P2X7 Receptors Regulate Phagocytosis and Proliferation in Adult Hippocampal and SVZ Neural Progenitor Cells: Implications for Inflammation in Neurogenesis

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ABSTRACT

Identifying the signaling mechanisms that regulate adult neurogenesis is essential to understanding how the brain may respond to neuro-inflammatory events. P2X7 receptors can regulate pro-inflammatory responses, and in addition to their role as cation channels they can trigger cell death and mediate phagocytosis. How P2X7 receptors may regulate adult neurogenesis is currently unclear. Here, neural progenitor cells (NPCs) derived from adult murine hippocampal subgranular (SGZ) and cerebral subventricular (SVZ) zones were utilized to characterize the roles of P2X7 in adult neurogenesis, and assess the effects of high extracellular ATP, characteristic of inflammation, on NPCs. Immunocytochemistry found NPCs in vivo and in vitro expressed P2X7, and the activity of P2X7 in culture was demonstrated using calcium influx and pore formation assays. Live cell and confocal microscopy, in conjunction with flow cytometry, revealed P2X7+ NPCs were able to phagocytose fluorescent beads, and this was inhibited by ATP, indicative of P2X7 involvement. Furthermore, P2X7 receptors were activated with ATP or BzATP, and 5-ethyl-2'-deoxyuridine (EdU) used to observe a dose-dependent decrease in NPC proliferation. A role for P2X7 in decreased NPC proliferation was confirmed using chemical inhibition and NPCs from P2X7−/− mice. Together, these data present three distinct roles for P2X7 during adult neurogenesis, depending on extracellular ATP concentrations: (a) P2X7 receptors can form transmembrane pores leading to cell death, (b) P2X7 receptors can regulate rates of proliferation, likely via calcium signaling, and (c) P2X7 can function as scavenger receptors in the absence of ATP, allowing NPCs to phagocytose apoptotic NPCs during neurogenesis. STEM CELLS 2018;36:1764–1777

SIGNIFICANCE STATEMENT

High concentrations of extracellular ATP are a hallmark of neuroinflammatory and neurodegenerative disorders. By activating P2X7 receptors, ATP can exacerbate the initial insult, furthering cell death. Results of this study demonstrated that P2X7 has multiple roles in neural progenitor cells. As a calcium channel, P2X7 signaling may negatively regulate neural progenitor cell proliferation, while retaining its canonical role as a death receptor via the formation of transmembrane pores. P2X7 may also contribute to niche maintenance by facilitating phagocytosis of apoptotic cell bodies. The presence of P2X7 within the adult neurogenic niches is of importance given the therapeutic potential of some antagonists following ischemic injury.

INTRODUCTION

Adult neurogenesis occurs in at least two stem cell niches, the subgranular zone (SGZ) of the hippocampal dentate gyrus and the subventricular zone (SVZ) that lines the walls of the anterior lateral ventricles [1]. Neural progenitor cells (NPCs) in both niches proliferate to generate large progenitor pools. In the hippocampus, these NPCs mature into granule neurons, and the newly integrated neurons exhibit an enhanced plasticity for a short period that is vitally important for the formation and retrieval of memories [2, 3]. In the SVZ, NPCs give rise to neuroblasts that migrate via the rostral migratory stream to the olfactory bulb and differentiate into GABAergic interneurons that participate in olfaction [4]. Neurogenesis...
requires tight regulation of cell numbers and proliferation is balanced by programmed cell death (PCD), allowing selection of only the most appropriate cells to participate in synaptic competition and integration. Apoptotic cells must be removed to prevent buildup and inflammation in the neurogenic environment [5, 6].

Purinergic signaling has emerged as an important regulator of proliferation, PCD, and lineage maturation during adult neurogenesis [7, 8]. P2X7 is unique among the P2X receptors in its ability to subserve a number of these physiological needs: (a) as an ion channel, P2X7 is able to regulate proliferation, (b) as a transmembrane pore, it can induce cell death, and (c) as a scavenger receptor, it is able to remove cell corpses by facilitating phagocytosis [9]. In addition to these roles, P2X7 is well known to mediate inflammatory responses and cytokine release [10, 11].

Embryonic stem cells can utilize P2X7 receptors to regulate cell cycle progression, alter the expression of neuronal markers, and play a role in differentiation [12, 13]. Cultured neurons also had increased axonal growth and branching in conditions of P2X7 inhibition, highlighting the negative influence of extracellular ATP, and P2X7 activation, on neurons in the hippocampus [14]. In response to higher concentrations of ATP (≥1 mM, such as those found following inflammatory events), P2X7 receptors form large transmembrane pores permeable to molecules up to 800 Da, leading to osmotic dysregulation and cell death [15, 16]. P2X7 pore formation also plays a significant role in inflammatory processes during ischemic events, such as a stroke, where the large amounts of ATP released from dying cells can activate the P2X7 receptors on neighboring cells and exacerbate the initial infarct [17].

P2X7 receptors have been shown to facilitate phagocytosis in the strict absence of ATP [18, 19]. Disulfide bonds in the extracellular domain distinct from agonist binding sites have been identified as important for the recognition and binding of apoptotic cells [18]. Uptake occurs via P2X7 C-terminus association with the nonmuscle myosin complex in the membrane cytoskeleton. Agonist binding results in dissociation of the C-terminus from the cytoskeleton, thus inhibiting P2X7 mediated phagocytosis [20, 21]. We recently demonstrated that cultured human fetal NPCs utilize P2X7 to phagocytose cellular debris, possibly as a means of cell removal and maintenance in the developing neurogenic niches [6]. Interestingly, P2X7+ NPCs and neuroblasts appear earlier in the developing brain than microglia, the professional phagocytes, suggesting progenitor cells can self-regulate their niches by acting as nonprofessional phagocytes, a process that may persist into adulthood [22].

