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Freshly isolated retinal capillaries to determine efflux transporter function at the inner BRB

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ABSTRACT

Since it has been known that *in vitro* cell lines for analyzing drug transport at the inner blood-retinal barrier (BRB) do not completely retain several *in vivo* functions, new *ex vivo/in vitro* methods to evaluate drug transport across the inner BRB help us understand the role of this barrier in maintaining the homeostasis of vision and regulating drug distribution to the retina. To expand the limitations of existing *in vitro* approaches, we established a protocol to isolate fresh rat retinal capillaries as *ex vivo* model of the inner BRB. Fresh retinal capillaries were prepared by applying serial filtration steps and using density gradient centrifugation. We performed mRNA and protein analyses by reverse transcription-polymerase chain reaction and immunostaining that indicated expression of marker proteins such as facilitative glucose transporter 1 and claudin-5 in freshly isolated rat retinal capillaries. We also used fluorescent transporter substrates to characterize functional activity of organic anion transporter (Oat) 3, P-glycoprotein (P-gp), breast cancer resistance protein (Bcrp), and multidrug resistance-associated protein (Mrp) 4 in isolated retinal capillaries. Capillary luminal accumulation of fluorescent substrates of P-glycoprotein and Bcrp was decreased in the presence of transporter inhibitors. Moreover, luminal accumulation of the Oat3 and Mrp4 substrate, 8-(2-[fluoresceinyl]aminoethylthio) adenosine-3′,5′-cyclic monophosphate (8-[fluo]-cAMP), was reduced by substrates/inhibitors of Oat3 and Mrp4. In conclusion, our study shows that freshly isolated retinal capillaries retain marker protein expression and transporter functional activity. It is suggested that isolated retinal capillaries are a useful tool to study transport across the inner BRB. Using freshly isolated retinal capillaries, we anticipate applying this approach to determine the role of transporters at the inner BRB during pathophysiological states of the eye and evaluate the drug delivery to the retina.

1. Introduction

The inner blood-retinal barrier (BRB) consists of a complex structure of retinal capillary endothelial cells, retinal pericytes, and Müller cells [1]. Retinal pericytes and Müller cells support the structure and function of retinal capillary endothelial cells, which themselves are connected by tight- and adherent-junctions that restrict paracellular transport. In addition, transporters in the plasma membrane of retinal capillary endothelial cells actively and selectively control the distribution of solutes [2]. For example, nutrients such as amino acids are actively distributed to the retina by influx solute carrier (SLC) transporters at the inner BRB [3,4]. Moreover, several SLC and ATP-binding cassette (ABC)

Abbreviations: 8-[fluo]-cAMP, 8-(2-[fluoresceinyl]aminoethylthio) adenosine-3′,5′-cyclic monophosphate; α-SMA, α-smooth muscle actin; ABC, adenosine triphosphate-binding cassette; β2M, β2-microglobulin; BBB, blood-brain barrier; Bcrp, breast cancer resistance protein; BRB, blood-retinal barrier; BSA, bovine serum albumin; cAMP, cyclic AMP; CD, cluster of differentiation; Cy3, cyanine 3; DAPI, 4′,6-diamidino-2-phenylindole; DIC, differential interference contrast; FBS, fetal bovine serum; DPBS, Dulbecco's phosphate-buffered saline; Ficoll, Ficoll® PM 400; GLUT1, facilitative glucose transporter 1; GS, glutamine synthetase; HEPES, 2-[4-(2-hydroxyethyl)-1-piperazinyl]ethanesulfonic acid; λ_{em}, emission wavelength; λ_{ex}, excitation wavelength; Mrp4, multidrug resistance-associated protein 4; NBD, 7-nitro-2,1,3-benzoxadiazole; Nefh, neurofilament heavy chain; Oat3, organic anion transporter 3; PCR, polymerase chain reaction; P-gp, P-glycoprotein; THF, tetrahydrofuran; SD, standard deviation;; *X.L.*, *Xenopus Laevis*.

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transporters are critical in restricting solute distribution to the retina [3,5]. Therefore, understanding the molecular regulation of inner BRB transporters helps establish strategies that improve the use of central nervous system (CNS) and ophthalmic drugs.

Several *in vivo* methods exist that allow analyzing membrane transporters at the inner BRB in rodents. Specifically, to elucidate *in vivo* transport of compounds from blood to retina in rodents, brain perfusion and intracarotid bolus injection are used [3,5]. To examine *in vivo* retina-to-blood compound/drug transport, we established *in vivo* microdialysis analysis after intravitreous administration in rats [6,7]. These *in vivo* techniques, however, also measure solute transport across the outer BRB, which is formed by retinal pigment epithelial cells, as well as transport across the inner BRB [2]. Therefore, *in vitro* approaches are also used to elucidate the detailed molecular mechanisms involved in solute exchange between the retina and blood across the inner BRB.

One *in vitro* model of the inner BRB utilizes the conditionally immortalized rat retinal capillary endothelial cell line, TR-iBRB. This cell line is widely used [8] because *in vitro* transport data obtained from these cells can be correlated with *in vivo* transport data [9]. Despite the availability of the TR-iBRB cell line, not all transporter-mediated phenomena that occur at the BRB have been fully identified and characterized. For example, Shen et al. have reported that uptake of rhodamine 123, a fluorescent P-glycoprotein (P-gp/Abcb1a/Abcb1b) substrate, in TR-iBRB cells was not decreased in the presence of several P-gp substrates [10], including acebutolol [11]. In addition, it is well-established that endothelial cell lines *in vitro* do not retain expression and function of SLC and ABC transporters *in vivo*. Take benzylpenicillin as an example: this drug is cleared from the brain into the blood across the blood-brain barrier (BBB) by organic anion transporter 3 (Oat3/Slc22a8) [12] and is also a substrate of multidrug resistance-associated protein 4 (Mrp4/Abcc4) [13]. In a rat BBB cell line, however, the distribution volume of benzylpenicillin was lower than the intracellular volume of the cells [14,15]. Thus, existing *in vitro* approaches to elucidate solute transport across the BBB are limited.

To overcome these limitations, we established a protocol to isolate rat retinal capillaries from freshly collected retinae without the use of invasive enzymatic treatments or freezing procedures. By applying serial filtration steps and using density gradient centrifugation, we obtained fresh retinal capillaries. In addition, we analyzed freshly isolated retinal capillaries for marker protein expression and transporter functional activity. Data from studies using fluorescent transporter substrates such as 7-nitro-2,1,3-benzoxadiazole (NBD)-cyclosporin A, BODIPY™ FL prazosin, and 8-(2-[fluoresceinyl]aminoethylthio) adenosine-3′,5′-cyclic monophosphate (8-[fluo]-cAMP) showed that isolated retinal capillaries retain transport function of ABC and SLC transporters at the inner BRB. Together, freshly isolated retinal capillaries are a potentially powerful tool to elucidate the molecular mechanisms underlying transport phenomena at the inner BRB.

