



P2X7/P2X4 Receptors Mediate Proliferation and Migration of Retinal Microglia in Experimental Glaucoma in Mice

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Abstract Microglia are involved in the inflammatory response and retinal ganglion cell damage in glaucoma. Here, we investigated how microglia proliferate and migrate in a mouse model of chronic ocular hypertension (COH). In COH retinas, the microglial proliferation that occurred was inhibited by the P2X7 receptor (P2X7R) blocker BBG or P2X7R knockout, but not by the P2X4R blocker 5-BDBD. Treatment of primary cultured microglia with BzATP, a P2X7R agonist, mimicked the effects of cell proliferation and migration in COH retinas through the intracellular MEK/ERK signaling pathway. Transwell migration assays showed that the P2X4R agonist CTP induced microglial migration, which was completely blocked by 5-BDBD. *In vivo* and *in vitro* experiments demonstrated that ATP, released from activated Müller cells through connexin43 hemichannels, acted on P2X7R to induce microglial proliferation, and acted on P2X4R/P2X7R (mainly P2X4R) to induce microglial migration. Our results suggest that inhibiting the interaction of Müller cells and microglia may attenuate microglial proliferation and migration in glaucoma.

Keywords Glaucoma · Chronic ocular hypertension · Retinal microglia · Proliferation · Migration · P2X7R/P2X4R · Müller cells

Introduction

Glaucoma is a disease resulting in irreversible blindness; it is characterized by apoptotic death of retinal ganglion cells (RGCs). Although the pathogenesis of glaucoma is not completely understood, it is commonly believed that retinal glial cells are activated, and the activated glial cells participate in RGC damage by releasing a variety of harmful factors [1–5].

In the vertebrate retina, there are two types of glial cells: microglia and macroglia (Müller cells and astrocytes). Under normal conditions, retinal microglia are mostly distributed in the inner plexiform layer (IPL) and the outer plexiform layer (OPL). As antigen-presenting cells, microglia monitor the retinal microenvironment, migrate extensively, clear cellular debris, and participate in the immune-mediated network, thus maintaining retinal homeostasis [5, 6]. Müller cells are the principal macroglial cells in the retina. Their nuclei are located in the inner nuclear layer and their processes span the entire thickness of the retina, and support the functioning and metabolism of retinal neurons [2, 7]. Astrocytes are highly aggregated in the optic nerve head, playing intrinsic protective and supportive roles for RGCs [8].

In the glaucomatous retina, microglia are activated [9–13], as evidenced by up-regulated expression of translocator protein, a biomarker of microglial activation [14]. Activated microglia undergo a morphological change from a ramified to an amoeboid shape; at the same time, the cells show enhanced proliferation and migration to the

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ganglion cell layer (GCL). These microglia gathering in the GCL induce RGC damage by releasing pro-inflammatory factors, such as tumor necrosis factor- α (TNF- α), interleukins (ILs), and nitric oxide (NO) [12]. However, the mechanisms underlying microglial proliferation and migration to the GCL are largely unknown. There is evidence demonstrating that the interaction of Müller cells and microglia augments the retinal inflammatory response in a positive-feedback manner in retinal injury, in which ATP may be an important mediator [15–17]. Müller cells are potential sources of extracellular ATP [18]. In a chronic ocular hypertension (COH) experimental glaucoma model, we have shown that Müller cells are activated, which is characterized by up-regulated expression of glial fibrillary acidic protein [19, 20]. Activated Müller cells may promote ATP release [21]. Therefore, in this study, we investigated whether and how ATP regulates the proliferation and migration of retinal microglia in glaucoma.

Materials and Methods

Animals

All animal experiments were carried out according to the National Institutes of Health (NIH) guidelines for the Care and Use of Laboratory Animals and the guidelines of Fudan University on the Ethical Use of Animals and were approved by the Institutes of Brain Science of Fudan University. *P2X7R^{-/-}* mice were a generous gift from Dr. Yuqiu Zhang at Fudan University. Male C57BL/6 mice (weighing 18–20 g) were purchased from SLAC Laboratory Animal Co., Ltd. (Shanghai, China) and MacGreen mice from The Jackson Laboratory (Hancock County, USA). All mice were housed on a 12-h light/dark schedule, with standard food and water provided *ad libitum*.

Mouse COH Model

The mouse COH model was produced following the procedure previously described [22]. Briefly, under an OPMI VISU 140 microscope (Carl Zeiss, Jena, Germany), micro-magnetic beads (2 μ L, diameter \sim 10 μ m, BioMag[®]-Superparamagnetic Iron Oxide, Bangs Laboratories, Inc., IN, USA) were microinjected into the anterior chamber of anesthetized mice. Sham-operated mice were injected with 2 μ L of 0.9% NaCl.

Under general anesthesia (2% pentobarbital sodium, 40 mg/kg, i.p.), intraocular pressure (IOP) was measured using a handheld digital tonometer (TonoLab, Icare, Finland). The average value of five consecutive measurements with a deviation of $<$ 5% was accepted. All measurements were performed in the morning (09:00–10:00) to avoid possible

circadian differences. The IOPs of both eyes were measured before the injection (d0), and then on the next day of the operation (G1d), days 2, 3, 4, and 7 (G2d, G3d, G4d, and G1w), and weekly thereafter (G2w–G6w).

Immunofluorescence and Quantification of Microglia in the Retina

Immunofluorescence was assessed as previously described [23–25]. In brief, the eyes of anesthetized mice were removed quickly after perfusion fixation, further fixed with 4% paraformaldehyde in 0.1 mol/L phosphate buffer (pH 7.4) for 4 h at 4°C, and then dehydrated with graded sucrose solutions at 4°C. The eyecups were embedded in OCT compound (Tissue Tek, Torrance, USA), and vertically sectioned at 14 μ m on a freezing microtome (Leica, Nussloch, Germany). After blocking in 10% normal donkey serum (v/v) (Sigma-Aldrich, St. Louis, USA) in phosphate-buffered saline (PBS) plus 0.1% Triton X-100 for 2 h at room temperature, the retinal sections were incubated with the primary antibodies polyclonal goat-anti-Iba1 (ab5076, 1:1000 dilution, Abcam, Cambridge, USA), monoclonal rabbit-anti-GS (MAB302, 1:1000, Millipore, Billerica, USA), or monoclonal mouse-anti-Cx43 (SAB4200819, 1:1000, Sigma-Aldrich) overnight at 4°C. The secondary antibodies were Alexa Fluor 488 AffiniPure donkey anti-goat IgG, Alexa Fluor 488 AffiniPure donkey anti-rabbit IgG, or Alexa Fluor Cy3 AffiniPure donkey anti-mouse IgG (1:500, Jackson ImmunoResearch Laboratories, Inc., West Grove, USA). Signals were visualized with a confocal laser scanning microscope (Fluoview 1000, Olympus, Monolith, Tokyo, Japan) through a 40 \times objective.

Microglia were counted on vertical retinal sections following the procedure previously reported [23], with some modifications. In a single preparation, the whole retina was cut vertically into 128 sections 14 μ m thick. The first 8 sections were sequentially collected on 8 different glass slides, and the next 8 sections were collected in the same way, and so on. Therefore, on each of the 8 slides, there was a total of 16 sections covering different zones of the whole retina. We randomly chose one section to count all Iba1-positive microglia in the 16 sections.

Primary Retinal Microglia and Müller Cell Culture

Primary retinal microglia and Müller cells were cultured following procedures previously described [25, 26]. Briefly, retinas of newborn C57BL/6 mice (5 days old) were digested with 0.25% trypsin (Thermo Fisher Scientific, Rockford, USA). Dissociated retinal cells were then cultured in 75 cm² flasks containing Dulbecco's modified Eagle's medium nutrient mixture F12 (DMEM/F12, Thermo Fisher

Scientific) supplemented with 10% fetal bovine serum (Thermo Fisher Scientific), 100 U/mL penicillin, and 100 µg/mL streptomycin in a humidified 5% CO₂ incubator at 37°C. Microglial purification was conducted when mixed retinal cells were cultured for 11–13 days. Microglia, mainly growing on top of the cell layer, were detached by forcibly flapping the flasks. The detached cells, which were 95% microglia, were seeded into 24-well plates or the transwell system. The cells remaining in the flasks were purified Müller cells, and the third generation of these cells was used for experiments. The purity of microglia and Müller cells were determined by positive staining of ionized Ca²⁺-binding adaptor molecule 1 (Iba1) and glutamine synthase (GS), respectively.