From these reports, P2X7 receptors have the potential to play a number of regulatory roles depending on the extracellular environment within the neurogenic niche. This in vitro study characterizes P2X7 receptors in adult NPCs to elucidate their possible involvement in proliferation, cell death, and phagocytosis in neurogenic niches in the mammalian brain. Identifying the signaling mechanisms that regulate adult neurogenesis is an essential step towards understanding how pathological conditions resulting in high extracellular ATP may influence the neurogenic niches and more generally, the pathology of the adult central nervous system.

Materials and Methods

Dissection and Cell Culture

Primary cultures of adult NPCs were derived from the hippocampi and SVZ of female C57BL/6 or P2F7+/− [10] mice between 8 and 12 weeks of age. The tissue was minced using a scalp knife blade (30 seconds), trypsinized (30 minutes) and triturated as previously described [23, 24]. The resulting single cell suspension was cultured in NeuroCult Basal Medium with NeuroCult Proliferation Supplement (StemCell Technologies), glutamine (2 mM, Invitrogen), EGF and bFGF (10 ng/ml, Peprotech), and heparin (2 μg/ml). Neurospheres could be visualized by 7 days in vitro and the initial culture was passaged after 2–3 weeks. Cell cultures were maintained at 37°C, 5% CO2 and subsequently passaged every 7–10 days. Cultures used for experiments were between passages 2 and 6. Genotyping of P2X7+/− NPC cultures confirmed the expected deletion in the P2X7+/− gene, and the presence of the neomycin resistance gene used to disrupt the P2X7 gene. Primers were as follows: P2X7 forward, GCA GCC CAG CCC TGA TAC AGA CAT T; P2X7 reverse, TCG GGA CAG CAC GAG CTT ATG GA; neomycin forward, TGC TCC TGC CGA GAA AGT ATC CAT CAT GGC; neomycin reverse, CGC CAA GCT CTT CAG CAA TAT CAC GGG TAG. Reaction master mix was prepared with GoTaq Hot Start Polymerase (Promega) and reaction conditions were 35 cycles of 94°C for 60 seconds, 58°C for 72 seconds and 72°C for 75 seconds, followed by 72°C for 10 minutes. All reagents were obtained from Sigma-Aldrich unless otherwise stated.

Multi-Marker Immunocytochemistry of Adult Mouse Hippocampal and SVZ Neural Progenitor Cells

Adult NPCs were plated on poly-l-ornithine (5 μg/ml) and laminin (2.5 μg/ml, Thermo Fisher Scientific) coated glass coverslips and cultured for 3 days before fixation with 4% paraformaldehyde (PFA) followed by multiple-marker immunohistochemistry, modified from Weible and Chan-Ling [23]. Differentiated cultures were seeded at low density and 50% proliferation medium was supplemented with differentiation medium (NeuroCult Basal Media with NeuroCult Differentiation Supplement) daily for 7 days. For the detection of cytoplasmic antigens, cells were permeabilized with 0.5% Triton X-100 and blocked with 3% normal goat serum. Primary antibodies were applied for 16 hours at 4°C and included those raised against P2X7 (rabbit, 1:1000, Alomone Labs, Jerusalem, Israel intracellular: APR-004, and extracellular: APR-008, epitenope), PROX1 (mouse, 1:200), glial fibrillary acidic protein (GFAP, mouse conjuagate, 1:1000), nestin (rabbit, 1:200, Abcam, Cambridge, UK); SRY-related HMG BOX gene 2 (SOX2, rabbit, 1:2000, Abcam), vimentin (mouse, 1:1000), mammalian achaete-scute homolog 1 (MASH1, mouse, 1:500, BD Pharmingen, San Jose, CA), brain lipid-binding protein (BLBP, rabbit, 1:1000), and doublecortin (DCX, rabbit, 1:200, Cell Signaling Technology, Danvers, MA). Immune complexes were detected with goat antibodies to mouse IgG H + L, IgG2a, IgM or rabbit IgG H + L, conjugated to Alexa-Fluor 488, Cyanine3 or Cyanine5 (Thermo Fisher Scientific, Waltham, MA). Nuclei were counterstained with 4',6-diamidino-2-phenylindole dihydrochloride (DAPI). Isotype control and/or omission of secondary antibodies served as negative controls. Images were captured with an Olympus FV1000 confocal microscope. For immunohistochemistry, mice
were perfused with 4% PFA and brains drop-fixed for 4 hours at 4°C. Sections were permeabilized with 0.5% Triton X-100 for 30 minutes and blocked for 1 hour in 10% normal goat serum and 0.1% saponin before antibody application. Western blotting membranes were blocked with 5% skim milk and probed as above, and immune complexes were detected with horseradish peroxidase (HRP).

**Click-it EdU to Identify Proliferating Cells**

The thymidine analog 5-ethyl-2’-deoxyuridine (EdU) was used to quantify proliferating cells as per the manufacturer’s protocol (Click-it EdU Imaging Kit; Life Technologies, Carlsbad, CA). For in vitro assays, single cells were plated as above. Treatments, including purines and P2X7 inhibitors, were applied for 18 hours followed by a 4-hour incubation with EdU (10 μM). For live cell proliferation assays, treatments were applied for 24 hours and recorded using live cell microscopy. Cells were fixed, blocked and permeabilized as previously described. The click-it EdU reaction cocktail (consisting of [in mM]: PBS [137], CuSO4 [4], AlexaFluor-488 azide dye [0.02], ascorbic acid [11.4]) was applied for 30 minutes at room temperature [25]. DAPI was used as a nuclear counterstain. At least 10 randomly selected fields of view (FOV) per treatment were imaged and threshold intensity analysis was utilized to calculate the percentage of EdU positive cells per FOV. For immunohistochemistry, mice were given three intraperitoneal injections of EdU (50 mg/kg) and sacrificed on the 4th day. Sections were permeabilized and blocked as previously described, then incubated with click-it EdU cocktail for 30 minutes.

**Flow Cytometry**

All flow cytometry assays were performed on a Becton Dickinson FACS Calibur flow cytometer (Florey Institute, University of Melbourne) or a Beckman Coulter CyAn flow cytometer (Griffith University).