2. Materials and methods

2.1. Animal studies

Animal experiments in this study (#A2017PHA-5-6, A2020PHA-1, and A2020PHA-5; principal investigator: Ken-ichi Hosoya) were approved by the Animal Care Committee of the University of Toyama. Male Wistar/ST rats (~150 g/animal; Japan SLC, Hamamatsu, Japan) were maintained in a controlled environment: temperature = ~23°C; humidity = 40%–50%; and 12/12 h dark/light cycle. Mature female *Xenopus Laevis* (*X.L.*) frogs (~300 g/animal; Kato-S-Science, Chiba, Japan) were kept in a controlled environment: temperature = ~16°C; humidity = ~50%; and 12/12 h dark/light cycle. All animal experiments followed the Animal Research: Reporting *in Vivo* Experiments guidelines.

2.2. Reagents and supplies

Bovine serum albumin (BSA), 4′,6-diamidino-2-phenylindole (DAPI), and Ficoll® PM 400 (Ficoll) were obtained from Merck (Darmstadt, Germany). Cyclosporin A, 4-vinylbenzylamine, and 4-fluoro-NBD were purchased from Tokyo Chemical Industry (Tokyo, Japan). Ko143 and 2-[4-(2-hydroxyethyl)-1-piperazinyl]ethanesulfonic acid (HEPES), and 8-[fluo]-cyclic AMP (cAMP) were obtained from FUJIFILM Wako Pure Chemicals (Osaka, Japan), Cayman Chemical (Ann Arbor, MI, USA), and Nakarai Tesque (Kyoto, Japan), respectively. BODIPY™ FL prazosin, fetal bovine serum (FBS), and Grubbs II were purchased from ThermoFisher Scientific (Waltham, MA, USA). LLC-GA5-CoL300 cells were obtained from RIKEN BioResource Research Center (Ibaraki, Japan). All other chemicals and reagents were of analytical grade and were purchased from the manufacturers described above.

2.3. NBD-cyclosporin A synthesis

Cyclosporin A was labeled with NBD as shown in Fig. 2(a). A starting material **1** was prepared by the procedures in the literature by Gaali et al. [16]. To a solution of **1** (5.3 mg, 4.10 μmol) in tetrahydrofuran (THF; 2.5 mL) was added 4-fluoro-NBD (NBD-F; 0.825 mg, 4.51 μmol). The reaction mixture was stirred for 2 h at room temperature, concentrated using a rotary evaporator, and subjected to preparative thin-layer chromatography (eluent: CH_2Cl_2/MeOH = 5:1), which yielded NBD-cyclosporin A (2.3 mg, 39%) as a yellow solid. To confirm the synthesis of NBD- cyclosporin A, ESI-HRMS analyses were carried out on Thermo LTQ Orbitrap XL mass spectrometer (Thermo Fisher Scientific). Analytical HPLC was conducted using COSMOSIL $5C_{18}$-MS-II (4.6 × 150 mm; Nacalai Tesque, Kyoto Japan) with the conditions as follows: (MeCN/H_2O 0.1% TFA, 65–85% in 20 min): t_R = 13.7 min (flow rate = 1 mL/s); HRMS (ESI-Orbitrap-MS): calc. For $C_{74}H_{117}N_{15}NaO_{15}$ [M + Na]: 1478.8751 found 1487.8738, Purity (HPLC, 254 nm): 96%.

2.4. Isolation of retinal capillaries

Retinal capillaries were isolated according to previously published methods describing the isolation of fresh brain capillaries and retinal capillaries from frozen tissue [17]. Eight retinae (eyeballs from 4 animal resulting in ~100 mg retinal tissue) from Wistar/ST rats were harvested, collected and homogenized in isolation buffer (1% BSA, 1.8 mM $CaCl_2$, 10 mM D-glucose, 2.7 mM KCl, 1.5 mM KH_2PO_4, 0.65 mM $MgCl_2$, 137 mM NaCl, 8.1 mM Na_2HPO_4, and 2.0 mM sodium pyruvate; pH 7.4). After adding isolation buffer containing 30% Ficoll to result in a final Ficoll concentration of 15%, the homogenate was centrifuged at 4500 *g* and 4°C for 25 min. The resulting pellets were resuspended in isolation buffer and filtered through a 200 μm mesh. The flow-through was filtered through a 40 μm mesh, and retinal capillaries were then trapped on a 30 μm nylon mesh. Retinal capillaries were collected by washing the nylon mesh with isolation buffer, and a capillary pellet was obtained after centrifugation (1200 *g*, 4°C, 8 min). The supernatant was removed, and capillaries were resuspended in capillary buffer (in mM: 1.8 $CaCl_2$, 10 D-glucose, 137 NaCl, 8.1 Na_2HPO_4, 2.7 KCl, 1.5 KH_2PO_4, 0.65 $MgCl_2$, and 2.0 sodium pyruvate; pH 7.4) followed by centrifuged centrifugation (1200 *g*, 4°C, 7 min) to wash the capillaries. After an additional washing step, the resulting capillary suspension was used for experiments. We added trypan blue (final concentration of 0.2%) to the capillary suspension to visualize retinal capillaries microscopically.

2.5. Immunostaining

Retinal capillaries in suspension were transferred to glass coverslips where they attached to the glass surface during a 30 min incubation at ~20°C. Capillaries were fixed with Dulbecco's phosphate-buffered saline (DPBS; in mM: 137 NaCl, 8.1 Na_2HPO_4, 2.7 KCl, and 1.5 KH_2PO_4; pH 7.4) containing 0.25% glutaraldehyde and 3% paraformaldehyde for

20 min at ~20°C, and were then treated for 30 min with 0.5% Triton X-100-containing DPBS at ~20°C. Permeabilized capillaries were rinsed twice with DPBS and then blocked with 10% normal goat serum (Nichirei Biosciences; Tokyo, Japan) for 60 min at ~20°C. Capillaries were incubated with 0.5 μg/mL rabbit-derived anti-facilitative glucose transporter 1 (GLUT1) antibody [18] for 16 h at 4°C. After washing with DPBS, capillaries were treated with cyanine 3 (Cy3)-labeled secondary antibody for 90 min at room temperature and then 5 times rinsed with DPBS at ~20°C. Nuclei were counterstained with 1 μM DAPI for 10 min at ~20°C.