5-ethynyl-2'-deoxyuridine (EdU) Labeling, Immunofluorescence, and Quantification

The proliferation of primary cultured microglia was assayed by the EdU labeling method as described previously [27, 28], with some modifications. In short, EdU was added to the culture medium at a concentration of 10 µmol/L. After labeling, microglia (1×10^3) were fixed and permeabilized followed by washing two to three times with PBS. The EdU-labeled microglia were assessed by immunocytochemistry with a Click-iTTM EdU Alexa FluorTM 594 imaging kit (Thermo Fisher Scientific). Signals were visualized with a confocal laser scanning microscope through a 20× objective. Experiments were performed in triplicate. In each sample, we randomly chose 8 fields for counting the number of EdU and DAPI double-labeled cells.

Transwell Migration/co-culture Assay

Cell migration was analyzed using Costar Transwell plates (5 µm pore size, 3421, Corning, Bedford, USA), as described previously [29, 30], with some modifications. In brief, primary microglia at a density of 1×10^3 in 200 µL of serum-free medium were placed in the upper chamber, and complete medium (500 µL) with 10% fetal bovine serum was added to the lower chamber. After incubation for 6 or 24 h in the presence or absence of the migration-inducing factors (100 µmol/L BzATP, 1 mmol/L CTP or Müller cells), microglia attached to the other side of the insert were fixed in 90% ethanol and stained with 0.1% crystal violet. Migrated cells were observed and counted under a Nikon Eclipse Ti inverted microscope (Nikon, Tokyo, Japan) through a 20× objective. In each well, at least 5 different fields were chosen at random, and the number of migrated cells was averaged from these fields. Experiments were performed in triplicate.

Interaction between Müller cells and microglia was analyzed using Costar Transwell plates (0.4 µm pore size;

3470, Corning), as described previously [31]. Müller cells were seeded onto the upper insert of a 24-well Transwell at a density of 5×10^5 /mL, and microglia were placed on the lower chamber. After co-culture, changes of microglia were measured by Western blot or EdU labeling. Experiments were performed in triplicate.

Western Blot Analysis

Western blotting was performed according to previous studies [20, 24]. Total proteins of retinas or cultured cells were extracted using the lysis buffer, and protein concentrations were determined using a standard bicinchoninic acid assay kit (Pierce Biotechnology, Rockford, USA). After separation by SDS-PAGE gel, transfer to a PVDF membrane, and blocking in non-fat powdered milk, the samples were incubated with the primary antibodies polyclonal goat-anti-Iba1 (ab5076; 1:1000 dilution; Abcam), monoclonal rabbit-anti-GS (MAB302; 1:1000; Millipore), monoclonal mice-anti-Cx43 (SAB4200819; 1:1000; Sigma-Aldrich), monoclonal mice-anti-p-ERK1/2 (M9692; 1:1000; Sigma-Aldrich), or polyclonal rabbit-anti-ERK1/2 (M5670; 1:1000; Sigma-Aldrich). The secondary antibodies used in this study were horseradish peroxidase-conjugated anti-goat, anti-rabbit, anti-mouse IgG (705-165-003, 711-165-152, and 715-165-151; 1:800; Jackson ImmunoResearch Laboratories). The blots were visualized with the ECL enhanced chemifluorescent reagent (Thermo Scientific) and an Odyssey near-infrared imaging scanner (FluorChem E System, Protein Simple, San Francisco, USA). Experiments were performed in triplicate.

Flow Cytometry Assay

The retinas from anesthetized MacGreen mice were digested in an enzyme buffer containing 1 mg/mL collagenase D (#11088858001, Roche, Switzerland) and 30 U/mL DNase I (#18047019, Thermo Fisher Scientific, USA) in Hank's balanced salt solution for 60 min at 37°C, during which gentle shaking was applied every 15 min. Retinal cells were carefully dissociated using a fire-polished pipette and filtered through a 70-µm cell strainer (#352350, Corning). Retinal microglia carrying the GFP signal were detected by fluorescence-activated cell sorting (BD FACSCalibur, BD, USA) and analyzed by FlowJo V10.0 software (TreeStar, Ashland, USA).

Statistical Analysis

Data are expressed as the mean ± SEM and analyzed using SPSS 19.0 (SPSS Inc.©, Armonk, New York, USA). One-way ANOVA with Bonferroni's *post hoc* test (multiple

comparisons) was used. $P < 0.05$ was considered statistically significant.

Results

P2X7R Mediates Microglial Proliferation in COH Retina

The mouse COH model was successfully produced, and the changes of IOP were similar to our previous report [22] (Fig. S1). In this model, we quantified the dynamic changes in the number of microglia in a set of vertical retinal sections. In control mice, microglia were mainly distributed in the IPL and OPL (Fig. 1A, a1). IOP elevation induced an increase in the number of microglia, and their migration to the GCL and the nerve fiber layer (NFL) (Fig. 1A, a9–a16). As shown in Fig. 1B, the number of microglia started to increase at G1d ($n = 6$, $P < 0.01$), peaked at G2w ($n = 6$, $P < 0.001$), and then declined but remaining higher than the controls through G6w ($n = 6$, $P < 0.05$). We also counted the number of microglia in each of the NFL + GCL, IPL, and OPL. The patterns of increase in the numbers of microglia in these areas were similar to that of the total number in COH retinas (Fig. 1C–E). In addition, we explored changes in the number of microglia in COH retinas from MacGreen mice, in which the green fluorescent protein (GFP) was highly expressed in retinal microglia, using flow cytometry. The results showed that IOP elevation indeed increased the percentage of GFP-positive microglia in the total number of retinal cells (Fig. S2). Mouse retinal microglia are known to express two purinergic receptor subtypes, the P2X7 receptor (P2X7R) and P2X4R, but only P2X7R has been reported to be involved in RGC injury [32, 33]. Overexpression of P2X7R in rat primary hippocampal cultures drives microglial activation and proliferation [34]. We examined whether P2X7R is involved in the IOP-induced change of microglia. Intraperitoneal injection of brilliant blue G (BBG), an antagonist of P2X7R, significantly, but not completely, inhibited the IOP elevation-induced increase in the total number of microglia (Fig. 1A, a17–a24, and Fig. 1B) ($n = 6$, $P < 0.05$). It is interesting that the increase in the number of microglia in the IPL and OPL was completely blocked ($n = 6$, $P < 0.05$) (Fig. 1D, E), but only partially reduced in the NFL + GCL ($n = 6$, $P < 0.05$) (Fig. 1C). Since microglia of the mouse retina also express P2X4R, we tested whether this receptor is also involved in microglial proliferation. However, intravitreal injection of 5-BDBD, a P2X4R inhibitor, did not affect the increase in the number of microglia in COH retinas (Fig. 1F) ($n = 6$, $P > 0.05$). Furthermore, in COH retinas from P2X7R-knockout ($P2X7R^{-/-}$) mice, the increase in the number of

microglia was significantly suppressed (Fig. 1F) ($n = 6$, $P < 0.001$). These results suggest that P2X7R indeed mediates the proliferation of microglia in the COH retina, but is unlikely to be involved in their migration.

We then measured changes in the protein levels of Iba1 and P2X7R in COH retinas at G2w, and found that the levels of Iba1 were significantly increased in COH retinas, and this was inhibited by BBG (10 $\mu\text{mol/L}$) [$n = 6$, $P < 0.001$ vs COH; $P < 0.05$ vs control (Ctr)], but not by 5-BDBD (10 $\mu\text{mol/L}$) ($n = 6$, $P > 0.05$ vs COH; $P < 0.001$ vs Ctr) (Fig. 2A, B). P2X7R knockout did not affect the levels of Iba1. In COH retinas from $P2X7R^{-/-}$ mice, the levels of Iba1 were slightly increased, but were significantly lower than those from COH retinas ($n = 6$, $P < 0.001$ vs COH; $P < 0.05$ vs $P2X7R^{-/-}$ Ctr) (Fig. 2A, B). The changes of P2X7R protein levels were similar to those of Iba1 except in $P2X7R^{-/-}$ mice (Fig. 2A, C). We have shown that P2X4R expression in COH retinas does not significantly change, while in $P2X7R$ -knockout mice P2X4R expression increases in a compensatory manner in COH retinas [35]. These findings further demonstrate that microglial proliferation in the retinas of COH mice is mediated by P2X7R, but not P2X4R.