**Calcium Assays.** NPCs were suspended in 1 ml of Ca²⁺ free Na⁺ buffer (in mM: NaCl [140], NaOH [5], KCl [5], HEPES [10], glucose [5], BSA [0.1%]) and loaded with Fluo8-AM (2 ng/ml) and 5% pluronic acid (10 μl) for 30 minutes at 37 °C with gentle shaking. NPCs were washed twice with Ca²⁺/free Na⁺ buffer and allowed to de-esterify on ice for 30 minutes before resuspension in K⁺ buffer (in mM: KCl [145], KOH [5], HEPES [10], glucose [5], CaCl₂ [0.1], BSA [1%]) with or without preincubation with the P2X7 specific inhibitors AZ10606120 (15 minutes, 1 μM, Tocris Bioscience) or A438079 (30 minutes, 10 μM, Tocris Biosciences, Bristol). CaCl₂ (3 mM) was added prior to analysis by flow cytometry, followed by the addition of ATP (1 mM) or BzATP (100 μM) after 40 seconds of acquisition. Assays were run for 3 minutes.

**Ethidium Assays.** NPCs were resuspended in K⁺ buffer with or without AZ10606120 preincubation. EtBr (25 μM) was added before analysis by flow cytometry, followed by the addition of ATP or BzATP at the 40 seconds mark. Assays were run for 6 minutes.

**Apoptosis Assays.** NPCs were cultured overnight in the presence of ATP (100 μM), BzATP (100 μM), and staurosporine (0.2 μM). The cells were harvested, washed and resuspended at 1 x 10⁶ cells/mL in Annexin binding buffer (in mM: HEPES [10], NaCl [140], CaCl₂ [2.5]) according to the manufacturer’s protocol (Annexin V staining kit; Thermo Fisher Scientific). Annexin V-APC was incubated for 15 minutes at room temperature. NPCs were washed and resuspended in binding buffer, before the addition of 7-AAD and analysis by flow cytometry. Fluorescence intensity of ethidium bromide reaches a plateau at about 4–6 minutes following the addition of ATP. Necrotic or dead cells are excluded automatically as the fluorescence intensity of ethidium bromide in these cells reaches the maximum (256 channel). Baseline cell leakage was seen in all types of cells due to membrane blebbing and/or macropinocytosis, and is subtracted when calculating area under the ethidium uptake curve.

**Calcium Microscopy**

NPCs were loaded with 2 ng/ml Fluo-8 AM dye with 10 μl 5% pluronic acid in 250 μl of medium for 20 minutes and allowed to de-esterify for 10 minutes. Phenol red-free Neurobasal medium was used for imaging. ATP was applied at various concentrations and images were captured at 100 ms intervals over a 1-minute period. Approximately 30–50 regions of interest were selected at random from monolayer areas and the change in fluorescence (F/F₀) was recorded.

**Phagocytosis Assays**

Cellular engulfment of 1 μm carboxylated yellow-green (YG) beads was used to measure innate phagocytosis via P2X7 in a method adapted from Lovelace et al. [6]. For live cell microscopy, cells were labeled with LysoTracker Red (1 μM, Molecular Probes, Eugene, OR) to identify lysosomes or CellTracker Red (1 μM, Molecular Probes) to identify the cell body. Cultures were incubated with YG beads overnight in a Zeiss AxioObserver under physiologic live cell conditions. Confocal microscopy was used to confirm intracellular location. For flow cytometry analysis, NPCs were resuspended in Na⁺ buffer. YG beads were added to the FACS tube 20 sec following the start of acquisition and samples were run for 6 minutes. Treatments included ATP (1 mM; 15 minutes preincubation), Cytochalasin D (20 μM; 20 minutes preincubation), PFA (4%, 20 minutes preincubation), or serum (5%).

**Statistical Analysis**

Data were presented as mean values ± SEM. Statistical significance was determined by one-way ANOVA and post hoc analysis was carried out using Tukey HSD. In cases where Levene’s test for homogeneity showed an effect of variance, Welch’s one-way ANOVA was carried out and Games Howell post hoc test used. The alpha value was set to 0.05. Unless otherwise stated, for each experiment at least three biological repeats (N) were conducted with a minimum of three technical repeats, and (n) refers to the total number of cells counted. Effect size was determined by partial eta-squared (η²) analysis where 0.01–0.09 was small, 0.09–0.25 medium, and ≥ 0.25 was regarded as substantially large [26].

**RESULTS**

**EdU Positive Adult NPCs Express P2X7 Receptors In Vivo**

EdU incorporation in combination with immunohistochemistry (N = 3) was used to determine the presence of proliferative...
P2X7+ NPCs within the adult dentate gyrus and SVZ neurogenic niches. Hippocampal NPCs of the SGZ were identified by MASH1 immunoreactivity, indicative of NPC entry into the proliferative phase of neurogenesis [27] and were observed to express P2X7 receptors (Fig. 1A–1G). P2X7 immunoreactivity was detected in the cytoplasm and membrane of these NPCs (B,C), and occasionally in the nucleus of actively dividing cells (D–G). DAPI was used to label the nuclei. NPCs were also identified in sections of the subventricular zone (H–J) by EdU incorporation to label actively dividing cells. P2X7 immunoreactivity was identified in the ventricular layer and in EdU+ cells of the subventricular layer, separated by the short dashed line in I and J. The boundary with the lateral ventricles (LV) is also indicated, and the rostral migratory stream (RMS) can be seen in H. Scale bars represent 10 μm.

**Figure 1.** Adult hippocampal and SVZ NPCs express P2X7 receptors in vivo. NPCs were identified in sections of the hippocampal dentate gyrus (A, with insets B–C and D–G) by immunohistochemical staining for MASH1 in combination with EdU to label actively dividing cells. P2X7 immunoreactivity was detected in the cytoplasm and membrane of these NPCs (B,C), and occasionally in the nucleus of actively dividing cells (D–G). DAPI was used to label the nuclei. NPCs were also identified in sections of the subventricular zone (H–J) by EdU incorporation to label actively dividing cells. P2X7 immunoreactivity was identified in the ventricular layer and in EdU+ cells of the subventricular layer, separated by the short dashed line in I and J. The boundary with the lateral ventricles (LV) is also indicated, and the rostral migratory stream (RMS) can be seen in H. Scale bars represent 10 μm.

