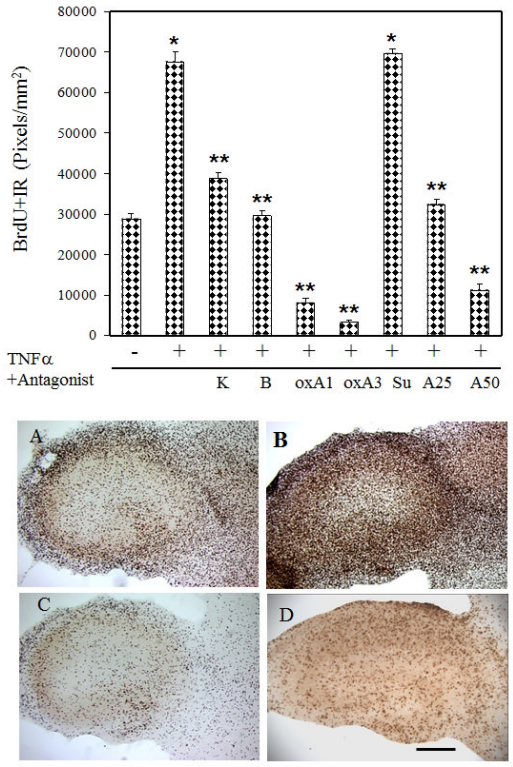

Figure 6. Proliferative action of TNFα is sensitive to the blockade of ATP- $P2X_7$ receptor signaling

Shown in bar graph (top) are mean ± SEM of BrdU+IR measured from each group (* p<0.0001, compared to control; ** p>0.001, compared with TNFα treatment (48 hr), n = 6-8). TNFα-increased proliferation of BrdU+ cells significantly blocked by $P2X_7R$ antagonists KN62 (K, 50 μM), BBG (B, 100 μM) and OxATP at 100 and 300 μM (oxA1 and OxA3) as well as by ATPase apyrase at 25 and 50 U/ml (A25 and A50), but not by $P2X_4R$ agonist suramin (Su: 100 μM). The experiments were repeated three times with similar designs and results. Representative images shown in bottom were taken from (A) control, (B) TNFα treatment, (C) TNFα + oxATP300 μM, and (D) TNFα+Apyrase 50U (Scale bar = 500 μm).

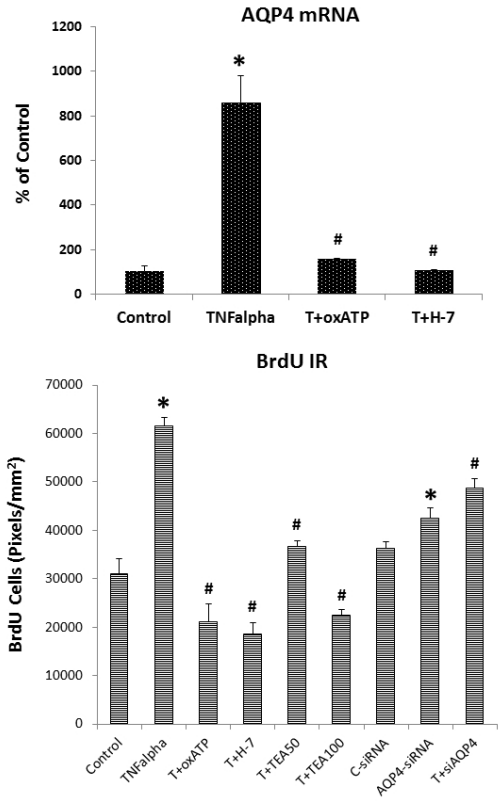

Figure 7. Involvement of AQP4 in proliferative actions of TNFα

Bar graph (top) showing strong induction of AQP4 mRNA by TNFα, which was blocked by the $P2X_7R$ antagonist oxATP and the PKC inhibitor H-7 (* $p<0.001$, compared to TNFα-treated group, n = 3). Bar graph in the bottom showing TNFα-stimulated increases in BrdU +IR was blocked by H-7 (50 μM) and AQP4 channel inhibitor TEA (50 and 100 μM) as well as AQP4 siRNA, suggesting that AQP4 is involved in TNFα regulation of BrdU+ progenitor cell proliferation through activation of $P2X_7Rs$. The experiments were repeated with similar designs and results.