We then explored how ATP/P2X7R mediates the microglial proliferation in the COH retina. Previous studies have shown that MEK/extracellular signal-regulated protein kinase (ERK) signaling is involved in microglial proliferation [36, 37]. We tested the possible involvement of this signaling in retinal microglia proliferation in primary purified cultured cells by EdU labeling, which showed that microglia treated with the P2X7R agonist BzATP (100 $\mu\text{mol/L}$) showed increased proliferation ($n = 3$, $P < 0.001$ vs Ctr) (Fig. 3Aa6, B), which was reduced by co-application of BBG (10 $\mu\text{mol/L}$) ($n = 3$, $P < 0.01$ vs Ctr and $P < 0.001$ vs BzATP alone) (Fig. 3Aa7, B). BzATP treatment also induced morphological changes of microglia (Fig. 3Aa10, B), indicative of microglial activation. Co-application of the MEK/ERK antagonist PD98059 (10 $\mu\text{mol/L}$) significantly inhibited the BzATP-induced microglial proliferation ($n = 3$, $P < 0.001$ vs Ctr and $P < 0.001$ vs BzATP alone) (Fig. 3Aa8, B). It is noted that stimulation of P2X7R also induced microglial activation, which resulted in morphological changes of microglia (Fig. 3Aa10), similar to our previous study [35]. Inhibition of P2X7R by BBG reversed these changes (Fig. 3Aa11). Although inhibition of ERK1/2 activation blocked microglial proliferation, their morphological changes were not completely blocked by the ERK1/2 inhibitor PD98059 (Fig. 3Aa12). We then examined the changes in protein levels of p-ERK1/2 and ERK1/2 in microglia by Western blotting. Figure 3C and D show that BzATP treatment increased the p-ERK/ERK ratio ($n = 3$, $P < 0.001$ vs Ctr), which was reversed by co-application of BBG ($n = 3$, $P < 0.001$ vs

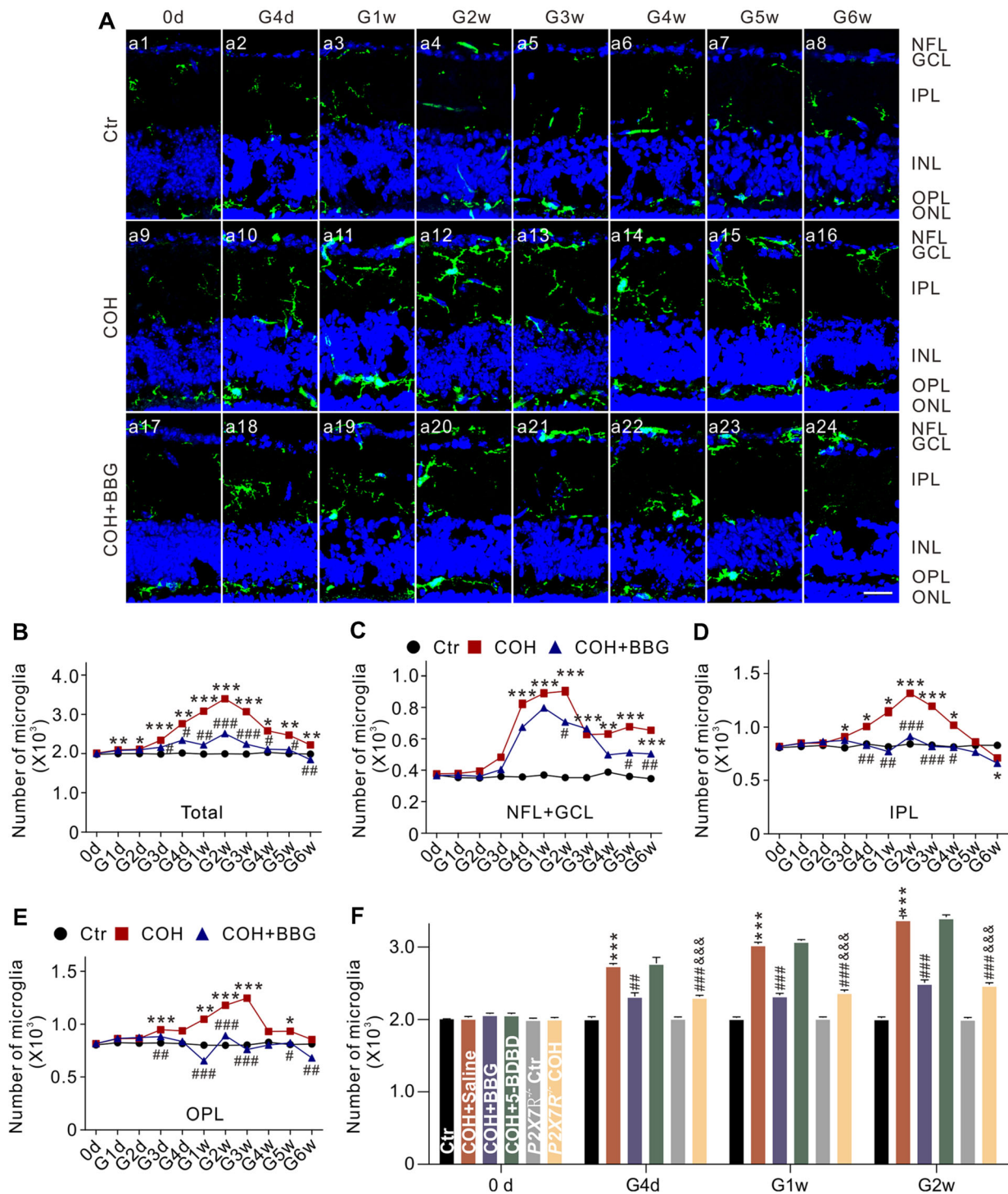


Fig. 1 P2X7R mediates the increased number of microglia in COH retinas. **A** Confocal laser microphotographs of vertical retinal sections stained with the antibody against Iba1 (green), showing the changes in the number of microglia in control (Ctr) (a1–a8), and in COH retinas at different post-operational times (G0d–G6w) without (COH group) (a9–a16) or with BBG injection (COH + BBG group) (a17–a24) (scale bar, 10 μ m). **B** Average numbers of microglia under different conditions as shown in **A**. **C–E** Average numbers of microglia in the NFL + GCL (**C**), IPL (**D**), and OPL (**E**) groups under different conditions as shown in **A**. $n = 6–10$. * $P < 0.05$, ** $P < 0.01$, and

*** $P < 0.001$ vs Ctr; # $P < 0.05$, ## $P < 0.01$ and ### $P < 0.001$ vs COH group. **F** Average numbers of microglia in COH retinas at different post-operational times (G0d–G2w) with intravitreal injections of saline (COH + Saline), BBG (COH + BBG), 5-BDBD (COH + 5-BDBD), or P2X7R knockout (P2X7R^{-/-} COH). $n = 6$. *** $P < 0.001$ vs Ctr, ## $P < 0.01$ and ### $P < 0.001$ vs COH + Saline, &&& $P < 0.001$ vs P2X7R^{-/-} Ctr. COH, chronic ocular hypertension; NFL, nerve fiber layer; GCL, ganglion cell layer; IPL, inner plexiform layer; OPL, outer plexiform layer; ONL, outer nuclear layer.