P2X7+ NPCs were identified as Type 2/Type C Intermediate Progenitors

Immuno-characterization of SGZ- and SVZ-derived NPCs cultured on glass coverslips found the majority of cells were GFAP−, nestin+, SOX2+, vimentin+, MASH1+, and BLBP+. SGZ NPCs were additionally Prox1+ and were identified as intermediate type 2 progenitors (Fig. 2A–2L), similar to previously described [31], whereas SVZ NPCs were defined as type C progenitors [32]. At 3 DIV, a small number of SGZ cells, 3.8 ± 0.8% (n = 1,887), were identified as possible type 1 NPCs by co-expression of GFAP and nestin (Fig. 2A–2C). MASH1 was observed to be negative in 13.9 ± 2.3% of cells (Fig. 2G, 2I; n = 507). When DCXhigh was used to identify type 3 neuroblasts they comprised <0.1% of the cells at 3 DIV (Supporting Information Fig. S1E–S1G). At 3 DIV, no cells were observed to express immature neuronal markers βIII tubulin, Map2a/b, or NeuN. When NPCs were maintained in differentiation media for 7 days, 10.1 ± 0.8% expressed βIII tubulin and 16.7 ± 0.9% expressed MAP2a/b (Supporting Information Fig. S1H–S1M, n > 7,000 each). The postmitotic neuronal marker NeuN was also observed in the nucleus of all cells (Supporting Information Fig. S1N–S1P) demonstrating the capability of the NPC cultures to differentiate into cells of the neuronal lineage. GFAP+ vimentin− cells in the...
differentiated culture also indicated the presence of astrocytic precursor cells and demonstrated multipotency, a key feature of NPCs (Supporting Information Fig. S1Q–S1S). O4 staining to detect oligodendrocyte precursor cells was negative in differentiated hippocampal cells, whereas 2.6/0.86% of differentiated SVZ cells were positive (Supporting Information Fig. S1T, S1U, respectively).

NPCs from both the SGZ and SVZ were observed to express P2X7 receptors in all cells when probed with antibodies against an intracellular epitope (Fig. 2J, 2K). NPCs examined without permeabilization displayed positive surface expression of P2X7 in 90.1 ± 2.5% of cells (n = 1,465, Fig. 2M–2O), whereas cells with absent surface expression (indicated by asterisk) retained intracellular P2X7 stores that were detected following permeabilization (Fig. 2P–2R). No staining was detected following exclusion of primary antibody (Supporting Information Fig. S1V). Immunochemical analysis of cryosectioned neurospheres found P2X7 was expressed homogenously throughout the spheres, confirming the presence in flask cultures (Supporting Information Fig. S2A). Western blotting also identified a band at ~85 kDa, consistent with the glycosylated form of P2X7 (Supporting Information Fig. S2B).

**Figure 2.** Adult NPCs were characterized as type 2/type C intermediate progenitor cells and express P2X7 receptors in vitro. Representative NPCs derived from the hippocampus and cultured in vitro were stained for GFAP and nestin (A–C) to identify type 1 NPCs. NPCs were also positive for the markers SOX2, vimentin (D–F), and BLBP (G–H). MASH1 was used to identify type 2 NPCs (G, I) and had a variable expression from low (indicated by hash) to high (asterisk). NPCs derived from the hippocampus expressed Prox1, a marker for granule cell neurons, and all NPCs expressed intracellular P2X7 receptors (J–L). Approximately 10% of representative hippocampal NPCs displayed negative P2X7 immunoreactivity under nonpermeabilized conditions, for example, they did not express P2X7 receptors on the membrane surface, indicated by an asterisk (M–O). Dual labeling against intracellular P2X7 and extracellular P2X7 revealed cells with negative surface expression retained intracellular stores (P–R). N ≥ 3; scale bars represent 10 μm.

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**Full Length P2X7 Receptors on Adult NPCs Function as Calcium Channels and Can Form Transmembrane Pores**

P2X7 receptor function as a calcium channel was assessed using time resolved flow cytometry. Adult NPCs were loaded with Fluo-8 calcium indicator dye, and calcium influx was observed upon P2X7 activation. Application of the general P2 agonist ATP (1 mM) and P2X7 agonist BzATP (100 μM) evoked calcium influx in hippocampal (Fig. 3A, N = 4) and SVZ (Fig. 3B, N = 4) derived NPCs. Calcium influx was reduced by preincubation with the P2X7 specific inhibitors AZ10606120 (1 μM, an allosteric antagonist) and A438079 (10 μM, a competitive antagonist),
Figure 3. Hippocampal and SVZ derived NPCs express functional P2X7 receptors. P2X7 receptor calcium channel function was demonstrated in hippocampal (A) and SVZ (B) derived NPCs by a change in relative Fluo-8 fluorescence over time following application of the general P2X agonist ATP and the P2X7 agonist BzATP. Using real-time flow cytometry, both agonists were observed to result in calcium influx that could be blocked with the P2X7-specific inhibitors AZ10606120 (AZ10) and A438079 (A438). P2X7 transmembrane pore formation was demonstrated by ethidium bromide uptake for both hippocampal (C) and SVZ (D) derived NPC, and was assessed by real-time flow cytometry. ATP and BzATP elicited P2X7 receptor pore formation, allowing ethidium bromide uptake, and the P2X7 inhibitor AZ10606120 attenuated this phenomenon. ATP dose response assays (E) demonstrated significant pore formation at 500 μM and 1 mM (F; N = 8). Flow cytometry was also used to assess the involvement of P2X7 receptors in phagocytosis. YG bead uptake in both hippocampal (G) and SVZ (H) derived NPCs was measured, and phagocytosis was notably higher in SVZ derived NPCs. Application of ATP blocked P2X7 mediated phagocytosis in NPCs derived from both niches, and similar findings were produced when using nonspecific inhibitors of phagocytosis included PFA, cytochalasin D (CytD), whereas 5% serum abolished all bead uptake (N > 3).
indicating presence of functional P2X7. Calcium microscopy was used to verify ATP-induced calcium influx in hippocampal NPCs (Supporting Information Fig. S3A–S3H), and a dose-dependent increase in cytosolic calcium concentration in response to increasing ATP concentration was observed \[Welch’s F(8, 650) = 455.0, p < 0.01; Supporting Information Fig. S3I\].