Capillary images were captured using a TCS-SP5 confocal laser microscope (Leica, Heidelberg, Germany) equipped with a blue diode/argon/green diode laser system, HCX PLAPO lambda blue 63× 1.2 water objective, and LAS AF software (Leica). DAPI and Cy3 were excited sequentially using the 405 and 561 nm excitation laser lines, respectively. 8-bit, 1024 × 1024 frame images were acquired using a pinhole setting to obtain 1 Airy unit.

2.6. mRNA expression analysis

Total RNA from retinal capillaries and intact retinae was isolated using an RNeasy Micro kit (QIAGEN, Hilden, Germany) following the manufacturer's protocol. Total RNA was used to synthesize complementary DNA using 15-mer oligo dT primers in the presence or absence of ReverTra Ace (TOYOBO, Osaka, Japan). Polymerase chain reaction (PCR) was performed using the primers shown in Table 1 and Ex Taq DNA polymerase (Takara Bio, Shiga, Japan). The thermal protocol used was as follows: 1 cycle of 95°C for 2 min and 30 cycles of 95°C for 0.5 min, 60°C for 0.5 min (β2-microglobulin (β2M): 55°C for 0.5 min), and 72°C for 0.75 min. The amplified PCR products were separated by electrophoresis on a 3% ethidium bromide-stained agarose gel and were visualized using ultraviolet light.

2.7. Uptake study using P-gp-overexpressing LLC-GA5-Col300 cells

LLC-GA5-Col300 cells were maintained in Medium 199 (ThermoFisher Scientific) containing 10% FBS, 1× Penicillin-Streptomycin solution (ThermoFisher Scientific), and 300 ng/mL colchicine. Cells were seeded at 7.2×10^5 cells/well onto collagen type I-coated 24-well plates and cultured at 37°C for 72 h in 5% CO_2/air. After a 6 h preincubation in Medium 199 containing 10% FBS and 1× Penicillin-Streptomycin at 37°C in 5% CO_2/air, the cells were rinsed three times with 37°C warm cell-uptake medium (in mM; 1.4 $CaCl_2$, 10 D-glucose, 10 HEPES-NaOH, 3 KCl, 0.4 K_2HPO_4, 1.2 $MgSO_4$, 122 NaCl, 25 $NaHCO_3$, pH 7.4). Then, cell uptake medium containing 1 μM NBD-cyclosporin A was added in the absence (control) or presence of 100 μM inhibitor. After a 1 h incubation, the cells were washed 3 times with 4°C cell-uptake medium. The protein amount in each well was determined using the Bio-Rad *DC* Protein Assay Kit II (Bio-Rad, Hercules, CA, USA). NBD-derived fluorescence (excitation wavelength (λ_{ex}), 470 nm; emission wavelength (λ_{em}), 530 nm) was measured with a SpectraMax i3 microplate reader (Molecular Devices, Sunnyvale, CA, USA). Changes in NBD-cyclosporin A uptake represented as the distribution volume (μL/mg protein; eq. 1), were expressed as the percentage of control and mean ± standard deviation (SD).

$$\text{Distributionvolume} = \frac{\text{NBD-cyclosporinAamountinthecell(pmol/mgprotein)}}{\text{NBD-cyclosporinAconcentrationinthemedium(pmol/}\mu\text{L)}} \quad (1)$$

Table 1
Primer sequences.

Target (GenBank Accession #)	Primer sequences (Upper, forward primer; lower, reverse primer)	Product size (bp)
Claudin-5 (NM_031701)	5′-gca gag cac cgg gca cat gc-3′ 5′-tag ttc ttc ttg tcg taa tcg cc-3′	483
GS (NM_017073)	5′-tac ccg agt gga act ttg atg-3′ 5′-taa agt tgg tgt ggc agc ctg-3′	600
Nefh (NM_012607)	5′-ttg gac cga ctc tca gag gca g-3′ 5′-caa tcc gac act ctt cgc ctt cc-3′	352
α-SMA (NM_031004)	5′-tat gtg tga aga gga aga ca-3′ 5′-cac aat acc agt tgt acg tc-3′	463
β2M (NM_012512)	5′-tca cct ggg acc gag aca t-3′ 5′-gag ggt ggg gga ttg aac tg-3′	455

GS, glutamine synthetase; Nefh, neurofilament heavy chain; α-SMA, α-smooth muscle actin; β2M, β2-microglobulin.

2.8. Transport of fluorescent substrates in retinal capillaries

Freshly isolated retinal capillaries were transferred to glass coverslips and let settle down for at least 30 min at room temperature. Then, 2 μM NBD-cyclosporin A, 5 μM BODIPY™ FL prazosin, or 5 μM 8-[fluo]-cAMP was added with or without inhibitors, and capillaries were incubated for 30 min (8-[fluo]-cAMP) or 60 min (NBD-cyclosporin A, BODIPY™ FL prazosin). Eight-bit color images were acquired of ~10 retinal capillaries using a TCS-SP5 confocal microscope equipped with a HCX PLAPO lambda blue 63× 1.2 water objective and a 488 nm Argon laser to excite NBD-cyclosporin, BODIPY FL prazosin, or 8-[fluo]-cAMP. Fluorescence in the lumen of retinal capillaries was analyzed with ImageJ 1.52u software (Wayne Rasband, National Institutes of Health, USA). Luminal fluorescence was quantitated by subtracting background fluorescence from fluorescence measured in the capillary lumen and averaging the values per capillary. Data are presented as the mean ± SD from ~10 capillaries.

2.9. Xenopus Laevis (X.L.) oocyte uptake studies

Rat Oat3- and Oatp1a4-expressing oocytes were generated as described in our previous report [19] and were kept for 4 days in culture medium (1.8 mM $CaCl_2$, 2.0 mM KCl, 1.0 mM $MgCl_2$, 100 mM NaCl, 2.5 mM pyruvic acid, 5.0 mM HEPES-NaOH, 25 μg/mL gentamicin sulfate, 1% BSA; pH 7.5). Oocytes were treated for 5 min at 20°C with oocyte uptake buffer: (in mM) 1.8 $CaCl_2$, 5 HEPES-NaOH, 2 KCl, 1 $MgCl_2$, and 96 NaCl, pH 7.4. The uptake experiment was started by adding 200 μL of oocyte uptake buffer containing 10 μM 8-[fluo]-cAMP or 5 μM SR-101 at 20°C. The uptake experiment was stopped by washing oocytes 4 times with ice-cold oocyte uptake buffer. Oocytes were solubilized with 5% sodium dodecyl sulfate solution for 3 h at ~20°C. Fluorescence from 8-[fluo]-cAMP (λ_{ex}, 480 nm; λ_{em}, 520 nm) or SR-101 (λ_{ex}, 590 nm; λ_{em}, 615 nm) was measured with a SpectraMax i3 microplate reader (Molecular Devices). Oocyte uptake of 8-[fluo]-cAMP or SR-101 was expressed as the distribution volume (μL/oocyte), namely oocyte/medium ratio (eq. 2) and was represented as mean ± SD.