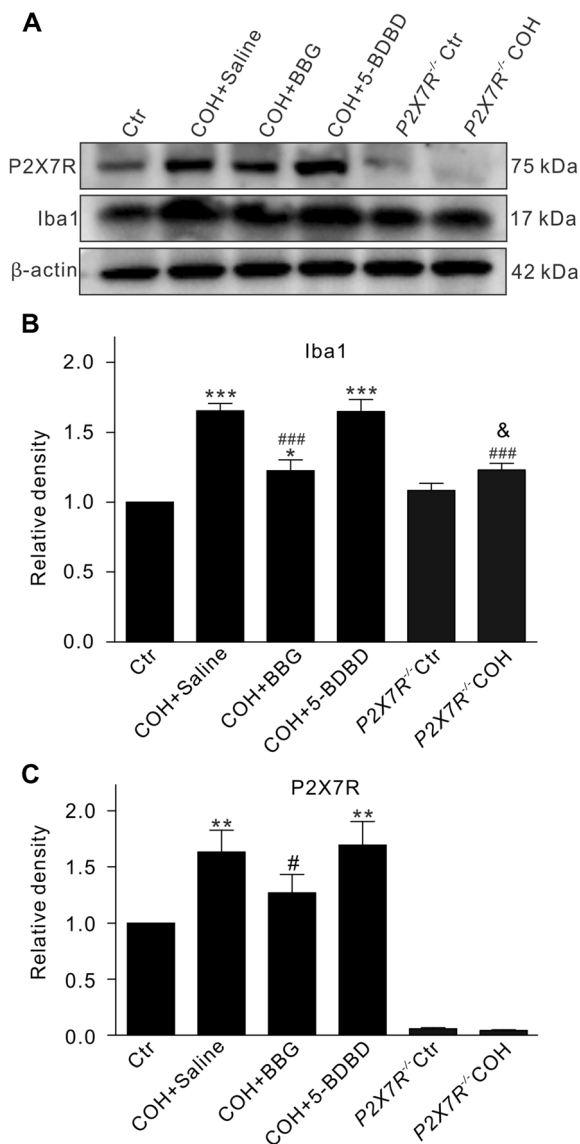


Fig. 2 Changes in protein levels of Iba1 and P2X7R in retinas of COH mice. **A** Representative immunoblots showing the changes in Iba1 and P2X7R levels in retinas from control (Ctr) mice or COH mice with intravitreal injection of saline (COH + Saline), BBG (COH + BBG), 5-BDBD (COH + 5-BDBD), or P2X7R knockout (P2X7R^{-/-} COH). **B, C** Average densitometric quantification of immunoreactive bands of Iba1 (**B**) and P2X7R (**C**) under different conditions as shown in **A**. All data are normalized to their corresponding β -actin and then to Ctr. $n = 6$ per group. * $P < 0.05$, ** $P < 0.01$, and *** $P < 0.001$ vs Ctr; # $P < 0.05$ and ### $P < 0.001$ vs COH + Saline; & $P < 0.05$ vs P2X7R^{-/-} Ctr.

BzATP alone). The MEK/ERK antagonist PD98059 also reduced the BzATP-induced increase of the p-ERK/ERK ratio ($n = 3$, $P < 0.001$ vs Ctr; $P < 0.001$ vs BzATP alone). In contrast, CTP (1 mmol/L), a P2X4R agonist, treatment of primary cultured microglia had no effect on their proliferation (Fig. S3). Moreover, intravitreal injection of BzATP (100 μ mol/L, 2 μ L) induced an increase in the number of Iba1-positive microglia ($n = 6$ – 9 , $P < 0.05$ vs

Ctr), while injection of CTP (1 mmol/L, 2 μ L) failed to change the number of microglia ($n = 6$, $P > 0.05$ vs Ctr) (Fig. S4). These results indicate that the ATP/P2X7R/MEK/ERK signaling pathway mediates microglial proliferation in the COH retina.

Activated Müller Cells Mediate Microglial Proliferation by Releasing ATP in COH Retina

Our previous studies have shown that Müller cell activation is mediated by group I metabotropic glutamate receptor I (mGluR I) in the COH retina [20, 24]. Activated Müller cells release ATP through connexin43 (Cx43) hemichannels [18, 21, 38, 39]. Indeed, treatment of primary cultured Müller cells with DHPG (100 μ mol/L), an mGluR I agonist, intravitreal injection of DHPG (100 μ mol/L, 2 μ L), or IOP elevation in COH retina increased the expression of Cx43, which was mainly localized at the endfeet of Müller cells, suggesting that there may be a higher ATP concentration in the NFL + GCL (Fig. S5). Although mGluR I is also expressed in microglia [40, 41], their treatment with DHPG (100 μ mol/L) for different times did not affect the number of EdU-labeled microglia ($n = 3$, $P > 0.05$ vs Ctr), suggesting that activation of mGluR I has no direct effect on microglial proliferation (Fig. S6). However, intravitreal injection of DHPG (100 μ mol/L, 2 μ L) to activate Müller cells [19, 20] significantly increased the total number of microglia at 4 days to 2 weeks after the injection ($n = 6$, P all < 0.001 vs saline-injected group), indicating that activated Müller cells induce microglial proliferation (Fig. 4). Co-injection of the Cx43 blocker Gap26 (200 μ mol/L, 2 μ L) or BBG (10 μ mol/L, 2 μ L) reversed the DHPG-induced changes in the total number of microglia ($n = 6$, $P < 0.01$ or 0.001 vs DHPG alone), while co-injection of 5-BDBD (10 μ mol/L, 2 μ L) failed to do so (Fig. 4). These results indicate that activated Müller cells may induce microglial proliferation by releasing ATP, which then acts on P2X7Rs expressed on microglia.

A transwell co-culture system was used to further confirm the Müller cell-mediated proliferation of microglia. As shown in Fig. 5A and C, co-culture of purified primary retinal microglia with normal Müller cells resulted in an increase in microglial proliferation ($n = 3$, $P < 0.001$ vs Empty transwell), which was further boosted when co-cultured with Müller cells activated by 100 μ mol/L DHPG ($n = 3$, $P < 0.001$ vs Empty transwell, and $P < 0.001$ vs normal Müller cells). When activated Müller cells were pre-treated with Gap26 (200 μ mol/L) and then co-cultured with microglia, activated Müller cell-induced microglial proliferation was blocked ($n = 3$, $P < 0.001$ vs activated Müller cells alone). Similarly, when microglia were pre-treated with BBG (10 μ mol/L) or PD98059 (10 μ mol/L) and then co-cultured with activated Müller cells, the microglial proliferation was

