

RESEARCH ARTICLE



Polarized effects of adenosine on blood–brain barrier integrity: Tightening from the luminal and opening from the abluminal side

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Funding information

Universidad Autónoma Metropolitana, Grant/Award Number: UAM-14407033; National Research, Development and Innovation Office of Hungary, Grant/Award Numbers: 2024-1.2.2-ERA_NET-2024-00018, K143766; Hungarian Academy of Sciences, Grant/Award Number: NAP2022-I-6/2022; Hungarian Ministry of Culture and Innovation; HORIZON EUROPE Marie Skłodowska-Curie Actions, Grant/Award Number: 101034252

Background and Purpose: Adenosine as a signalling molecule modifies blood–brain barrier (BBB) tightness in pathological conditions. Our aim was to investigate the direct and polarized effects of adenosine on the BBB using co-culture and in vivo models.

Experimental Approach: The expression of adenosine receptors was measured by qRT-PCR. BBB models were prepared from primary rat brain endothelial cells, pericytes and glial cells and were treated with adenosine and A_{2A} receptor antagonist SCH-58261 in the luminal and abluminal compartments. Barrier function in cultures was tested by impedance kinetics, electrical resistance and fluorescein permeability. Acute effects of intracardiac or intracerebroventricular adenosine were measured on the BBB permeability for fluorescein in the brain cortex and hippocampus of rats and mice.

Key Results: Adenosine receptors were present in BBB cells and brain endothelial cells expressed predominantly A_{2A} receptors. Adenosine exerted a polarized effect on both in vitro and in vivo models; it tightened the paracellular barrier from the luminal side and increased fluorescein permeability from the abluminal or brain side. The abluminal, but not the luminal effect, was observed in sleep and in sleep deprivation. Based on the receptor profile, the results of co-culture experiments and the blocking effects of SCH-58261, the effect is mainly mediated luminally on brain endothelial, abluminally on brain endothelial and pericyte adenosine receptors.

Abbreviations: BBB, blood–brain barrier; bFGF, basic fibroblast growth factor; DMEM/F-12, Dulbecco's modified Eagle medium/Nutrient mixture F-12; i.c.v., intracardiac administration; i.c.v., intracerebrovascular administration; ITS, insulin–transferrin–selenium; NECA, N-ethylcarboxamidoadenosine; P_{app} , apparent permeability coefficient; PDS, plasma-derived serum; PET, polyethylene terephthalate; SF, sodium fluorescein; TEER, transendothelial electrical resistance; VEGFA, vascular endothelial growth factor A.

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Conclusion and Implications: Adenosine had a polarized circadian effect on BBB permeability, from the luminal side tightened the barrier in culture models and in vivo, whereas it opened the barrier from the abluminal side.

KEYWORDS

adenosine, adenosine A_{2A} receptor, blood–brain barrier, pericyte, permeability, polarized effect, sleep

1 | INTRODUCTION

Adenosine is an important signalling molecule both in the periphery and the CNS, exerting cardiovascular effects in the former and inhibitory effect on neuronal activity and inducing sleep in the latter (Lopes et al., 2011). Adenosine receptors are ubiquitously expressed but with a different pattern of the four G-protein coupled subtypes A₁, A_{2A}, A_{2B} and A₃ on various cells, resulting in unique pharmacological profiles in different tissues (Jacobson, 2009).

In the CNS, adenosine and its receptors participate in the control of physiological processes such as sleep (Reichert et al., 2022) but are also involved in pathological conditions such as Alzheimer's disease (Merighi et al., 2021) and ischaemia (Pedata et al., 2014). Adenosine is accumulated in the brain during wakefulness (Porkka-Heiskanen & Kalinchuk, 2011) and is a somnogenic signal via A₁ receptors in astrocytes (Lazarus et al., 2019). During sleep deprivation, adenosine levels in the brain further increase, activate A_{2A} adenosine receptors and contribute to cognitive impairment (Florian et al., 2011). Cytokines and neuroinflammation also play key roles in the induction of blood–brain barrier (BBB) dysfunction during sleep restriction, and these effects could be attenuated by A_{2A} adenosine receptor antagonists (Hurtado-Alvarado et al., 2016 and 2018), indicating an effect for adenosine on the cells of the BBB and on BBB permeability from the abluminal, brain side.