$$\text{Oocyte}\Big/\text{mediumratio} = \frac{\text{Compoundamountinanoocyte(pmol/oocyte)}}{\text{Compoundconcentrationintheuptakebuffer(pmol/}\mu\text{L)}} \quad (2)$$

2.10. Statistics

Statistical differences between the means of two (Fig. 5) or more than two groups (Figs. 2–5) were determined using the unpaired two-tailed Student's *t*-test or Dunnett's test following one-way analysis of variance, respectively.

3. Results

3.1. Isolation of fresh retinal capillaries

Fig. 1a shows a representative image of freshly isolated retinal capillaries stained with trypan blue. We used density-gradient

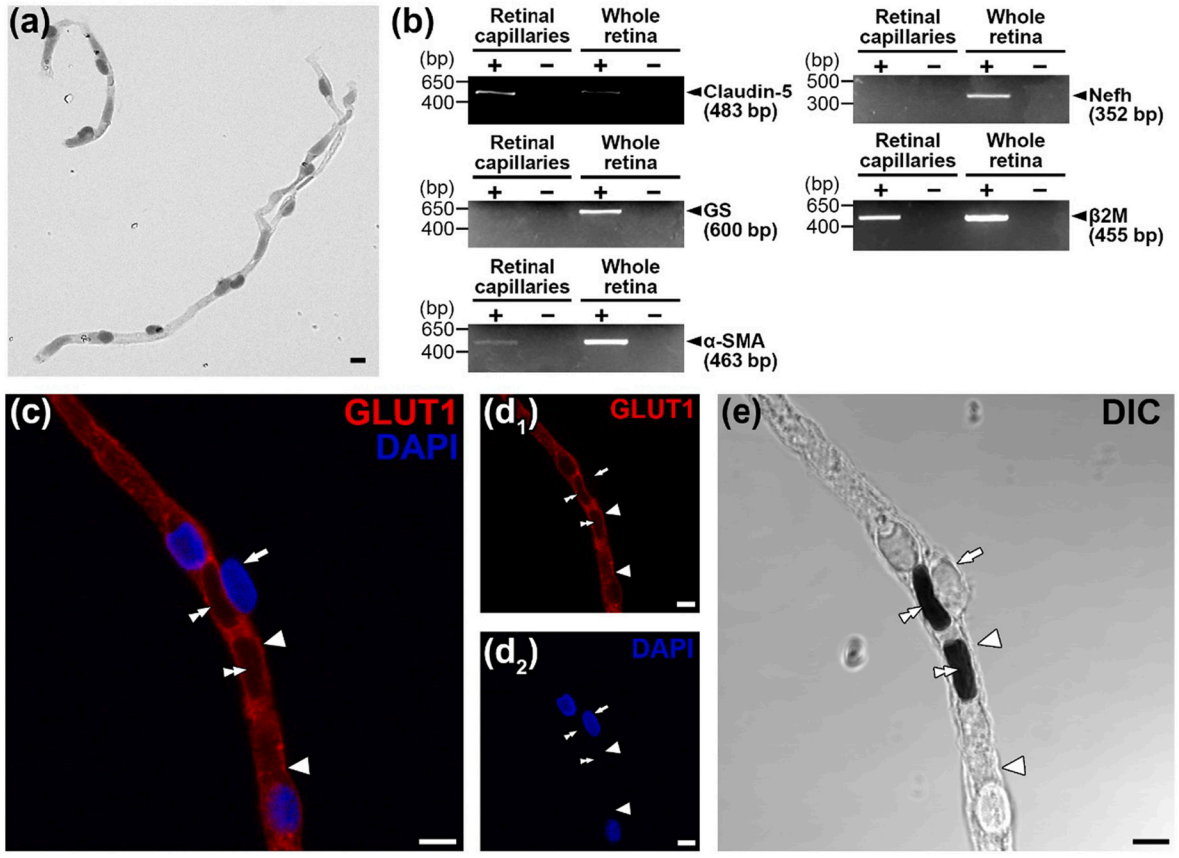

Fig. 1. Expression of endothelial marker proteins in isolated retinal capillaries.
a Representative image of freshly isolated capillaries from fresh rat retinae. Retinal capillaries were stained with 0.2% trypan blue solution. Scale bar: 10 μm. **b** mRNA expression in freshly isolated retinal capillaries and retinae from rat using PCR in the presence (+) or absence (−) of reverse transcriptase. GS, glutamine synthetase; α-SMA, α-smooth muscle actin; Nefh, neurofilament heavy chain; β2M, β2-microglobulin. **c**–**e** Representative image of freshly isolated rat retinal capillary showing immunostaining of facilitative glucose transporter 1 (GLUT1), a marker protein of retinal capillary endothelial cells. GLUT1 (red in **c and d_1**) was detected in the plasma membrane of retinal capillaries (arrowheads in **c**), but not in retinal pericytes (arrows in **c and d_1**) that are attached to capillaries. Nuclei were counterstained with DAPI (blue in **c and d_2**). Scale bar: 10 μm. **e** Differential interference contrast (DIC) image showing the morphology of the representative retinal capillary. Red blood cells were also observed in the lumen of retinal capillaries (double arrowheads).

centrifugation followed by a serial-mesh filtration that yielded retinal microvessels with a diameter of less than 10 μm, indicating they are capillaries. To characterize the retinal capillaries in the collected fraction, we examined mRNA expression of several marker genes (Fig. 1b). We detected stronger mRNA expression of the tight junction protein claudin-5 [20] in isolated retinal capillaries compared to whole retina. In contrast, mRNA expression of glutamine synthetase (GS, a marker molecule for Müller cells and retinal astrocytes) [21] and neurofilament heavy chain (Nefh, a marker molecule for retinal neurons) [22] was only detected in whole retinae but not in retinal capillaries. We also detected strong mRNA expression of β2-microglobulin (positive control) in both retinal capillaries and whole retinae, as well as stronger mRNA expression of α-smooth muscle actin (α-SMA, a marker molecule for retinal pericytes and smooth muscles cells [23,24]) in whole retinae compared to retinal capillaries.