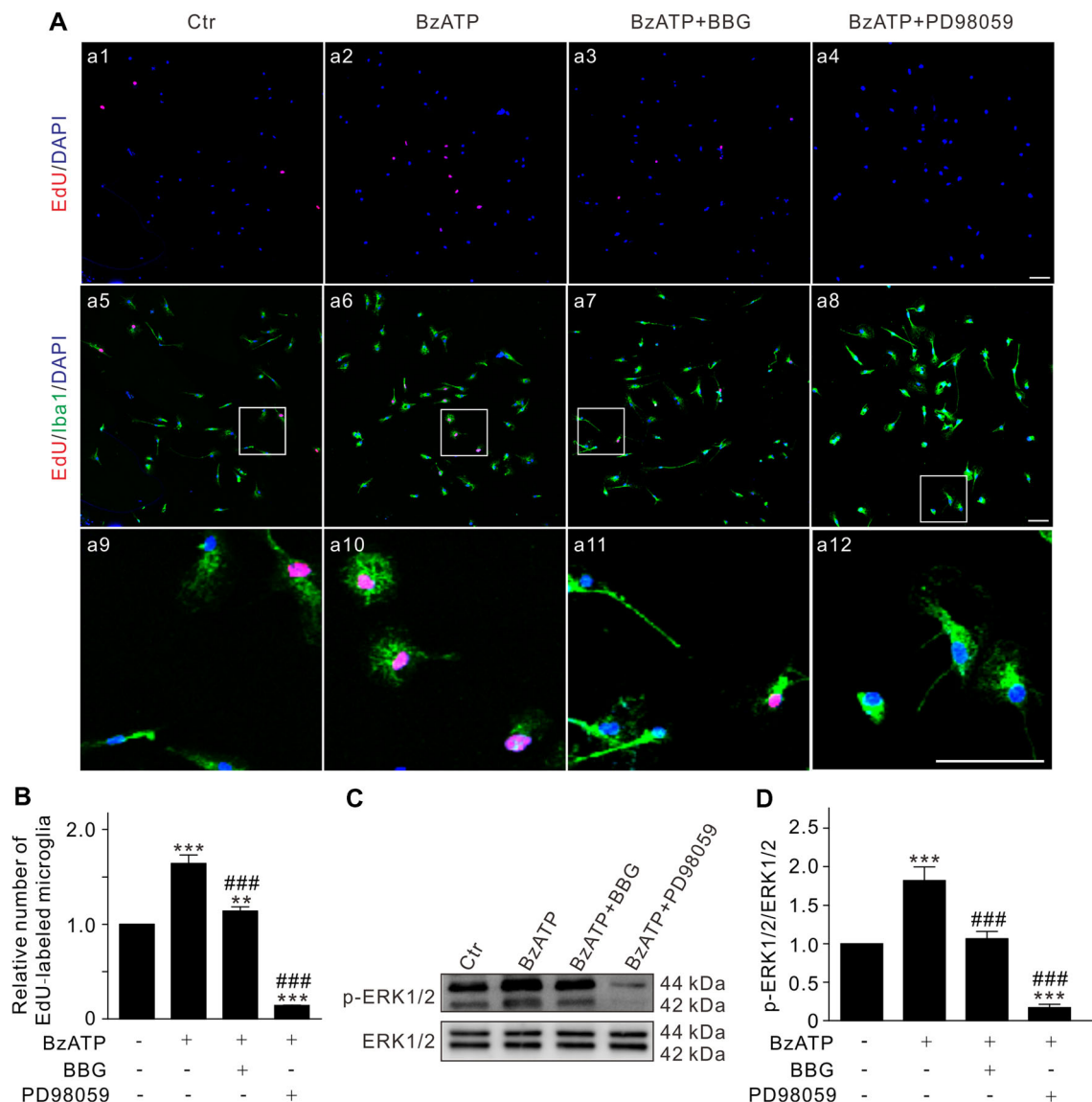


Fig. 3 Activation of P2X7R induces retinal microglia proliferation. **A** Representative images showing double immunofluorescent staining of Iba1 (green) and EdU (red) in primary cultured microglia in controls (Ctr) (a5), and after treatment with BzATP (a6), BzATP + BBG (a7), and BzATP + PD98059 (a8). Nuclei are stained with DAPI. a9–a12 are enlarged images of the white squares in a5–8. Scale bars, 50 μ m. **B** Bar charts summarizing the average relative number of Iba1 and EdU double-labeled microglia under different conditions.

All data are normalized to Ctr. $n = 3$ biological replicates \times 3 technical replicates. **C** Representative immunoblots showing the changes in protein levels of p-ERK1/2 and ERK1/2 in primary cultured microglia in controls (Ctr), and after treatment with BzATP, BzATP + BBG, or BzATP + PD98059. **D** Ratios of p-ERK/ERK under different conditions as shown in **C**. All data are normalized to Ctr. $n = 3$ biological replicates \times 3 technical replicates. *** $P < 0.001$ vs Ctr; ### $P < 0.001$ vs BzATP alone.

reversed ($n = 3$, P all < 0.001 vs activated Müller cells alone), while pre-treatment with 5-BDBD (10 μ mol/L) did not affect microglial proliferation ($n = 3$, $P > 0.05$ vs activated Müller cells alone) (Fig. 5A, C). Changes in the ratio of p-ERK1/2 to ERK1/2, tested by Western blotting, were basically similar to those of microglial proliferation (Fig. 5D, E). These results provide further evidence showing that activated Müller cells induce microglial proliferation through the Cx43/ATP/P2X7R/MEK/ERK pathway in the COH retina. It should be noted that in this study, the *in vitro* experiments were done

in the presence of serum. The higher level of basal p-ERK1/2 may be a result of stimulation from the serum.

P2X4R/P2X7R Mediates Microglial Migration in Retina of COH Mice

The results in Fig. 1 showed that BBG completely blocked the increase in the number of microglia in the IPL and OPL, but partially blocked the increase in the NFL + GCL, suggesting that microglia migrate to this area, so we explored the

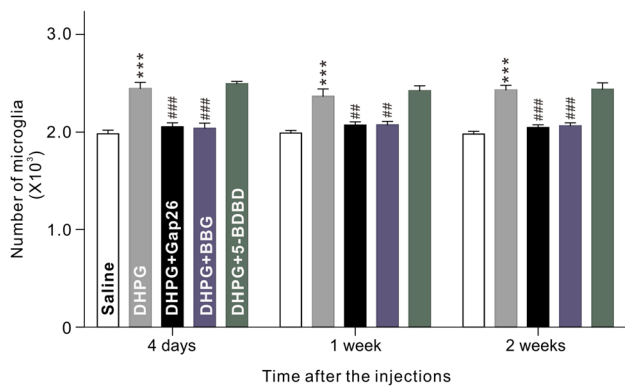


Fig. 4 Activation of Müller cells induces proliferation of microglia through ATP/P2X7R. Total numbers of microglia in vertical retinal sections taken from mice injected intravitreally with saline (control), DHPG, DHPG + Gap26, DHPG + BBG, or DHPG + 5-BDBD at 4 days, 1 week, and 2 weeks after injection. $n = 6$ per group. *** $P < 0.001$ vs Saline, ## $P < 0.01$ and ### $P < 0.001$ vs DHPG alone.

mechanisms of microglial migration by transwell assay. As shown in Fig. 6A and 6B, BzATP (100 $\mu\text{mol/L}$) treatment of primary cultured microglia for 6 h induced a significant increase in cell migration ($n = 3$, $P < 0.001$ vs Ctr), which was partially inhibited by pre-application of BBG (10 $\mu\text{mol/L}$) ($n = 3$, $P < 0.05$ vs BzATP alone; $P < 0.001$ vs Ctr). CTP (1 mmol/L) treatment of primary cultured microglia for 6 h also induced a significant increase of cell migration ($n = 3$, $P < 0.001$ vs Ctr), which was completely blocked by 5-BDBD (10 $\mu\text{mol/L}$) ($n = 3$, $P < 0.001$ vs CTP alone; $P > 0.05$ vs Ctr) (Fig. 6C, D). Furthermore, in COH retinas, the number of microglia in the NFL + GCL were counted after different treatments (Fig. 6E). The changes in microglial number were similar to that shown in Fig. 1 when BBG (10 $\mu\text{mol/L}$, 2 μL) was injected. Similarly, in COH retinas from $P2X7R^{-/-}$ mice, the number of microglia was also significantly reduced ($n = 6$, $P < 0.001$ vs COH saline). Interestingly, intravitreal injection of 5-BDBD (10 $\mu\text{mol/L}$, 2 μL) significantly reduced the number of microglia in the NFL + GCL ($n = 6$, $P < 0.01$ vs COH saline) (Fig. 6F). These results indicate that the increase of microglial in the NFL + GCL may be due to both cell proliferation and migration in COH mice, and this is mediated by both P2X7R and P2X4R, the latter being predominant.

Activated Müller Cells Mediate Microglial Migration in COH Retina

We then tested whether activated Müller cells mediate microglial migration by counting the number of microglia in the NFL + GCL. Intravitreal injection of DHPG (100 $\mu\text{mol/L}$, 2 μL) in normal mice significantly increased the number of microglia in the NFL + GCL [$n = 6$, $P < 0.001$ vs Ctr (saline)], and this was completely blocked by co-application of Gap26 (200 $\mu\text{mol/L}$, 2 μL) ($n = 6$, $P < 0.01$ vs DHPG alone) or BBG (10 $\mu\text{mol/L}$, 2 μL) ($n = 6$, $P <$

0.001 vs DHPG group alone), and partially blocked by co-application of 5-BDBD (10 $\mu\text{mol/L}$, 2 μL) ($n = 6$, $P < 0.001$ vs DHPG alone) (Fig. 7A, B). These results indicate that ATP, released from activated Müller cells through the Cx43 hemichannel, induces the accumulation of microglia (including proliferation and migration) in the NFL + GCL through activating P2X7R/P2X4R.

The transwell chemotaxis assay was applied to further confirm the effect of activated Müller cells on microglial migration. As shown in Fig. 8A–C, no change of microglial migration was found when co-cultured with normal Müller cells for 24 h ($n = 3$, $P > 0.05$ vs Empty group), while a significant increase in microglial migration occurred when co-cultured with Müller cells activated by 100 $\mu\text{mol/L}$ DHPG ($n = 3$, $P < 0.001$ vs Empty group). Activated Müller cells were pre-treated with Gap26 (200 $\mu\text{mol/L}$) for 2 h (Activated Müller cells + Gap26 group) or microglia were pre-treated with 5-BDBD (10 $\mu\text{mol/L}$) for 2 h (Activated Müller cells + 5-BDBD group), and then these cells were co-cultured. The increased microglia migration was no longer found ($n = 3$, P all < 0.001 vs Activated Müller cell group) (Fig. 8A, C). However, BBG (10 $\mu\text{mol/L}$) pre-treatment of microglia did not block the effect of activated Müller cells ($n = 3$, $P > 0.05$ vs Activated Müller cells group; $P < 0.001$ vs Empty group) (Fig. 8A, C).