BBB transporters, receptors and enzymes that regulate homeostatic and protective functions are expressed in a polarized way at the luminal and abluminal membranes of the cerebral endothelium facing blood and brain parenchyma, respectively (Abbott et al., 2006). Efflux pumps, such as **P-glycoprotein/ABCB1**, are asymmetrically expressed at the luminal membrane of brain endothelial cells, which helps in the vectorial transport of drugs and xenobiotics in the brain to the blood (Chaves et al., 2014). The signalling effects of **vascular endothelial growth factor A (VEGFA)** in the cerebral and retinal microvasculature are also polarized. VEGFA induces a cytoprotective response from the luminal side and barrier leakage from the abluminal side (Dragoni & Turowski, 2018; Hudson et al., 2014).

The expression of adenosine receptors was demonstrated on brain endothelial cells, but the adenosine receptor profile of the BBB cell types, including brain pericytes and astrocytes, and how adenosine regulates BBB permeability, remain unclear (Mills et al., 2011; Schaddelee, Voorwinden, van Tilburg, et al., 2003). In sleep-restricted rats, adenosine increases BBB permeability that can be blocked by A_{2A} receptor antagonists (Hurtado-Alvarado et al., 2016 and 2017). In

What is already known

- Adenosine contributes to increased BBB permeability in brain pathological conditions.
- Signalling molecules have polarized effects on BBB permeability, which has not been investigated for adenosine.

What does this study add

- Adenosine lumenally tightens the BBB and from the abluminal side increases paracellular permeability.
- Adenosine has a polarized effect on BBB permeability acting on brain endothelial cells and pericytes.

What is the clinical significance

- Acute luminal adenosine does not decrease BBB paracellular integrity.
- Adenosine A_{2A} receptor blockers may protect against BBB opening in pathologies such as sleep deprivation.

some studies, A_{2A} receptor agonists increased BBB permeability via actin-cytoskeletal reorganization and modulation of tight junctions (Carman et al., 2011), whereas in others, A_{2A} receptor agonists enhanced BBB properties (Chen et al., 2020; Liu et al., 2018). These data suggest that adenosine may regulate BBB permeability by action on A_{2A} receptors, but its direct and potentially polarized effects on the BBB have not been studied yet.

In the work reported here, we first investigated the adenosine receptor profile in primary cultures of rat brain endothelial cells, pericytes, astrocytes and rat brain cortex samples. To reveal the polarized effects of adenosine, we measured BBB permeability changes after luminal or abluminal administration of the molecule using in vitro models. To explore the role of pericytes and astrocytes in the adenosine induced changes on brain endothelial integrity, we compared monocultures and co-cultures using two and three cell types. In the in vivo studies, we injected adenosine intracardially and intracerebroventricularly to rats and mice and measured BBB permeability for the

small paracellular marker fluorescein in the cortex, hippocampus and cerebellum. Finally, we examined the effect of circadian rhythm and sleep deprivation on the adenosine-induced BBB permeability changes.

2 | METHODS

2.1 | Animals

All animal care and experimental procedures complied with the Guidelines for the Care and Use of Mammals in Neuroscience and Behavioural Research (National Research Council, 2010) and were approved by the Academic Ethic Committee of the Biological Science Division of the Universidad Autónoma Metropolitana, Unidad Iztapalapa and from the Universidad Nacional Autónoma de México (039-CIC-2023). Animal studies are reported in compliance with the ARRIVE guidelines (Percie du Sert et al., 2020) and with the recommendations made by the *British Journal of Pharmacology* (Lilley et al., 2020).