To confirm that the isolated blood vessels were indeed capillaries from the rat retina, we performed immunostaining for GLUT1, a marker protein for retinal capillaries [25]. Fig. 1c–e **(arrowheads)** show GLUT1 staining (red) in the plasma membrane of retinal microvessels; cell nuclei were counterstained with DAPI (blue). There was no GLUT1 immunosignal in the plasma membrane of retinal pericytes that were attached to the microvessels (Fig. 1c–e**, arrows**) or in red blood cells in the capillary lumen (Fig. 1c–e**, double arrowheads**).

3.2. Synthesis of the fluorescent P-gp substrate NBD-cyclosporin A

To visualize P-gp-mediated transport, we synthesized the fluorescent P-gp substrate NBD-cyclosporin A that is transported by P-gp [26–28]. For the present study, we synthesized cyclosporin A to which NBD was covalently bound, as shown in Fig. 2a. To test if P-glycoprotein transports NBD-cyclosporin A, we examined NBD-cyclosporin A uptake by P-gp-overexpressing LLC-GA5-Col300 cells. As shown in Fig. 2b, in the control group, NBD-cyclosporin A uptake reached 88.2 ± 3.4 μL/mg protein (n = 8) after 60 min. In the presence of the competitive P-gp substrates [29] verapamil (100 μM) and quinidine (100 μM), NBD-cyclosporin A uptake was increased 1.7-fold and 1.4-fold compared to the control, respectively. In contrast, probenecid and *p*-aminohippurate (PAH) had no statistically significant effect on NBD-cyclosporin A uptake.

3.3. NBD-cyclosporin A transport in retinal capillaries

To evaluate P-gp-mediated transport in retinal capillaries, we examined accumulation of NBD-cyclosporin A in the lumen of retinal capillaries. Fig. 3a–d show representative confocal images of retinal capillaries incubated for 60 min with 2 μM NBD-cyclosporin A in the absence (control; Fig. 3a) or presence of verapamil (Fig. 3b), quinidine (Fig. 3c), or probenecid (Fig. 3d) at a concentration of 100 μM. Luminal NBD-cyclosporin A accumulation was determined by measuring NBD-

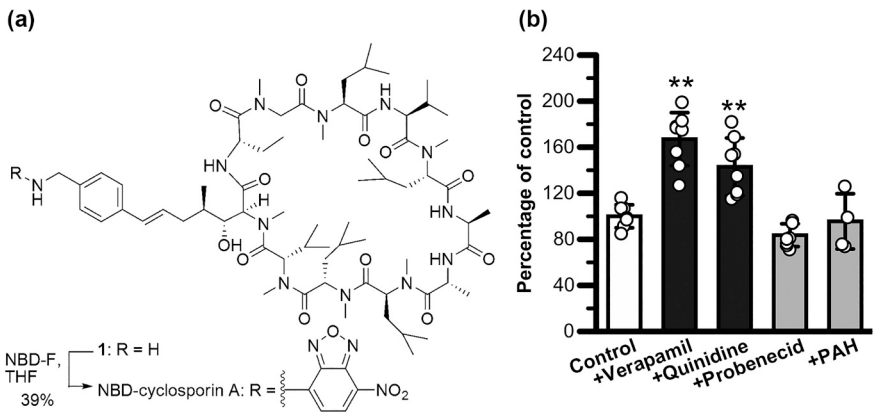

Fig. 2. NBD-cyclosporin A synthesis and evaluation as P-gp substrate.
a Synthetic diagram of NBD-cyclosporin A. **b** NBD-cyclosporin A (1 μM) uptake by P-gp-overexpressing LLC-GA5-Col300 cells after 60 min in the absence (control) or presence of transporter modulators (100 μM). The open circles represent individual data points. Each column represents the mean ± SD (n = 4–8). ** $p < 0.01$ using Dunnett's test. PAH, *p*-aminohippurate.

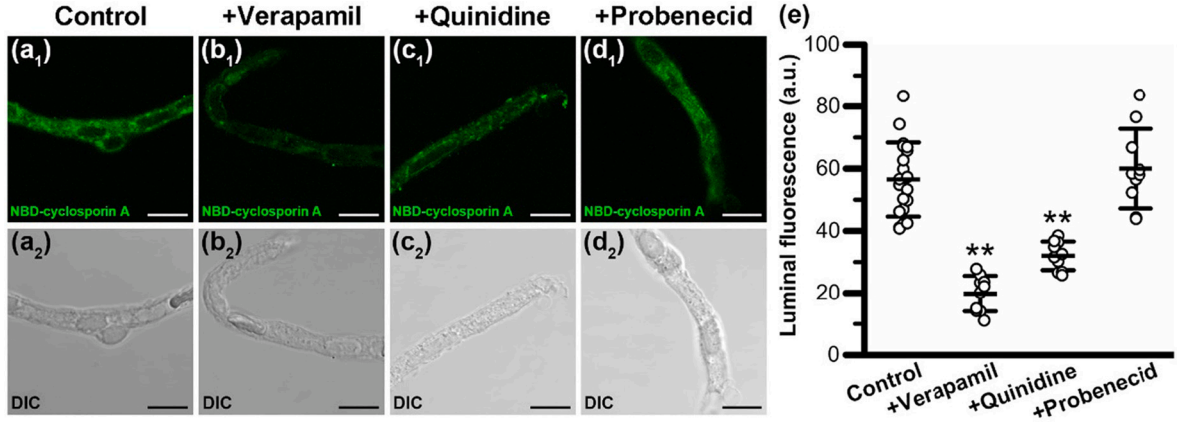

Fig. 3. *Ex vivo* P-gp-mediated transport of NBD-cyclosporin A in freshly isolated retinal capillaries.
a_1–d_1 Representative confocal images of retinal capillaries exposed to 2 μM NBD-cyclosporin A (green) for 60 min in the absence (control) or presence of modulator (100 μM). **a_2–d_2** Corresponding differential interference contrast (DIC) images of the same retinal capillaries. Scale bar: 10 μm. **e** Quantification of NBD-cyclosporin A fluorescence in the lumen of retinal capillaries. Open circles represent the individual data points used to calculate the mean ± SD (scale, 0–255; n = 10–20). ** $p < 0.01$ using Dunnett's test.

cyclosporin A fluorescence in the lumen of retinal capillaries (Fig. 3e). Luminal fluorescence was significantly decreased by 65% and 43% in the presence of verapamil and quinidine, respectively. In contrast, 100 μM probenecid did not have a significant effect on luminal NBD-cyclosporin A fluorescence.