Time-resolved flow cytometry was also used to assess the pore-forming capabilities of P2X7 receptors in adult NPCs. Ethidium bromide was used to measure pore formation and is excluded from cells lacking the large transmembrane pore. Application of the agonists ATP and BzATP evoked pore formation in both hippocampal (Fig. 3C) and SVZ (Fig. 3D) derived NPCs. This effect was attenuated by the P2X7 specific inhibitor AZ10606120. Dose-response assays (Fig. 3E) demonstrated that pore formation occurred in response to concentrations of 500 μM ATP and above (quantified in Fig. 3F; \(N = 4\) each of SVZ and SGZ \(Welch’s F[5,27] = 15.6, p = .002\), partial \(\eta^2 = 0.80\)). The ethidium bromide uptake assays demonstrate the expression of functional P2X7 with a full-length C-terminus, as opposed to splice variants lacking the complete C-terminus, which are unable to associate with the membrane cytoskeleton and do not form transmembrane pores [33].

P2X7 Receptors Facilitate Phagocytosis by Adult NPCs within the Neurogenic Niches

In the absence of ATP, P2X7 can also function as a scavenger receptor to facilitate phagocytosis [18, 19, 21, 34]. The carboxyl terminus of P2X7 is associated with the heavy chain of non-muscle myosin IIA (NMMHC-IIA); it is the activity of this motor protein that enables the cytoskeletal rearrangements required for phagosome formation and particle engulfment. Our group has previously demonstrated that activation of P2X7 by extracellular ATP results in conformational changes which open the selective cation channel/pore and also dissociate the P2X7 C-terminus from the NMMHC-IIA in the underlying actin cytoskeleton, thereby abolishing its phagocytic function [9, 20, 21].

To determine the potential of adult NPCs to phagocytose, and if P2X7 can facilitate this process, we used live cell flow cytometry, as well as live cell and confocal microscopy, to measure uptake of fluorescent YG latex beads. NPCs from the hippocampus (Fig. 3G) and SVZ (Fig. 3H, \(N = 2\)) were observed to phagocytose beads over a time period of 6 minutes. NPCs derived from the SVZ displayed a greater capacity to phagocytose than that
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observed for hippocampal cultures. Preincubation with ATP inhibited phagocytosis of YG beads to the same extent as PFA and actin polymerization inhibitor Cytochalasin D, whereas 5% serum completely abolished all innate phagocytosis. These data strongly suggest P2X7 receptors can play a role in facilitating phagocytosis by adult NPCs within the neurogenic niches.

To confirm the intracellular localization of phagocytosed beads, hippocampal NPC cultures were stained with LysoTracker (red) to identify lysosomes and incubated overnight with YG beads (green). Live cell imaging captured NPCs phagocytosing YG beads in real-time, showing internalization, followed by docking with lysosomes and trafficking inside the cell (Fig. 4A, 4B, frame rate is 5 minutes). Engulfed beads could be identified as yellow (indicated by white arrowheads and indicative of co-localization in lysosomes) whereas non-engulfed beads were green. The total percentage of NPCs containing phagocytosed beads was 62 ± 3.3% with an average of 4.5 ± 0.3 beads per cell (n = 491). Orthogonal reconstruction using confocal microscopy further exemplified intracellular localization of YG beads in NPCs stained red with CellTracker (Fig. 4C, planes of projection indicated by yellow cross hairs). NPC cultures were probed for microglial marker Iba1 to confirm the absence of professional phagocytes. No Iba1* cells were observed in NPC cultures (Fig. 4D, 4E), whereas Iba1+ microglia is shown in Figure 4F as a positive control.

Activation of Hippocampal and SVZ P2X7 Receptors Negatively Regulates Proliferation of NPCs: Evidence from Inhibition and Knock-Out Studies

The effects of purinergic signaling on NPC proliferation were investigated using EdU incorporation to identify proliferative cells, with DAPI used as a nuclear counterstain. A negative dose-dependent correlation was demonstrated between increasing exogenous ATP concentrations and percentage of total EdU+ proliferating NPCs (Fig. 5A) with a large effect size (Welch’s $F_{[9,136]} = 125.0, p < .001$, partial $\eta^2 = 0.70, N = 6$). Post hoc analysis revealed the maximal response to be observed at 100 μM with a decrease of 24.9 ± 2.7% (p < 0.001). A small reversal of the decreasing trend in proliferation was observed at 500 μM, possibly due to the recruitment of multiple purinergic signaling pathways. Purines ADP, UTP, and UDP (100 μM) were tested in comparison to ATP, to gauge the possible involvement of multiple purinergic receptors in regulating proliferation (Fig. 5B). Application of ADP also resulted in a decrease (23.6 ± 2.8%, p < .001) in NPC proliferation, though UTP and UDP showed no effect, indicating other ionotropic or metabotropic purinergic receptors may be involved. Cell death was not observed upon application of ATP (100 μM) using live cell microscopy (Supporting Information Movie S1) and recovery assays demonstrated NPC proliferation returned to basal level following ATP washout and overnight recovery, indicating the reduction in proliferation was not due to activation of irreversible apoptotic or necrotic pathways (Fig. 5C).