Three-month-old male Wistar rats ($n = 40$) and 3-month-old male C57BL/6 mice ($n = 40$) were from Charles River Laboratories, Mexico City, Mexico; they were randomly divided into four groups: control (C), adenosine (A), adenosine plus SCH-58261 (A + S) and SCH-58261 alone (S). Animals were housed in standard conditions in the laboratory vivarium under a 12-h light-dark cycle (lights on at 7:00 AM) at a room temperature of 20–25°C, with 40–60% humidity. There were 5 rats per cage and 10 mice per cage. Commercial rat chow (LabDiet 5001, PetFoods, Mexico City, Mexico) and tap water were provided ad libitum.

2.2 | Cell cultures and BBB model

Primary cultures of brain endothelial cells, glia and pericytes, and construction of the in vitro BBB model were prepared as described previously (Barna et al., 2020; Nakagawa et al., 2009). After isolation, cells were seeded on collagen type IV ($100 \mu\text{g}\cdot\text{mL}^{-1}$) and fibronectin ($25 \mu\text{g}\cdot\text{mL}^{-1}$) coated culture dishes (Corning, Costar, New York, NY, USA). Brain endothelial cells were cultured in Dulbecco's Modified Eagle's Medium/Nutrient Mixture F-12 (DMEM/F-12, Gibco, Life Technologies, Carlsbad, CA, USA) supplemented with 15% plasma-derived serum (PDS, First Link, Wolverhampton, UK), $100 \mu\text{g}\cdot\text{mL}^{-1}$ heparin, $5 \mu\text{g}\cdot\text{mL}^{-1}$ insulin, $5 \mu\text{g}\cdot\text{mL}^{-1}$ transferrin, $5 \text{ ng}\cdot\text{mL}^{-1}$ sodium selenite (ITS, Pan-Biotech GmbH, Aidenbach, Germany), $1 \text{ ng}\cdot\text{mL}^{-1}$ basic fibroblast growth factor (bFGF, Roche, Basel, Switzerland) and $50 \mu\text{g}\cdot\text{mL}^{-1}$ gentamycin. To eliminate P-glycoprotein-negative contaminating cell types, the medium of brain endothelial cells was supplemented with $3 \mu\text{g}\cdot\text{mL}^{-1}$ puromycin during the first 3 days of the culture (Perrière et al., 2005). During the isolation of primary rat brain pericytes, the same method was followed as for brain endothelial cells, except that pericytes were plated onto uncoated culture dishes and did not receive puromycin. Primary rat mixed glial cultures were passaged to 12-well plates (Corning, Costar) coated with collagen type IV ($100 \mu\text{g}\cdot\text{mL}^{-1}$). Glial cells were cultured for 2 weeks before using them

in the co-culture BBB model. Pericytes and glial cells were cultured in DMEM supplemented with 10% foetal bovine serum (Pan-Biotech GmbH) and $50 \mu\text{g}\cdot\text{mL}^{-1}$ gentamycin. To prepare the co-culture model, pericytes were seeded to the bottom side of the tissue culture inserts coated with collagen IV (Transwell, PET membrane, $0.4 \mu\text{m}$ pore size, Corning, Costar), and brain endothelial cells were passaged to the upper side of the collagen IV and fibronectin-coated culture insert membranes. Then, the inserts containing brain endothelial cell and pericyte layers on the two sides of the membrane were placed in 12-well plates in which glial cells were grown at the bottom. Both upper and lower fluid compartments received endothelial culture medium supplemented with hydrocortisone (550 nM), and the three types of cells were cultured together for 4 days before permeability experiments (Barna et al., 2020; Nakagawa et al., 2009). In addition to the co-culture BBB model described above, to observe the effect of adenosine on the different cell types of the BBB, experiments were performed also on a monoculture model containing only brain endothelial cells and on a co-culture model of brain endothelial and glial cells.