Discussion

ATP/P2X7R/MEK/ERK Pathway Mediates Microglial Proliferation in COH Retina

Neuroinflammation is one of the important factors in the pathogenesis of glaucoma [42], and is mainly mediated by activated retinal glial cells. Activated retinal glial cells promote widespread inflammatory responses by releasing excessive pro-inflammatory factors, such as TNF- α , ILs, and NO, thus aggravating a cytotoxic reaction and RGC injury [1–5, 43]. As the resident immune cells in the retina, microglia are activated in the glaucomatous retina, and these activated glial cells are the main source of inflammatory factors [9–11, 13, 44]. Microglial proliferation has also been reported in retinas with experimental glaucoma [28, 45]. Similarly, in this study, we showed that IOP elevation in COH mice induced an increase in the number of EdU-labeled microglia, suggestive of cell proliferation. This may be one of the important factors for the retinal inflammatory response in glaucoma. We provide evidence *in vivo* and *in vitro* demonstrating that it was ATP, released by the activated Müller cells, that induced microglial proliferation through activating P2X7Rs. First, intraperitoneal or intravitreal injection of the P2X7R blocker BBG significantly inhibited the proliferation of microglia induced by IOP elevation.

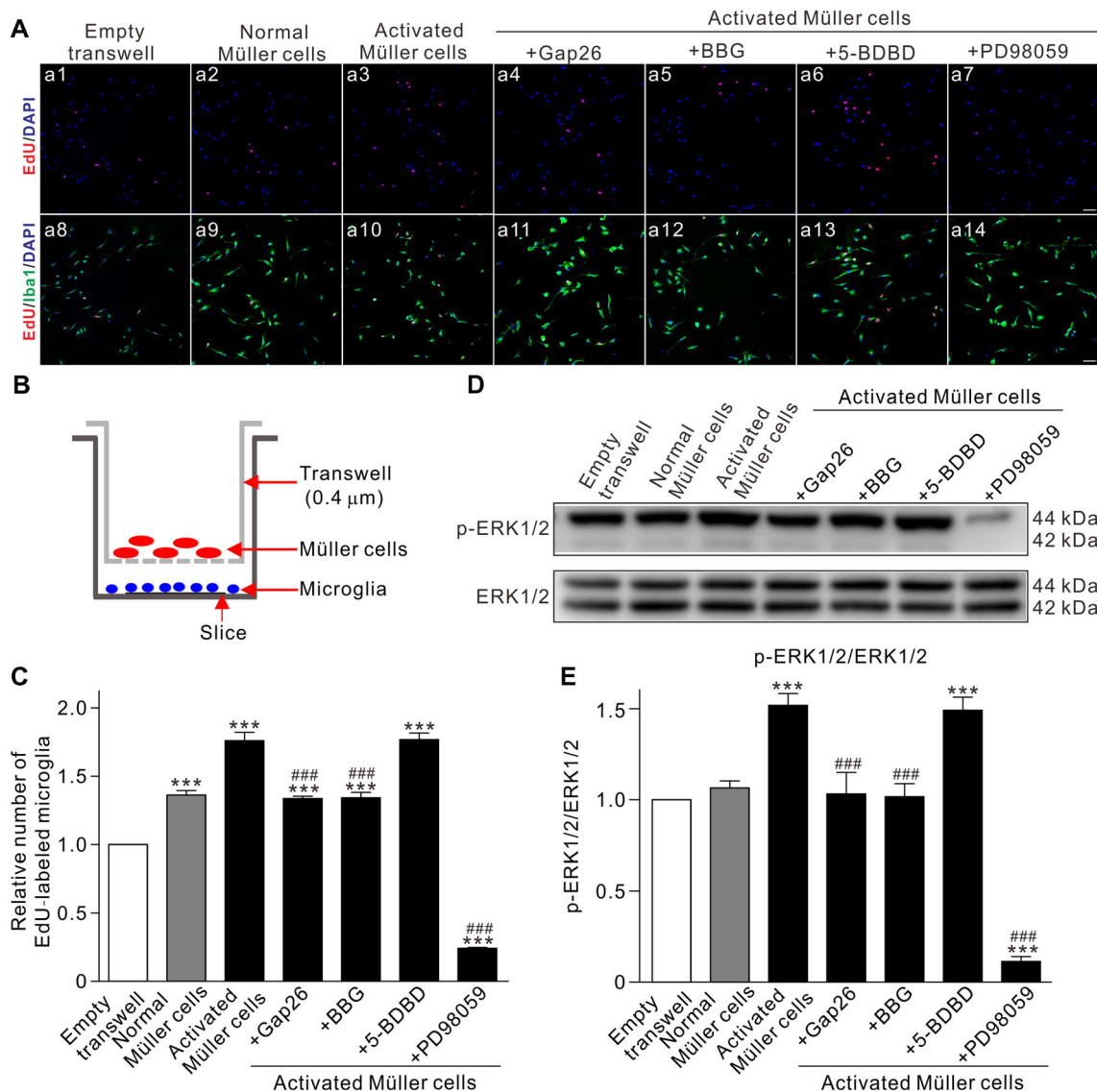


Fig. 5 Activated Müller cells induce proliferation in primary cultured microglia *via* the ATP/P2X7R/MEK/ERK pathway. **A** Representative images showing double immunofluorescent staining of Iba1 (green) and EdU (red) in primary cultured microglia in the transwell co-culture system. Microglia are co-cultured with Empty Transwell (control) (a8), normal Müller cells (a9), activated Müller cells without (a10) or with Gap26 pre-treatment (a11), BBG (a12), 5-BDBD (a13), and PD98059 (a14). Nuclei are stained with DAPI. Scale bars, 50 μm. **B** Diagrammatic sketch showing the transwell co-culture system.

C Relative average numbers of Iba1 and Edu double-labeled microglia under different conditions as shown in **A**. **D** Representative immunoblots showing the changes in protein levels of p-ERK1/2 and ERK1/2 in primary cultured microglia under different treatments similar to **A**. **E** Average ratios of p-ERK/ERK. All data are normalized to the Empty Transwell group. *n* = 3 biological replicates × 3 technical replicates. ****P* < 0.001 vs Empty Transwell, ###*P* < 0.001 vs Activated Müller cells group.

Knockout of P2X7R had a similar effect. Although P2X4Rs were also expressed in microglia, this receptor was unlikely to be involved in microglial proliferation because the P2X4R blocker 5-BDBD had almost no effect on the increased number of microglia in COH retinas (Fig. 1). It should be noted that either BBG or knockout of P2X7R did not completely inhibit the proliferation of microglia, suggesting that other factors may be involved in the proliferation in addition to the ATP/P2X7R signaling pathway. On the other

hand, previous studies have shown that under some pathological conditions or eliminating retinal resident microglia, peripheral monocytes/macrophages or residual microglia in the optic nerve can infiltrate the retina [46, 47]. Therefore, the increased number of Iba1⁺ cells in the NFL/GCL in the COH retina may include infiltrated peripheral monocytes/macrophages. These issues will be explored in the future. Second, in the normal retina, intravitreal injection of the P2X7R agonist BzATP, but not the P2X4R agonist CTP,