To determine any specific involvement of P2X7 signaling in the regulation of NPC proliferation, P2X7 agonists and antagonists were utilized. BzATP application (Fig. 5D) also produced a significant negative dose–response relationship [$F_{[7,143]} = 130.1, p < .001$, partial $\eta^2 = 0.86, N = 4$]. The negative effects of ATP on the proliferative potential of NPCs were significantly blocked by preincubation with the P2X7 antagonist A438079 (10 and 25 μM) prior to the application of ATP, Fig. 5E (Welch’s $F_{[5,168]} = 85.5, p < .001$, partial $\eta^2 = 0.68$). Finally, hippocampal NPC cultures generated from knockout mice (P2X7−/−) were also utilized to confirm P2X7 receptor involvement in proliferation and were confirmed to be lacking P2X7 receptors using PCR (Fig. 5F). P2X7−/− hippocampal NPC cultures had significantly higher proliferation rates when cultured in the presence of ATP, compared with the wild-type NPCs (Fig. 5G [Welch’s F $(9,298) = 182.9, p < .001$, partial $\eta^2 = 0.68$]). Similar results were observed for SVZ P2X7−/− cultures (Supporting Information Fig. S3; [Welch’s F$(3,125) = 189.2, p < .001$, partial $\eta^2 = 0.75$]). P2X7−/− NPC culture proliferation was significantly negatively impacted by exogenous ATP application, indicating the involvement of other purinergic receptors in modulating proliferation and aligning with data presented in Figure 5B.

Purinergic Signaling in Adult NPCs Can Influence Neuronal Differentiation

P2X7 receptors have previously been shown to regulate differentiation of embryonic NPCs [13]. We investigated if purinergic signaling in adult NPCs could also influence neuronal differentiation. Lineage investigations following application of ATP over a 3-day period induced a small but significant increase in the number of DCX+ neuroblasts generated in wild-type hippocampal NPC cultures ($Welch’s F_{[1,78]} = 38.3, p < .001$, partial $\eta^2 = 0.33, n = 41,389$) correlating with decreased proliferation (Fig. 5H).

ATP Treatment Negatively Regulates NPC Proliferation without Increasing Cell Death

Application of ATP at 100 μM overnight decreased cell proliferation without significant effects on cell death (N = 10). NPCs were treated with ATP (100 μM), BzATP (100 μM), and staurosporine (0.2 μM), and were assessed by flow cytometry for initiation of apoptosis, as detected by Annexin V binding. Viability marker 7-AAD was used to distinguish early apoptosis (7-AAD negative) from late apoptosis or nonviable cells (7-AAD positive). Cell
populations were gated (Fig. 6A) and Annexin V fluorescence was plotted against 7-AAD, to identify region 1 (R1; Annexin V positive, 7-AAD positive) and region 2 (R2; Annexin V positive, 7-AAD negative). Staurosporine significantly increased apoptosis, but not cell death, and increased Annexin V binding above control, whereas ATP and BzATP remained unchanged (Fig. 6B). Importantly, there was no significant difference between control and ATP or BzATP in the percent of late apoptotic or nonviable cells (R1; [Welch’s F(2,27) = 1.85, p = .20]; Fig. 6C) or early apoptotic cells (R2; [F(2,27) = 1.57, p = .23]; Fig. 6D).

**DISCUSSION**

High concentrations of extracellular ATP are a hallmark of neuroinflammation resulting from various ischemic and neurodegenerative disorders [8, 35]. Our studies demonstrated P2X7 receptor signaling may have significant effects on adult neurogenesis, particularly in the aftermath of inflammatory events. Using immunohistochemistry, we found EdU+ cells were evident in both adult neurogenic niches and displayed positive immunoreactivity against P2X7 receptors. P2X7 was mostly observed on the membrane or within the cytoplasm of NPCs but was also detected at the nucleus of some MASH1+/EdU+ dividing precursors (Fig. 1D–1G). This nuclear staining was similar to observations made of mouse retinal ganglion cells [29] and is congruent with our work on postmortem human brain tissue where P2X7 can be detected on the nuclear envelope of microglia and astrocytes, but not mature neurons (unpublished observations). In vitro cultures derived from these niches were characterized as intermediate neural progenitors (type 2 from the SGZ of the dentate gyrus and type C from the SVZ), and were also P2X7 receptor immunoreactive.

Functional analysis by calcium influx and ethidium bromide uptake utilized the P2X7 agonist BzATP and the specific P2X7 inhibitor AZ10606120 and found that P2X7 receptors expressed in NPC cultures were able to form both cation-selective channels and transmembrane pores. This confirmed the presence of a full-length functional receptor, as opposed to the Δ-C splice variant displaying a deleted C-terminus that is also present in the brain [33]. Expression of multiple variants may account for a number of observational discrepancies in previous studies, such as the absence of pore functionality in embryonic NPCs [36]. Our data aligns with a study by Messemer and colleagues, who used patch clamping to report functional P2X7 in SVZ NPCs [16].

Numerous studies report the existence of P2X7 receptors in mature neurons [37], although this remains debated [38]. Currently, however, evidence of receptor presence in adult hippocampal NPCs is limited to reports of immunohistochemistry and membrane current recordings [39, 40]. Our study confirms and builds on these observations. Our functional investigations demonstrate P2X7 receptors could subserve a number of physiological functions in the NPCs within both the hippocampal and subventricular neurogenic niches, depending on the concentrations of extracellular ATP. Through its function as a calcium channel, P2X7 signaling may govern the size of the adult progenitor pools by negatively regulating cell proliferation, whereas retaining its canonical role as a death receptor via the formation of transmembrane pores. P2X7 receptors may also contribute to niche maintenance by facilitating phagocytic clearance of apoptotic cell bodies. Figure 7 is a schematic representation of the roles subserved by P2X7 based on findings from the present study as well as earlier investigators [6, 9, 13, 15, 16, 21]. The presence of P2X7 receptors within the adult neurogenic niches is of importance given the therapeutic potential of some antagonists following ischemic injury [41–43].