2.3 | Quantitative real-time polymerase chain reaction

Brain cortex samples, primary cultures of brain endothelial cells, pericytes and astrocytes were homogenized in TRI Reagent, and then, $0.5 \mu\text{g}$ RNA from each sample was transcribed to complementary DNA by High Capacity cDNA Reverse Transcription Kit (Applied Biosystems, Waltham, MA, USA) according to the manufacturer's protocol. The quantitative real-time polymerase chain reaction (qRT-PCR) was performed with TaqMan Gene Expression Master Mix (Life Technologies) in a Bio-Rad C1000 Touch Thermal Cycler mounted with a CFX96 Touch Real-Time PCR Detection System (Bio-Rad Laboratories, Hercules, CA, USA). The adenosine receptor genes were investigated by the following inventoried TaqMan Gene Expression Assays (Life Technologies): Adora1 Rn00567668_m1, Adora2a Rn00583935_m1, Adora2b Rn00567697_m1 and Adora3 Rn00563680_m1. After heat activation at 95°C for 3 min, the cycling conditions were the following: denaturation for 30 s at 95°C , annealing and polymerization for 30 s at 60°C (40 cycles). Fluorescent signals were collected after each extension step. The qRT-PCR data were analysed by Bio-Rad CFX Maestro software (Bio-Rad Laboratories). The expression of genes was normalized to an endogenous control gene in all samples (18S rRNA , $\Delta\text{Ct} = \text{Ct}_{\text{gene}} - \text{Ct}_{18\text{S rRNA}}$). Expression values of the studied genes were determined based on the normalized expression of genes calculated with the $2^{-\Delta\text{Ct}}$ formula.

2.4 | Cell impedance measurements by real-time cell microelectronic sensing

An impedance-based label-free technique was used for dynamic monitoring of living cells and non-invasively quantifying proliferation, viability and barrier integrity of adherent cells (Harazin et al., 2018;

Sato et al., 2022). The RTCA-SP instrument (Agilent Technologies, Santa Clara, CA, USA) utilizes 96-well plates containing gold micro-electronic sensor arrays (Agilent). The interaction between cells and electrodes generates an impedance response that correlates linearly with cell number, growth, adherence and barrier properties. This method is more sensitive and informative than colorimetric end-point assays to test damage or functional changes in brain endothelial cells (Tóth et al., 2014). The E-plates were coated with collagen IV ($100 \mu\text{g}\cdot\text{ml}^{-1}$) and fibronectin ($25 \mu\text{g}\cdot\text{ml}^{-1}$) for 20 min at 37°C . Culture medium ($50 \mu\text{l}$) was added to each well for background readings, and then, $50 \mu\text{l}$ of rat brain endothelial cell suspension was dispensed at the density of 6×10^3 cells per well. The cells were grown for 3–5 days until reaching confluency, indicated by the plateau phase of the registered cell index. The cells in the E-plate were kept in an incubator at 37°C for 24 h and monitored every 10 min. The cell index at each time point was defined as $(R_n - R_b)/15$, where R_n is the cell-electrode impedance of the well when it contains cells and R_b is the background impedance of the well with the medium alone.

2.5 | Measurement of the integrity of the paracellular barrier

Transendothelial electrical resistance (TEER) shows the ionic permeability of interendothelial tight junctions; therefore, it is a key parameter to validate BBB tightness in cell culture models. TEER was measured by epithelial-volt-ohm metre and Endohm-12 chamber electrodes (World Precision Instruments, Sarasota, FL, USA) and expressed relative to the surface area of the monolayers ($\Omega \times \text{cm}^2$). The background values of inserts without cells were subtracted from the measured TEER values. TEER was measured every day until reaching confluency.