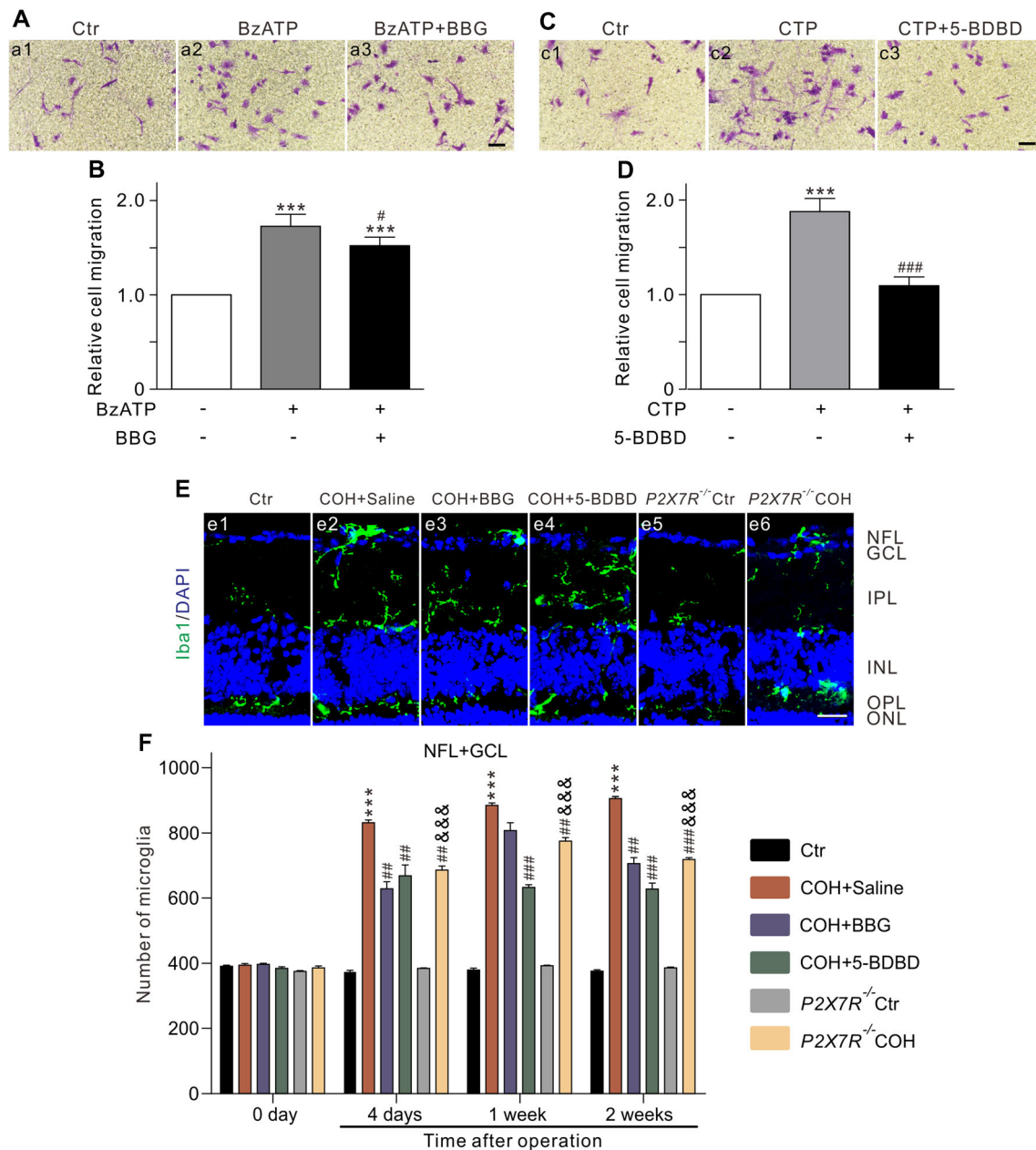


Fig. 6 P2X4R/P2X7R mediates microglial migration in the COH retina. **A** Representative images of transwell migration assays showing changes in microglial migration in control (Ctr), BzATP, and BzATP+BBG treatment groups (scale bar, 20 μ m). **B** Average relative migration of microglia under different conditions as shown in **A**. **C** Representative images showing changes in microglial migration in control (Ctr), CTP, and CTP + 5-BDBD treatment groups, respectively (scale bar, 20 μ m). **D** Average relative migration of microglia under different conditions as shown in **C**. All data are normalized to the Ctr group. $n = 3$ biological replicates \times 3 technical replicates. *** $P < 0.001$ vs Ctr; # $P < 0.05$ vs CTP. **E** Confocal laser

microphotographs of vertical retinal sections stained with the antibody against Iba1 (green), showing changes in the numbers of microglia in control (Ctr) (e1), and COH retinas after intravitreal injection of saline (COH saline) (e2) or BBG (COH + BBG) (e3), or 5-BDBD (COH + 5-BDBD) (e4), and in *P2X7R*^{-/-} mice without COH (*P2X7R*^{-/-} Ctr) (e5) or with COH (*P2X7R*^{-/-} COH) (e6) (scale bar, 10 μ m). **F** Average numbers of microglia in the NFL + GCL under different conditions as shown in **E**. $n = 6$ per group. *** $P < 0.001$ vs Ctr; ## $P < 0.01$ and ### $P < 0.001$ vs COH + Saline; &&& $P < 0.001$ vs *P2X7R*^{-/-} Ctr.

mimicked the effect of COH on microglial proliferation (Fig. S3). Indeed, it has been reported that overexpression of P2X7R in rat primary hippocampal cultures is enough to induce cell proliferation [34]. Elevated expression of P2X7R

was found in our COH retinas. Furthermore, intravitreal injection of DHPG to activate Müller cells increased the proliferation of microglia, which was inhibited by Gap26 and BBG, but not by 5-BDBD (Fig. 4). This was further

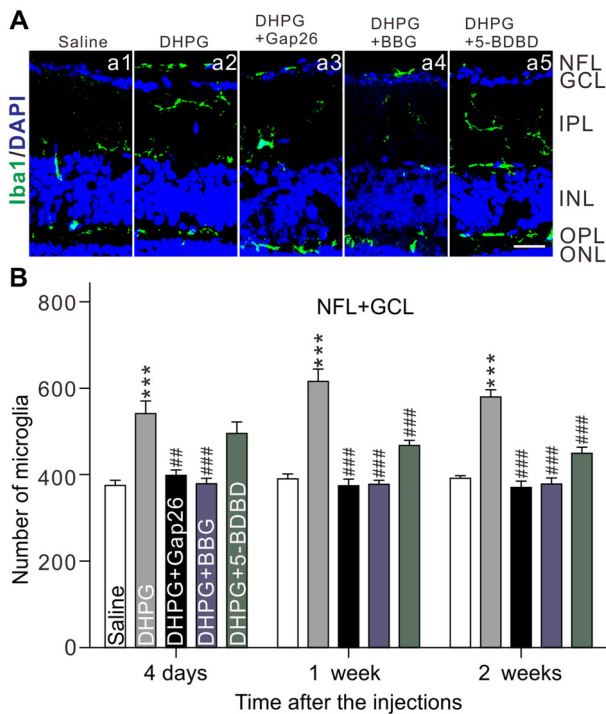


Fig. 7 Activation of Müller cells induces microglia migration in retinas through P2X4R/P2X7R. **A** Representative microphotographs of vertical retinal sections stained with the antibody against Iba1 (green), showing changes in the numbers of microglia in retinas after intravitreal injection of saline (control) (a1), DHPG (a2), DHPG + Gap26 (a3), DHPG + BBG (a4), or DHPG + 5-BDBD (a5) (scale bar, 10 μ m). **B** Average numbers of microglia in the NFL + GCL under different conditions as shown in **A**. $n = 6$. *** $P < 0.001$ vs Saline group, ### $P < 0.001$ vs DHPG alone.

confirmed in the transwell co-culture system *in vitro* (Fig. 5). Moreover, in line with previous reports [36, 37], our results showed that the MEK/ERK signaling pathway is involved in the proliferation of microglia in COH retinas. It is interesting that the MEK/ERK signaling blocker PD98059 not only completely inhibited the P2X7R-mediated microglial proliferation, but also reduced the number of microglia to levels lower than that of controls. We speculate that basal levels of MEK/ERK in microglia play an important role in maintaining normal cell proliferation. MEK/ERK activation may be mediated by a Ca^{2+} -dependent pathway. In a recent study, we have shown that activation of P2X7R increases intracellular Ca^{2+} levels [35]. Evidence also shows that stimulation of P2X7R elevates the levels of intracellular Ca^{2+} , thus activating the MEK/ERK signaling pathway [48, 49].

P2X4R/P2X7R Mediates Microglial Migration in COH Retina

Cell migration is a complex process that plays an important role in a variety of physiological and pathological processes [50]. In this study, we showed that microglia