**P2X7 Receptors Facilitate Phagocytosis by Adult NPCs in the Absence of ATP**

During neurogenesis, cell proliferation is balanced by large amounts of cell death both in the niche (target-independent PCD) and at the site of neural integration (target-dependent PCD), allowing for the refinement of new neurons and synapses [44]. These apoptotic cells must be removed to prevent buildup and inflammation. Both professional phagocytes (microglia) and nonprofessional phagocytes contribute to this process; resident NPCs and neuroblasts have been demonstrated to engulf the debris of neighboring apoptotic cells via innate phagocytosis. In 2011, Lu and colleagues demonstrated DCX positive adult NPCs to have a phagocytic role within both the hippocampal and subventricular neurogenic niches [22]. Recently, human neuroblasts with high expression of both DCX and P2X7 had the greatest phagocytic capability of developing CNS cells, and DCX野生型 NPCs a lesser capability [6]. P2X7 receptors were identified as the scavenger receptor responsible for phagocytosis of beads, apoptotic neuroblasts, and apoptotic ReN line neural stem cells [6]. Traditionally, the role of phagocyte belonged exclusively to microglia, generally requiring “activation” by an inflammatory stimuli [45]. The presence of scavenger receptors on nonprofessional phagocytes, such as NPCs and astrocytes alleviates, the responsibility of niche maintenance from microglia alone, and allows the clearance of debris without activation of resident microglia.

In the present study, we also demonstrate that adult NPCs derived from both the hippocampus and SVZ were capable of phagocytosis via P2X7 receptors, despite low levels of DCX expression. NPCs isolated from the SVZ had greater basal rates of proliferation than those derived from the SGZ; this potentially reflects the need for the greater phagocytic capacity that was also observed in the SVZ, to remove cell corpses generated by the higher basal proliferation rates. Phagocytosis was inhibited by the presence of ATP or serum as previously observed in human NPCs [6]. This is of physiological significance as though P2X7 receptors are best known for their role in inflammation, under normal conditions ATP levels in the cerebrospinal fluid are between 8 and 16 nM [46]. Even in the event of astrocyte stimulation, which can result in ATP release, luciferase probe assays report values no higher than 80 nM [47]. Given that micromolar concentrations of extracellular ATP are required to activate the P2X7 receptor [15] it is unlikely that ATP could reach the required concentrations except under inflammatory or pathological conditions such as trauma or infection, where high levels of ATP can result in further damage and hinder innate repair mechanisms. This permits a potential role for P2X7 in adult NPCs under normal physiological conditions, which is to facilitate the phagocytosis of apoptotic progenitors in the neurogenic niches. Although other scavenger receptors, for example MerTK, have been identified within SVZ cells, MerTK blocking antibody did not completely abrogate phagocytosis [48]. This suggests that
Figure 6. ATP does not cause significant cell death at 100 μM. Flow cytometry was used to determine if ATP-induced decreases in proliferation were resulting from undetected cell death. NPCs were cultured overnight with ATP (100 μM), BzATP (100 μM), and staurosporine (0.2 μM). NPCs were harvested and assessed for Annexin V fluorescence. Region 1 (R1) gated 7-AAD positive Annexin positive cells and represents late apoptotic and nonviable cells. Region 2 (R2) gated 7-AAD negative Annexin positive cells and represents cells in the early stages of apoptosis. Histogram of Annexin V fluorescence (B) shows an increase in Annexin V binding following staurosporine treatment, but no difference following ATP or BzATP treatment. Quantification of the mean percentage of G1 for regions 1 and 2 (C and D respectively; N = 10) found no effect of ATP or BzATP treatment (p = .196 and p = .227 for R1 and R2 respectively), whereas staurosporine-induced early apoptosis (D, * indicates p = .018), but not late apoptosis (C, p = 1.00).

**P2X7 Receptor Regulation of NPCs in the Presence of High Extracellular ATP: Relevance to Neuroinflammation**

P2X7 function in ischemic brain injury, epilepsy, and stroke has recently gained momentum in the light of a number of studies demonstrating conferral of neuroprotection by modulation of P2X7 activity, alleviating the detrimental effects of excess extracellular ATP [11, 42, 49]. Antagonism of P2X7 receptors assisted functional recovery in spinal cord injury, possibly by decreasing secondary cell death caused by excessive ATP release, and was also shown to decrease pro-inflammatory mediators and increase neuronal survival rates in the striatum [50, 51]. Supporting this observation, status epilepticus (prolonged seizures) increased levels of P2X7 in the granule neurons of the dentate gyrus, and antagonizing the receptor reduced both seizure duration and subsequent neuronal death [52]. P2X7 receptors have also been associated with numerous neurodegenerative diseases including Alzheimer’s disease [53, 54], Multiple Sclerosis [55, 56], age-related macular degeneration [57], and were shown to be significantly increased in the hippocampus of rats suffering from cognitive dysfunction [58]. Subsequently, the neuroprotective effects of P2X7 antagonists have led to drug candidate studies [42, 43]. Given the potential P2X7 receptors hold as a therapeutic target, their function in SGZ- and SVZ-derived NPCs was confirmed. We demonstrated adult NPCs exposed to high ATP concentrations (500 μM and 1 mM) formed P2X7-mediated transmembrane pores. This was reduced by preincubation with the specific P2X7 inhibitor AZ10606120 and highlights the vulnerability of adult NPCs to cell death following neuro-inflammatory events.