To measure paracellular permeability, the hydrophilic sodium fluorescein (SF, 376 Da ; $10 \mu\text{g}\cdot\text{ml}^{-1}$) dye was added to the upper, luminal compartments, as described before (Veszeka et al., 2018). The assay medium was phenol red-free DMEM/F12 containing 10% PDS and supplements as described for brain endothelial cell culture medium. The inserts were kept for 30 min in 12-well plates in a CO_2 incubator on a horizontal shaker (100 rpm). Samples were collected from both compartments, and the concentrations of the marker molecule were determined by a fluorescence multiwell plate reader (Fluostar Optima, BMG Labtechnologies, Offenburg, Germany; excitation wavelength: 485 nm , emission wavelength: 535 nm). From the concentration values, the apparent permeability coefficients (P_{app}) were calculated by the following equation (Harazin et al., 2018):

$$P_{\text{app}} = \frac{[C]_A \times V_A}{A \times [C]_L \times t}$$

based on the concentration of the tracer in the luminal compartments ($[C]_L$) at 0 h and abluminal compartments at 30 min ($[C]_A$), the volume of the abluminal compartment (V_A ; 1.5 ml) and the surface area available for permeability (A ; 1.12 cm^2).

2.6 | Immunocytochemistry

To evaluate the morphology of interendothelial junctions, rat brain endothelial cells were immunostained for claudin-5 junctional protein. The cell layers were washed in phosphate-buffered saline (PBS), fixed with ice-cold acetone-methanol solution (1:1) for 2 min, and the non-specific binding sites were blocked with 3% bovine serum albumin (BSA) in PBS for 1 h. Cells were incubated with rabbit anti-claudin-5 antibody (Sigma, cat. SAB4502981, [RRID: AB_10753223](#); $2.5 \mu\text{g}\cdot\text{ml}^{-1}$ in 3% BSA-PBS) overnight at 4°C . Then, cells were incubated with secondary antibody Cy3-labelled anti-rabbit IgG (Sigma, cat. C23061, [RRID: AB_258792](#); $2 \mu\text{g}\cdot\text{ml}^{-1}$ in PBS), and Hoechst dye 33,342 ($1 \mu\text{g}\cdot\text{ml}^{-1}$) to stain cell nuclei, for 1 h at room temperature. Cells were washed three times with PBS after each step. Stained cells were mounted in Fluoromount-G (Southern Biotech, Birmingham, AL, USA) and staining was visualized by a Leica TCS SP5 confocal laser-scanning microscope (Leica Microsystems, Wetzlar, Germany). The tight junction staining was analysed with MATLAB software (MathWorks, Natick, MA, USA). The background was determined for each picture and was subtracted to eliminate the incidental non-uniform background. The resulting grayscale images were converted to binary, and the intercellular tight junction pattern was complemented with the surface area of the structure. Small-sized, non-connecting objects were eliminated. The pixel number of this image gave the area of the whole structure. Then the binary image was used as a mask on the grayscale image without the non-uniform background to calculate the overall intensity. The average intensity of each image was calculated by taking the ratio of the intensity and the area of the structure.

2.7 | In vivo experiments

Experiments were performed between 10 and 11 AM (zeitgeber time 0 = lights on, ZT3–4). Animals were anaesthetized with sodium pentobarbital i.p. ($200 \text{ mg}\cdot\text{kg}^{-1}$ for both rats and mice), and a 0.5-cm thoracic incision was made. A cocktail containing marker molecule SF ($10 \text{ mg}\cdot\text{ml}^{-1}$) and adenosine ($150 \mu\text{g}\cdot\text{kg}^{-1}$) or adenosine and SCH-58261 ($100 \mu\text{g}\cdot\text{kg}^{-1}$) or SCH-58261 was diluted in 0.9% saline and administered by intracardiac (i.c.) injection (0.2 ml per 100 g) and left to circulate for 2 min. Other groups of anaesthetized animals received intracerebroventricular (i.c.v.) injections using Hamilton syringes with the following coordinates: AP = -0.5 mm ; L = 1.0 mm ; depth 2.0 mm ; relative to the bregma. Two microlitres of a cocktail containing marker molecule SF ($10 \text{ mg}\cdot\text{ml}^{-1}$) and adenosine ($1.5 \mu\text{g}\cdot\text{kg}^{-1}$) or adenosine and SCH-58261 ($100 \mu\text{g}\cdot\text{kg}^{-1}$) or SCH-58261 was diluted in 0.9% saline and administered by i.c.v. A thoracic incision was made and at the time of the i.c.v. injections, the animals received i.c. SF injections, as described before.

