Supplementary materials

Supplementary materials and methods

Primary culture of rat Müller cells

Rat Müller cells were isolated as described previously with minor modification. In brief, rat eyes were enucleated into Dulbecco’s modified Eagle’s medium (Nissui Pharmaceuticals, Tokyo, Japan) supplemented with 20 mM NaHCO₃, 25 mM D-glucose, 100 U/mL benzylpenicillin, 100 mg/mL streptomycin, and 2.5 μg/mL amphotericin B (growth medium) to be stored overnight at room temperature in the dark. Intact eyes were incubated in growth medium containing 70 U/mL collagenase type II (Worthington Biochemical, Lakewood, NJ) and 0.1% trypsin (Life Technologies, Carlsbad, CA) for 1 h at 37°C. Retinas were separated from the retinal pigment epithelial cells layer, dissected into small pieces, and seeded onto a culture dish (BD Biosciences, Bedford, MA). The cells were cultured in growth medium containing 10% FBS at 37°C in a humidified atmosphere of 5% CO₂/air. The cells were cultured for 5–7 days with several passages to remove retinal aggregates, debris and neural cells.

HPLC analysis

TR-MUL5 cells (1 x 10⁵ cells/cm²) were cultured at 33°C for 2 days on a 24-well tissue culture plate (BD Biosciences, Bedford, MA) and washed with extracellular fluid (ECF) buffer (122 mM NaCl, 25 mM NaHCO₃, 3 mM KCl, 1.4 mM CaCl₂, 1.2 mM MgSO₄, 0.4 mM K₂HPO₄, 10 mM D-glucose and 10 mM HEPES, pH 7.4) at 37°C. The cells were incubated in 200 μL ECF buffer containing 0.1 μCi [³H]adenosine for 5 minutes. The cells were then rinsed 3 times with 37°C ECF buffer, and the release was initiated by applying 200 μL ECF buffer (as an efflux medium). After incubating for 3 hours, the efflux medium was collected, and then the cells were
washed three times with ice-cold ECF buffer. The efflux medium was mixed with 2.4 volumes methanol, and the cells were homogenized in 70% methanol. These samples were centrifuged at 8,000 × g for 5 minutes at 4°C, and the supernatant was collected. Each sample was dried under nitrogen, and dissolved in 55 μL mobile phase (acetonitrile: methanol: water = 7: 5: 88 (v: v: v)). Then, 50 μL was subjected to HPLC analysis. The HPLC system consisted of a pump (EP-300, Eicom, Kyoto, Japan), a column oven (ATC-300, Eicom) and a UV monitor (NOD-10, Eicom). The HPLC analytical column was an Inertsil ODS-2 (5 μm, 4.6 × 150 mm, BL Sciences, Tokyo, Japan) and the mobile phase was pumped through the column at a rate of 0.8 mL/minute at 20°C. Samples of eluate were collected in scintillation counting vials, and the ³H-radioactivity in each fraction (0.3-0.4 mL) was determined in a liquid scintillation counter (LSC-5200, Aloka, Tokyo, Japan).

³H]Adenosine and ³H]hypoxanthine uptake by TR-MUL5 cells and TR-iBRB2 cells

TR-MUL5 cells (1 × 10⁵ cells/cm²) were cultured at 33°C for 48 hours on 24-well tissue culture plates (BD Biosciences), and primary-cultured rat Müller cells (1 × 10⁵ cells/cm²) were cultured at 37°C for 48 hours on 24-well tissue culture plate (BD Biosciences). TR-iBRB2 cells (0.5 × 10⁵ cells/cm²) were cultured at 33°C for 48 hours on rat tail collagen type I-coated 24-well tissue cultured plates (BD Biosciences). Cultured cells were washed with ECF buffer at 37°C, and uptake was initiated by applying 200 μL ECF buffer containing 0.1 μCi [³H]adenosine (13 nM) or 0.1 μCi [³H]hypoxanthine (16 nM) in the presence or absence of inhibitors. Na⁺-free ECF buffers were prepared by equimolar replacement of NaCl and NaHCO₃ with choline chloride and choline bicarbonate. After a predetermined period, uptake was terminated...
by removing the solution, and cells were rinsed three times with ice-cold ECF buffer. The cells were then solubilized in 1 N NaOH and subsequently neutralized with 1 N HCl. Cell-associated radioactivity and protein content were assayed by liquid scintillation counting and detergent compatible protein assay (a DC protein assay kit, Bio-Rad, Hercules, CA) with bovine serum albumin as a standard.