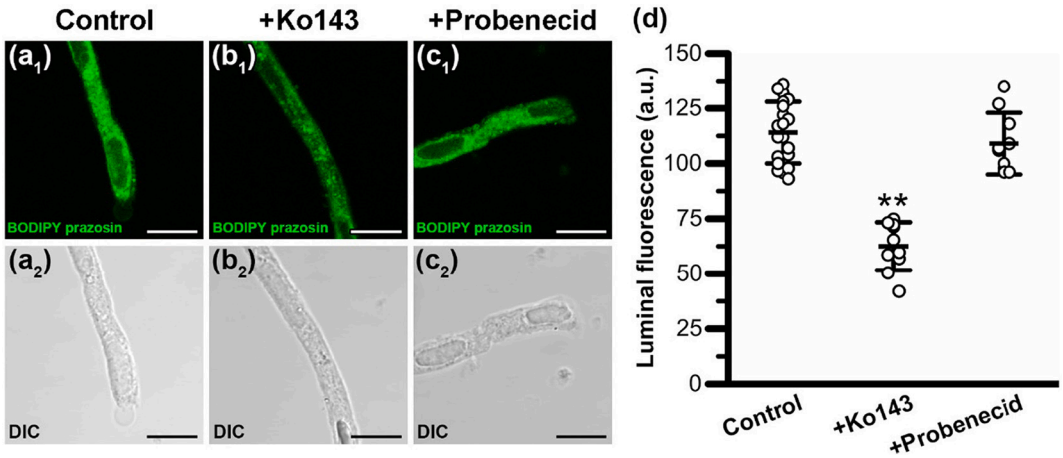

Fig. 4. *Ex vivo* Bcrp-mediated transport of BODIPY™ FL prazosin in freshly isolated rat retinal capillaries.
a_1–c_1 Representative confocal images of retinal capillaries exposed to 5 μM BODIPY™ FL prazosin (green) for 60 min in the absence (control) or presence of 250 nM Ko143 or 10 μM probenecid. **a_2–c_2** Corresponding differential interference contrast (DIC) images of the same retinal capillaries. Scale bar: 10 μm. **d** Quantification of BODIPY™ FL prazosin fluorescence in the lumen of the retinal capillaries. Open circles represent the individual data points used to calculate the mean ± SD (scale, 0–255; n = 10–20). ** $p < 0.01$ using Dunnett's test.

3.4. BODIPY™ FL prazosin transport retinal capillaries

In addition to P-gp, breast cancer resistance protein (Bcrp/Abcg2) is another efflux transporter that pumps its substrates from the cytosol of retinal capillary endothelial cells into the circulating blood [30,31]. To examine Bcrp functional expression in freshly-isolated retinal capillaries, we examined accumulation of the fluorescent Bcrp substrate BODIPY™ FL prazosin [32] in the lumen of retinal capillaries. As shown in Fig. 4a, b, and d, BODIPY™ FL prazosin fluorescence was significantly decreased by 45% in the presence of 250 nM Ko143, a reported BCRP inhibitor [32]. On the other hand, luminal fluorescence was not significantly altered by 10 μM probenecid (Fig. 4a, c, and d).

3.5. Oat3-mediated 8-[fluo]-cAMP transport in vitro

To test if 8-[fluo]-cAMP is transported by anionic SLC transporters such as Oat3 and organic anion transporting polypeptide 1a4 (Oatp1a4/Slco1a4) [6,25], we first performed experiments using rat Oat3- or