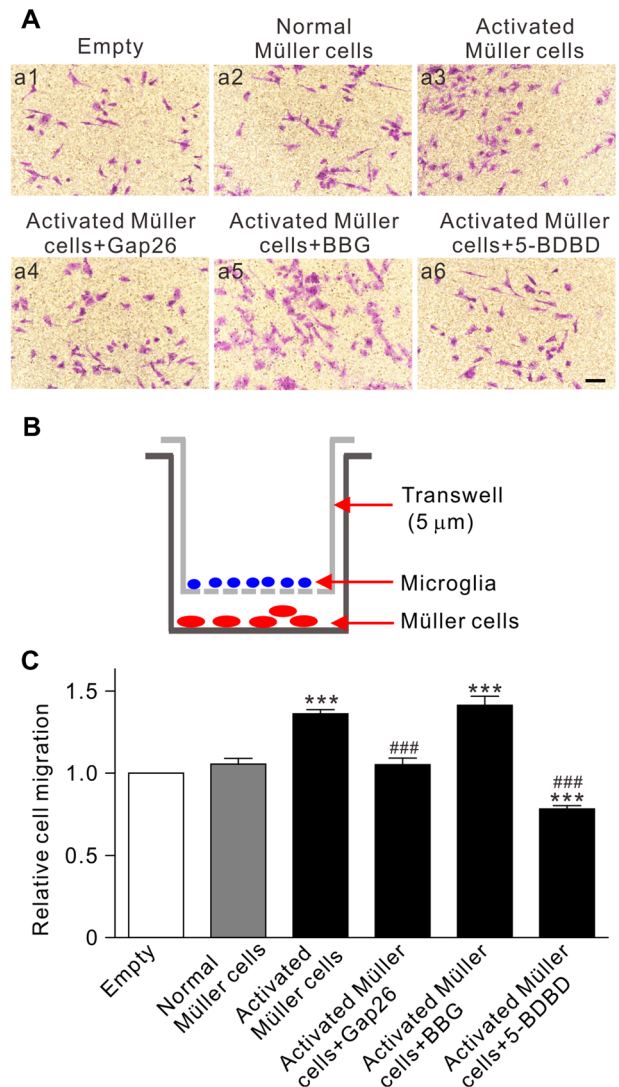


Fig. 8 Activated Müller cells mediate the migration of primary cultured microglia. **A** Representative images showing changes in the numbers of primary cultured microglia migrating in the transwell co-culture system. Microglia are co-cultured with Empty (control) (a1), normal Müller cells (a2), activated Müller cells without (a3) or with pre-treatment of Gap26 (a4), BBG (a5), and 5-BDBD (a6) (scale bar, 20 μ m). **B** Diagrammatic sketch showing the transwell migration assay system. **C** Average relative migration of microglia under different conditions as shown in **A**. All data are normalized to the Empty group. $n = 3$ biological replicates \times 3 technical replicates. *** $P < 0.001$ vs Empty group, ### $P < 0.001$ vs Activated Müller cell group.

migrated to the NFL + GCL in the COH retina, in which glaucoma-induced RGCs occurred, similar to other reports [12]. The migrated and proliferated microglia in the NFL + GCL, together with microglial activation, may enhance phagocytosis, and they may secrete cytokines, chemokines, and neurotoxins, thus contributing to RGC damage. Our present results showed that both P2X4R and P2X7R participated in the microglial migration to the NFL +

GCL in experimental glaucoma, P2X4R dominating, as evidenced by *in vivo* and *in vitro* experiments. First, in the COH retinas, BBG or P2X7R knockout completely blocked the increased number of microglia in the IPL and OPL, but partially in the NFL+GCL (Figs 1 and 6), suggesting that the increased number of microglia in the NFL + GCL may be due to both the migration and proliferation of cells. Intravitreal injection of 5-BDBD did not inhibit the total number of microglia (Fig. 1), but partially reduced the number in the NFL + GCL (Fig. 6). Considering the fact that P2X4R does not participate in the proliferation of microglia, it is likely that P2X4R mediated the migration of microglia in the COH retina. Second, the transwell chemotaxis assay showed that CTP treatment significantly increased the migration of primary cultured microglia, and the effects were fully reversed by 5-BDBD. Interestingly, the P2X7R agonist BzATP also induced microglial migration, but was only slightly suppressed by BBG (Fig. 6). This result suggests that P2X7R is also involved in microglia migration to a certain extent. Another possibility is that BzATP may have a weak effect on P2X4Rs [51, 52]. Nevertheless, the P2X4R is the dominant factor that mediates the migration of microglia. Similarly, P2X4R-mediated chemotaxis has been reported in primary cultured microglia from the rat cerebral cortex [53]. Finally, activated Müller cells mediated microglial migration through releasing ATP. We showed that the expression of Cx43 was increased at the endfeet of activated Müller cells, and this may lead to an ATP concentration gradient to induce microglial migration to the NFL + GCL (Fig. S4). We did find that intravitreal injection of DHPG induced an increase in the number of microglia in the NFL + GCL that was completely inhibited by Gap26 and partially inhibited by 5-BDBD (Fig. 7). In the transwell chemotaxis assay, activated Müller cells significantly increased microglial migration, which was completely inhibited by Gap26 and 5-BDBD. Indeed, in the retina Cx43 is mainly expressed in glial cells; it consists of both connexon and hemichannel. Under pathological conditions, including glaucoma and diabetic retinopathy, up-regulated Cx43 expression and hemichannel opening have been implicated in retinal neuronal death through activating glial cells, dysfunction of vascular integrity, and retinal edema [54–57]. A question is how Müller cells sense mechanical information in the COH retina. In the retina, Müller cells express mechanosensitive ion channels, such as transient vanilloid isoform 4 and pannexin-1. We speculate that IOP elevation can activate these channels, thus mediating Ca^{2+} influx and inducing ATP release [58–61]. In addition, we have previously demonstrated that in the COH retina, Müller cell gliosis is mediated by activation of mGluR I through the intracellular Ca^{2+} -dependent PLC/IP₃-ryanodine/PKC signaling pathway

[20]. Treatment of purified cultured Müller cells with the mGluR I agonist DHPG induced an increase in ATP release, which is mediated by the mGluR5/Gq/PI-PLC/PKC signaling pathway [21]. Therefore, it is possible that increased PKC activity when mGluR I is activated may regulate Cx43-mediated ATP release. The detailed mechanisms will be explored in future studies. It should be noted that BBG completely blocked the DHPG-induced increase in the number of microglia in the NFL + GCL and 5-BDBD did not significantly reduce the number at 4 days after the DHPG injection (Fig. 7). In addition, in the transwell chemotaxis assay, BBG did not affect the microglial migration induced by pre-activated Müller cells (Fig. 8). We speculate that activated Müller cells induced by DHPG injection mainly induce microglial proliferation at day 4, while at 1 and 2 weeks, both the proliferation and migration of microglia occur and proliferation may be the primary event. It should be noted that in the central nervous system, P2Y12Rs may play important roles in microglial activation and translocation, in addition to P2X7R [53, 62, 63]. Although P2X12R is also expressed in mouse retinal microglia [64], it seems unlikely that this receptor is involved in microglial proliferation and migration because the P2X7R antagonist BBG and the P2X4R antagonist 5-BDBD completely blocked the activated Müller cell-induced microglial proliferation and migration, respectively. In addition, the migration of microglia needs complex interactions between cells and intercellular molecules, such as chemokines [12], which may be elucidated in future studies.

Apoptotic death of RGCs is a fundamental characteristic of glaucoma. We have previously shown RGC apoptotic death in mouse COH experimental glaucoma [22]. It should be noted that ATP released from Müller cells can directly activate P2X7Rs expressed on RGCs and trigger RGC death [21, 65, 66], in addition to the induction of microglial proliferation. Increasing evidence has demonstrated that glial responses (such as activation, proliferation, migration, and interaction among glial cells) in glaucomatous retinas are involved in RGC damage [1–4, 12, 16, 17, 67]. Many studies, including our work [35], have shown that P2X7R plays an important role in microglial activation [68, 69] and the interaction of Müller cells and microglia, which could aggravate the retinal inflammatory response and RGC injury in the COH retina [33, 35]. Therefore, in our opinion, P2X7Rs expressed in both RGCs and microglia play an important role in glaucoma pathology. Inhibition of Müller cell activation by the mGluR5 antagonist MPEP or inhibition of microglial activation/proliferation by the P2X7R antagonist BBG indeed reduces RGC apoptosis in the COH retina [35]. In a rat COH model, there is also evidence showing that the P2X7R antagonist BBG reduces RGC apoptosis by

inhibiting microglial activation [69]. In purified cultured Müller cells, the mGluR I agonist DHPG treatment induces an increase in ATP release, which up-regulates P2X7R expression and induces RGC death [21, 67]. All these studies, together with our present work, suggest that activation of Müller cells in the COH retina induces microglial proliferation and migration through ATP-P2X7/P2X4R signaling pathways, thus contributing to RGC damage.

In summary, our results demonstrate that in the retinas of COH experimental glaucoma mice, activated Müller cells release ATP through Cx43 hemichannel; in turn, ATP acts on P2X7Rs to induce microglial proliferation through the intracellular MEK/ERK signaling pathway. At the same time, ATP acts on P2X4Rs and P2X7Rs (mainly P2X4Rs) to induce microglial migration. Inhibiting the interaction of Müller cells and microglia may be an effective strategy to attenuate microglial proliferation and migration, thus reducing the RGC damage in glaucoma.

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Conflict of interest The authors declare no potential conflict of interest.

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