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

Total RNA was prepared from phosphate-buffered saline (PBS)-rinsed TR-MUL5 cells and primary-cultured rat Müller cells using an RNeasy® Mini Kit (Qiagen, Hilden, Germany). Single-strand cDNA was synthesized from 1 µg total RNA by RT using oligo dT primer. PCR was performed using a GeneAmp PCR system 9700 (Applied Biosystems, Foster City, CA) with TaKaRa ExTaq® (Takara Bio, Shiga, Japan), and specific primers ([Supplementary Table S1](#)) through 35 cycles at 94°C for 30 seconds, 62°C for CNT2 or 65°C for 30 seconds for the others, and at 72°C for 30 seconds for ENTs or at 72°C for 1 minute for CNTs. The amplified products were separated by electrophoresis on a 2.0% agarose gel in the presence of ethidium bromide and visualized under ultraviolet light. The products of the expected length were then cloned into a plasmid vector using pGEM-T Easy Vector System I (Promega, Madison, WI) and amplified in *Escherichia coli*. Several clones were then sequenced in both directions using a DNA sequencer (ABI PRISM 3100; Applied Biosystems).

**Microdialysis study**

Rats were anesthetized with an intraperitoneal injection of sodium pentobarbital (60 mg/kg) and their heads were mounted on a stereotaxic frame (SR-5R, Narishige, Tokyo, Japan). Their eyelids were locally anesthetized by instillation of 2%...
xylocaine and fixed using surgical sutures in order to prevent blinking. A 25 gauge needle was inserted about 1 mm below the corneal scleral limbus through the pars plana at a depth of 3.0 mm. The needle was then removed and $[^3]$Hhypoxanthine (2 $\mu$Ci) and $[^1]$H$[^4]$C-D-mannitol (0.2 $\mu$Ci) dissolved in 1.0 $\mu$L Ringer-HEPES solution (141 mM NaCl, 4.0 mM KCl, 2.8 mM CaCl$_2$, 10.0 mM HEPES-NaOH, pH 7.4) were administered using a 10-$\mu$L microsyringe (Hamilton, Reno, NE) at a depth of 3.0 mm from the surface of the eye. The microdialysis probe (TEP-50, Eicom, Kyoto, Japan) was implanted immediately into the vitreous chamber and fixed with surgical glue (Aron Alpha A, Daiichi Sankyo, Tokyo, Japan) on the surface of the eye. Ringer-HEPES solution at 37°C was delivered to the probe continuously at 2 $\mu$L/minute via polyethylene tubing (SP19, inner diameter 0.35 mm, outer diameter 1.05 mm, Natsume, Tokyo, Japan) connected to an infusion pump (model 11, Harvard, Holliston, MA). The dialysate sampling and determination of radioactivity were performed according to a previous report. For the experiment conducted in the presence of inhibitors, each inhibitor was dissolved in Ringer-HEPES solution and delivered to the probe as described above.

References


## Supplementary Table S1 Oligonucleotide primers for RT-PCR analysis

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<th>Target</th>
<th>GenBank Accession No.</th>
<th>Primer sequences</th>
<th>Product size (bp)</th>
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Supplementary Figure S1. [³H]Adenosine uptake by primary-cultured rat Müller cells. A, Time-course of [³H]adenosine uptake by primary-cultured rat Müller cells. [³H]Adenosine uptake (13 nM) was measured at 37°C for the indicated times. The solid line was fitted using a nonlinear least-squares regression analysis program. Each point represents the mean ± S.E.M. (n = 3). B, Na⁺-independence and inhibitory effect of unlabeled adenosine on [³H]adenosine uptake by primary-cultured rat Müller cells. [³H]Adenosine uptake was performed at 37°C for 3 minutes in the presence (control) or absence of Na⁺ or in the absence (control) or presence of 2 mM unlabeled adenosine. Each column represents the mean ± S.E.M. (n = 3) and is expressed as a percentage of the control. **p<0.01, significantly different from the control.