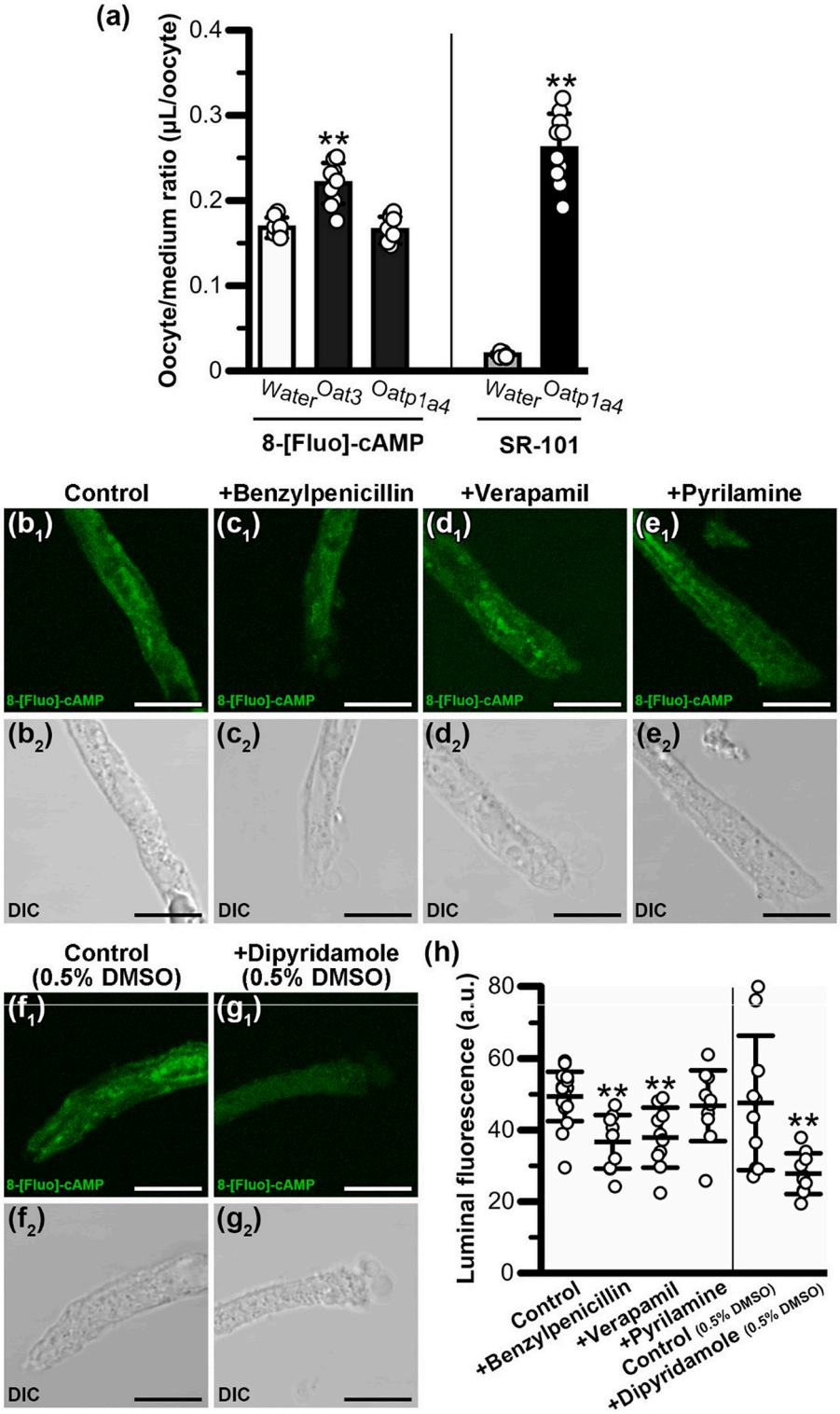

Fig. 5. 8-[Fluo]-cAMP transport in transporter-overexpressing *X.L.* oocytes and freshly isolated rat retinal capillaries.
a Uptake of 10 μM 8-[fluo]-cAMP (rat Oat3 substrate) and 5 μM SR-101 (rat Oatp1a4 substrate) in rat Oat3 or Oatp1a4 cRNA-injected oocytes (180 min). Open circles represent the individual data points that were used to calculate the mean ± SD ($n = 10$). ** $p < 0.01$ using Dunnett's test (8-[fluo]-cAMP) or unpaired *t*-test (SR-101). **b_1–g_1** Representative confocal images of retinal capillaries exposed to 5 μM 8-[fluo]-cAMP (green) for 30 min in the absence (control) or presence of 1 mM benzylpenicillin, 10 μM verapamil, 100 μM pyrilamine, or 50 μM dipyridamole w/wo 0.5% DMSO. **b_2–g_2** Corresponding differential interference contrast (DIC) images of the same retinal capillaries. Scale bar: 10 μm. **h** Quantification of 8-[fluo]-cAMP fluorescence in the lumen of retinal capillaries. Open circles represent the individual data points that were used to calculate the mean ± SD (scale, 0–255; $n = 10$–20). ** $p < 0.01$ using Dunnett's test or unpaired *t*-test.

Oatp1a4-expressing *X.L.* oocytes (Fig. 5a). In *X.L.* oocytes injected with rat Oat3 cRNA, uptake of 8-[fluo]-cAMP after 180 min was significantly increased by 1.3-fold compared to water-injected *X.L.* oocytes (control). However, in *X.L.* oocytes injected with rat Oatp1a4 cRNA, 8-[fluo]-cAMP was the same as in water-injected *X.L.* oocytes. In contrast, after 180 min, uptake of the fluorescent Oatp1a4 substrate SR-101 [33] in *X.L.* oocytes injected with rat Oatp1a4 cRNA was 13.7-fold greater than in water-injected *X.L.* oocytes.

3.6. Carrier-mediated 8-[fluo]-cAMP accumulation in retinal capillaries

Lastly, we examined luminal accumulation of 8-[fluo]-cAMP in freshly-isolated capillaries prepared from rat retinae. Fig. 5b–h depict representative images of isolated rat retinal capillaries exposed to 8-[fluo]-cAMP with or without transporter modulators. The Oat3 and Mrp4 substrate benzylpenicillin (1 mM; [13,34]) had a significant inhibitory effect and reduced 8-[fluo]-cAMP fluorescence in the lumen of rat retinal capillaries by 26% (Fig. 5c and h). Luminal 8-[fluo]-cAMP fluorescence was also significantly reduced by 23% and 42% in the presence of 10 μM verapamil (Fig. 5d and h) and 50 μM dipyridamole (Fig. 5g and h), both of which inhibit Mrp4 [34,35]. In contrast, 100 μM pyrilamine did not affect luminal 8-[fluo]-cAMP fluorescence compared to control (Fig. 5e and h).

4. Discussion

In this study, we established a procedure to isolate retinal capillaries from rat retinae using density gradient centrifugation and serial filtration. Several methods for isolating retinal capillaries from rodent retinae have been previously established. Traditionally, retinal capillaries have been isolated following enzymatic treatment of whole retinae with collagenase [36]. A further development from this procedure was digestion of retinae with collagenase/DNase followed by a series of filtration steps and collection of retinal capillary endothelial cells with magnetic bead-coated anti-cluster of differentiation (CD) 31 antibodies [37,38]. Recently, Li et al. described a retinal capillary isolation protocol by combining tissue freezing, density gradient centrifugation, and capture of capillaries with a nylon mesh [17]. Compared to these relatively harsh methods, the protocol used in the present study did not employ freezing or collagenase treatment and is thus a relatively mild procedure to isolate retinal capillaries. Therefore, we expect that changes in the physiological response of retinal capillaries to the isolation process are minimized. In this regard, Fig. 1a and e show that freshly isolated retinal capillaries were morphologically intact and that the preparation most free of visible contaminations. Fig. 1a and c–e also show that freshly isolated retinal capillaries were GLUT1-positive, indicating that the capillaries were biochemically intact. Furthermore, RT-PCR analysis for markers of retinal neurons and glial cells (Fig. 1b) shows that retinal capillaries obtained with the procedure described here are free of these cell types. We did, however, detect the amplified products of retinal pericyte and smooth muscle cell markers (Fig. 1b). This was confirmed by differential interference contrast (DIC) images (Fig. 1c–e) showing pericytes attached to isolated retinal capillaries and is likely due to the lack of an enzymatic digestion step that results in the isolation of morphologically intact retinal capillaries. In contrast, retinal capillaries isolated using collagenase treatment combined with anti-CD31 antibodies rarely contain perivascular cells such as retinal pericytes [38]. Thus, to evaluate the molecular expression signature of the inner BRB capillary fraction, combining capillary isolation methods with enzymatic treatment to remove perivascular cells is a useful approach.

From the DIC images (Fig. 1e) [39,40], it is possible that the immunocytochemical localization of proteins on the plasma membrane of retinal capillaries and in attached retinal cells to the capillaries is determined (Fig. 1c and d). In this study, the expressional analysis of membrane proteins which are dominantly localized on the luminal and/or abluminal membranes of retinal capillary endothelial cells, such as P-gp and Bcrp, has not been performed. Nevertheless, utilizing our retinal capillaries, the co-immunostaining of marker proteins for expression of luminal and abluminal membrane of the inner BRB is expected to be helpful to determine the transporter protein localization at the inner BRB.