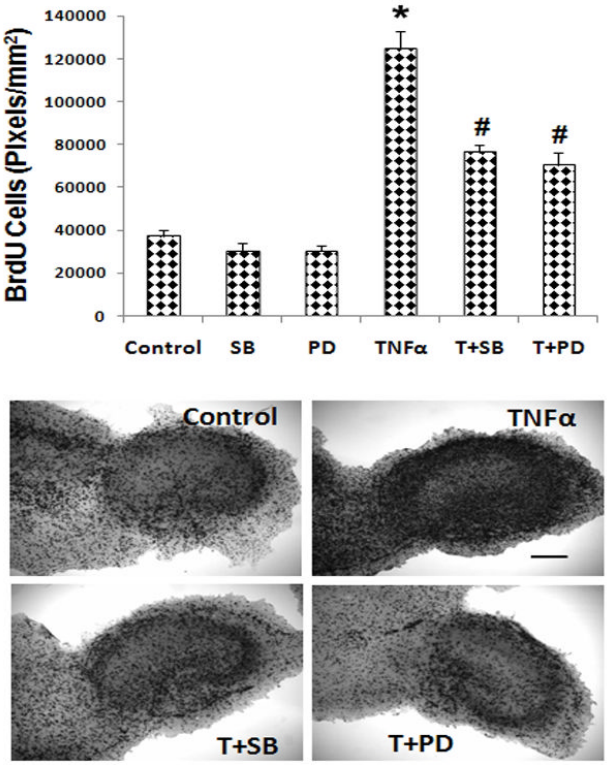

Figure 8. Blockade of downstream signaling cascade ERK and p38MAPK inhibits TNFα-stimulated BrdU+ cell proliferation

BrdU-treated slices were incubated with either p38MAPK inhibitor SB 203580 (SB: 50 μM) or the Erk inhibitor PD 98059 (50 μM) in the absence or presence of TNFα (25 ng/ml) for 48 hr and BrdU+IR determined. Shown in bar graph (top) are mean ± SEM of BrdU+IR measured from each group (* $p<0.0001$, compared to control; ** $p>0.001$, compared with TNFα treatment, n = 5-8). The experiments with inhibitors were repeated three times with the similar results. Representative images of BrdU+ IR were shown from each group as indicated (Scale bar = 500 μm).

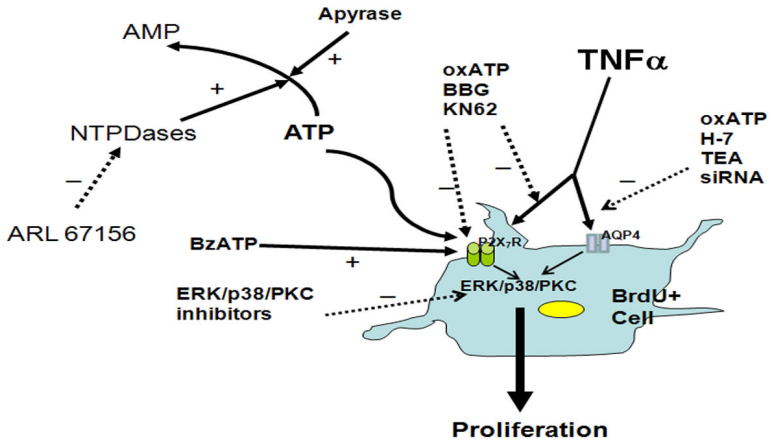

Figure 9. ATP-P2X$_7$ receptor signaling controls the proliferation of BrdU+ progenitors

ATP/BzATP activation of P2X$_7$ receptor-downstream signaling cascades promotes basal proliferation of BrdU+ cells. Apyrase functionally hydrolyses ATP to ADP/AMP (for review Fields and Burnstock, 2006) and thus lowers extracellular ATP level limiting action of P2X$_7$Rs leading to reduction of BrdU+ cell proliferation. NTPDase family also hydrolyses ATP to ADP/AMP (for review Fields and Burnstock, 2006)g to lower the extracellular ATP level. ARL 67156 is an inhibitor of NTPDase family and subsequently elevates extracellular ATP level leading to increased activation of P2X$_7$Rs and BrdU+ cell proliferation. Blockade of ATP-P2X$_7$ receptor by using the P2X$_7$R antagonists oxATP, BBG or KN62 can shut down proliferation of BrdU+ progenitors. TNFα strongly induces expression of P2X$_7$ R and AQP4 mRNA and in turn increases BrdU+ progenitor proliferation. Proliferative actions of TNFα can be abolished by blocking either P2X$_7$Rs or AQP4. TNFα-induced AQP4 expression may be influenced by P2X$_7$Rs because the P2X$_7$R antagonist oxATP can eliminate TNFα induction of AQP4 and subsequent BrdU+ cell proliferation. Inhibition of downstream signaling cascades abolishes proliferative actions of TNFα.