**P2X7 Receptor as a Negative Regulator of Proliferation in SVZ and Hippocampal: Evidence from ATP or BzATP Stimulation**

ATP has pleiotropic physiological effects and its actions may converge at multiple purinergic receptors to derive an overall phenotype. Recently, ATP release from hippocampal astrocytes has been shown to positively regulate neural stem cell proliferation [59], however this occurs via P2Y1 receptors. There currently exists a lack of consensus in the literature as to the nature of P2X7 involvement in the regulation of proliferation and differentiation. P2X7 signaling was found to maintain survival in mouse embryonic stem cells [60], as well as proliferation in neuroblastoma cells [61, 62] and glial cells [63]. Alternatively, ATP and BzATP application was observed to decrease proliferation in murine luteal cells, via P2X7 modulation of the p38 MAP kinase pathway, and this occurred without inducing apoptosis [64]. In embryonic NPCs, BzATP application also decreased cell counts and BrdU+ cell numbers and enhanced neuronal differentiation [12]. P2X7 inhibition in embryonic NPCs was further demonstrated to increase expression of the proliferation marker Ki-67 [13]. These differences may be attributed to agonist concentration or exposure times, as well as P2X7 expression levels or the presence of various splice variants. Cell type and function may also contribute to scavenger receptors like P2X7 could operate cooperatively within the SVZ microenvironment. Furthermore, it is possible that MerTK actively removes apoptotic cells in inflammatory environments where high extracellular ATP levels do not allow P2X7 to operate as a scavenger receptor.
those cells lost. Alternatively, maturation may be a default
tion pathways as a compensatory mechanism to replace
death event, where neighboring cells (who might only expe-

[12]. This may be a physiological response of NPCs to a cell

increased TUJ1 and MAP2 expression via P2X7 activation

receptor in the absence of their agonist, and likely engulf particles by modulating the actin cytoskeleton by the C-terminus association

with NMMHC-IIa. Activation of the receptor by agonist binding dissociates NMMHC-IIa and inhibits phagocytosis [21].

Figure 7. Diagrammatic depiction of the three signaling roles of P2X7 receptors in adult NPCs identified in this study. P2X7 receptors can play at least three main roles in adult NPCs, the first of these being the formation of a large transmembrane pore, which has significant implications following inflammatory events. The transmembrane pore allows macromolecule exchange and an overload in cytosolic calcium concentrations, resulting in cell death. The second role of P2X7 is as a cation channel. The influx of calcium in response to ATP signaling can activate downstream calcium-dependent signaling cascades and result in possible transcription factor activation to regulate proliferation and differentiation. The third function of P2X7 receptors is that of phagocytosis. P2X7 receptors can act as a scavenger receptor in the absence of their agonist, and likely engulf particles by modulating the actin cytoskeleton by the C-terminus association with NMMHC-IIa. Activation of the receptor by agonist binding dissociates NMMHC-IIa and inhibits phagocytosis [21].

differences, for example, proliferation in response to inflam-

mation is a general function of glial cell populations [65]. In

the current study, the application of exogenous ATP and BzATP decreased proliferation and preincubation with P2X7 inhibitor A438079 partially attenuated the effects of ATP on proliferation. P2X7−/− cultures treated with ATP did not demonstrate as substantial a reduction in proliferation as was observed in the wild-type cultures. This suggests P2X7 receptors can contribute to negative regulation of adult NPC proliferation in the neurogenic niches, particularly in the context of neuroinflammation, and align with findings by Tsao et al. [12].

It is of note that ATP application still resulted in a
decrease in proliferation in NPCs derived from P2X7−/− mice. This in combination with the finding that ADP also decreased proliferation in wild-type cultures, as well as ATP having a greater potency than BzATP at inhibiting proliferation, indicates that P2X7 receptors are not the only purinergic receptor present on the surface of adult NPCs that may exert regulatory effects over proliferation. A decrease in NPC proliferation following an inflammatory event may have severe implications in terms of adult neurogenesis, particularly in the hippocampus where new neurons are vital for memory formation [3]. Decreased hippocampal neurogenesis is associ-

ated with impaired memory, as well as major depression [66], whereas rodents provided with an enriched environment and/or exercise consistently show increased rates of neurogenesis and improved learning and memory performance [67–70].

In addition, we reported an increase in DCX positive

NPCs following ATP application over a 3-day period. This

aligns with findings that long-term BzATP application

increased TUJ1 and MAP2 expression via P2X7 activation

[12]. This may be a physiological response of NPCs to a cell
death event, where neighboring cells (who might only expe-

rience a decrease in proliferation) initiate lineage elabora-
tion pathways as a compensatory mechanism to replace
those cells lost. Alternatively, maturation may be a default

state for NPCs that cease to proliferate. As our studies were
undertaken in vitro in the absence of surrounding cells in
the intact niche, further work could explore if NPC counter-
parts in the intact niche behave similarly as reported in this
study.

CONCLUSION

Our data shows P2X7 receptors have the ability to subserve at least three distinct physiological roles in NPCs derived from the adult neurogenic niches, depending on extracellular ATP conditions in vitro. The first, in the presence of low to moderate levels of ATP, results in calcium influx and a decrease in NPC proliferation. The second function of P2X7 receptors is cell death via transmembrane pore formation in response to the high concentrations of ATP that may be present during an inflammatory event. The third role for P2X7 receptors in the absence of ATP is phagocytosis, which may contribute to the maintenance of the neurogenic niche by removal of apoptotic bodies. Our study demonstrates the complex roles of P2X7 receptors in adult hippocampal and subventricular NPCs and is an important step towards understanding how inflammation may regulate adult neurogenesis.

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**AUTHOR CONTRIBUTIONS**

H.L.: conception and design, collection and assembly of data, data analysis and interpretation, manuscript writing, final approval of manuscript; M.: collection and assembly of data, final approval of manuscript; T.C.-L.: provision of study materials, manuscript writing, final approval of manuscript; M.L.: manuscript writing, final approval of manuscript; J.B.: manuscript writing, final approval of manuscript; K.T.: collection of data, final approval of manuscript; B.G.: financial support, provision of study materials, collection and assembly of data, data analysis and interpretation, manuscript writing, final approval of manuscript; M.W.: conception and design, financial support, administrative support, provision of study materials, collection and assembly of data, data analysis and interpretation, manuscript writing, final approval of manuscript.

**DISlosure of Potential Conflicts of Interest**

T.C.-L. declared advisory role with Eyeco. The other authors indicated no potential conflicts of interest.

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