Cyclosporin A is a widely known substrate of P-gp [41]. Labeling cyclosporin A with a fluorescent compound such as NBD *via* a D-Lys8 linker generates the fluorescent P-gp substrate NBD-cyclosporin A, which can be used to determine P-gp mediated transport. Specifically, exposing isolated brain capillaries to NBD-cyclosporin A results in P-gp-mediated accumulation of fluorescence in the lumen of brain capillaries [42,43]. In the present study, we covalently linked cyclosporin A with NBD using 4-vinylbenzylamine (Fig. 2a) according to a report showing cyclosporin A conjugation with 5/6-carboxyfluorescein [16]. Data from the previous study demonstrate that the affinity of 5/6-carboxyfluorescein-labeled cyclosporin A to peptidyl-prolyl-isomerase is similar to that of intact cyclosporin A, implying that pharmacology of the labeled cyclosporin A is retained at least in part. However, it remains unclear whether this labeling procedure affects the recognition of the NBD-cyclosporin A for P-gp. To test this, we used P-gp-overexpressing LLC-GA5-Col300 cells (Fig. 2b) and found that cellular NBD-cyclosporin A uptake was significantly increased in the presence of the P-gp substrates verapamil and quinidine, indicating that P-gp recognizes the NBD-cyclosporin A we synthesized.

Using this NBD-cyclosporin A, we tested P-gp-mediated transport function in freshly isolated retinal capillaries. Previous studies show that accumulation of NBD-cyclosporin A in the lumen of brain capillaries is a measure of P-gp-mediated efflux transport across the BBB [42,44]. Here, we used the same approach with freshly isolated retinal capillaries. We quantified accumulation of NBD-cyclosporin A in retinal capillary lumens according to previous references [39, 41] and found that luminal NBD-cyclosporin A was significantly reduced by more than 40% in the presence of P-gp substrates (Fig. 3). In this regard, using our *in vivo* retinal uptake index method, we previously demonstrated that P-gp limits drugs that are P-gp substrates from distributing into the retina [3,45]. Consistent with these *in vivo* findings, the data presented here also indicate that freshly isolated retinal capillaries retain P-gp-mediated retina-to-blood transport.

In addition to P-gp, Bcrp is also localized in the luminal membrane of the inner BRB [32], and Bcrp-mediated transport has been observed in retinal capillaries [30]. Indeed, here we show that the Bcrp inhibitor Ko143 significantly reduced luminal accumulation of the fluorescent Bcrp substrate BODIPY™-prazosin in freshly isolated retinal capillaries (Fig. 4). Our data suggest that Ko143 inhibits Bcrp-mediated transport of BODIPY™-prazosin in the luminal membrane of retinal capillaries resulting in a decrease of luminal fluorescence. Thus, based on these data, we conclude that isolated retinal capillaries are helpful to determine P-gp and BCRP transport function at the inner BRB.

Although the functional study using our retinal capillaries was only utilized by using fluorescent drugs/compounds, the transporters which are expected to be down-regulated in the *in vitro* cell lines are able to be examined [14,15]. Indeed, we also used freshly isolated retinal capillaries to study, for the first time, the role of Oat3 and Mrp4 in retina-to-blood transport of anionic compounds at the inner BRB. We previously showed Oat3 expression in the abluminal membrane of the inner BRB and demonstrated carrier-mediated anionic compound/drug elimination from the vitreous humor/retina into the blood [6]. In the present study, we used the anionic fluorescent compound 8-[fluo]-cAMP that is a known substrate of the ABC transporter Mrp4 [46,47]. In addition, our data from *in vitro* uptake studies using *X.L.* oocytes demonstrate that Oat3, but not Oatp1a4, transports 8-[fluo]-cAMP (Fig. 5a). Thus, based on these data and previous reports [46,47], we conclude that 8-[fluo]-cAMP can be used as a fluorescent probe to assess Oat3- and Mrp4-mediated transport. Luminal accumulation of 8-[fluo]-cAMP in retinal capillaries was significantly decreased in the presence of the Oat3/Mrp4 substrate benzylpenicillin (Fig. 5c and h) [13,48]. In addition, the Mrp4

substrates/inhibitors verapamil and dipyridamole [34,35] blocked luminal 8-[fluo]-cAMP accumulation in retinal capillaries (Fig. 5d, g, and h). These data are consistent with reports showing that Mrp4 is expressed at the inner BRB [49]. However, Mrp4 localization has not yet been determined, but immunohistochemical analyses of brain sections indicate that Mrp4 expression levels are greater in the luminal membrane of brain capillaries than in the abluminal membrane [39,40]. These findings are consistent with our data showing inhibition of luminal 8-[fluo]-cAMP accumulation by Mrp4 inhibitors, suggesting that Mrp4 is localized on the blood side of the plasma membrane in retinal capillaries. Taken together, our transport studies in freshly isolated retinal capillaries using 8-[fluo]-cAMP suggest Oat3- and Mrp4-mediated vectorial transport of anionic compounds on the abluminal and luminal membranes, respectively, at the inner BRB.

Data from previous studies show that distribution of drugs and other compounds across the inner BRB is altered under several pathological conditions. Metrikin et al. [50] reported that inflammation induced by intravitreous injection of lipopolysaccharide disrupts tight junctions resulting in increased paracellular transport of compounds across the BRB. In brain capillaries, lipopolysaccharide induces down-regulation of P-gp expression and function within minutes after exposure, and this effect is mediated by signaling *via* a toll-like receptor 4/protein kinase C pathway [51]. In addition, data from our previous *in vivo* study demonstrate that Oat3- and Mrp4-mediated elimination of prostaglandin E_2 from the brain across the BBB was decreased by high glutamate brain levels [52]. Since retinal inflammation is involved in the development of diabetic retinopathy [53], it is possible that expression and function of inner BRB transporters are altered under this condition. The pathway that regulates these transporters at the BBB is conserved in brain capillaries *in vivo* [44]. Therefore, we anticipate that our *ex vivo* approach using fresh retinal capillaries is useful to evaluate the mechanisms underlying the regulation of inner BRB transporters, including P-gp, Bcrp, and Oat3/Mrp4 (Figs. 3–5). Such findings would help elucidate the role of the inner BRB in the development of retinal diseases and how drug exchange between blood and retina is regulated.

5. Conclusions

Here we present a novel method to isolate fresh capillaries from rat retina. Isolated retinal capillaries express marker proteins of the inner BRB and retain P-gp- and Bcrp-mediated transport activity. Moreover, we show, for the first time, *ex vivo* transport of anionic compounds in isolated retinal capillaries. Our findings suggest that both Mrp4 and Oat3 are involved in efflux transport of anionic compounds at the inner BRB. Using freshly isolated retinal capillaries *ex vivo*, we anticipate applying this approach to determine the role of transporters at the inner BRB during pathophysiological states of the eye and evaluate the drug delivery to the retina.

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Declaration of Competing Interest

The authors declare no competing interests.

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