2.8 | Sleep deprivation protocol

As levels of adenosine in the hippocampus are higher before sleep onset, we explored the effect of adenosine i.c. and i.c.v. at two time

points in mice: at ZT0 (daytime; $n = 40$) just before the sleep phase and at ZT12 (nighttime; $n = 40$) as the time of activity onset. In addition, a group with 12 h of sleep deprivation ($n = 40$ mice) was compared with the ZT12 group (well-rested mice). Sleep deprivation was performed by placing mice into a slow activity wheel (Omniaiva, Mexico City, Mexico) for 12 h (ZT0–ZT12) that allows the mice to eat and drink (Hurtado-Alvarado et al., 2023). The low-speed rotating cylinder for sleep deprivation studies in mice consists of five lanes (one per mouse), with a diameter of 20 cm. Each lane is 8 cm wide. Each lane contains a water bottle and a food pellet container. The cylinder is made of translucent red acrylic. The rotation speed is fixed: 1 revolution every 2.5 min.

2.9 | Analysis of fluorescein extravasation to brain regions

The circulation time for SF was 2 min in all experimental setups, after which animals were transcardially perfused with 0.9% saline solution (temperature of the solution: 25°C; rats: perfusion rate 15 ml·min⁻¹, total volume 30 ml; mice: perfusion rate 5 ml·min⁻¹, total volume 10 ml). The brains were removed, and the cerebral cortex, hippocampus and cerebellum were dissected. The samples were weighed, homogenized with cold PBS and centrifuged at 12,000 g for 10 min at 4°C. Supernatants (100 µl per sample) were collected, and methanol was added (100 µl per sample). Supernatants were mixed and centrifuged at 12,000 × g for 10 min at 4°C. The supernatants were placed in a 96-well plate, and absorbance values were measured in a plate reader HLAB (H Reader 1) for SF (excitation wavelength: 485 nm, emission wavelength: 535 nm). Each sample was quantified in duplicate. A standard curve of known concentrations of SF was prepared in duplicate, measured and used to calculate dye concentrations. Data are presented as a percentage of the control group.

2.10 | Data and statistical analysis

The data and statistical analysis in this study comply with the recommendations of the British Journal of Pharmacology on experimental design and analysis in pharmacology (Curtis et al., 2025). All data were obtained without knowledge of the group treatments. Results were analysed using GraphPad Prism software (GraphPad Software, San Diego, CA, USA). Data are presented as means ± SD or SEM. Significant differences between groups was determined using one-way or two-way ANOVA followed by Bonferroni post-tests. Differences were considered statistically significant at $P < 0.05$.

2.11 | Materials

All reagents were purchased from Sigma-Aldrich Corporation (subsidiary of Merck, Darmstadt, Germany), unless otherwise indicated. Adenosine was dissolved in distilled water and used at 1-, 3-, 10- and

30-µM concentrations in the cell impedance experiments and at 30 µM concentration in the permeability experiments. The non-selective adenosine agonist 5'-N-ethylcarboxamido-adenosine (NECA) and the A_{2A} receptor antagonist SCH-58261 (7-(2-phenylethyl)-5-amino-2-(2-furyl)-pyrazolo-[4,3-e]-1,2,4-triazolo[1,5-c]pyrimidine) were dissolved in DMSO and used at 1-µM concentration in the cell culture experiments. In the cell impedance experiments to tighten brain endothelial cell layers, CPT-cAMP (8-(4-chlorophenylthio)adenosine 3',5'-cyclic monophosphate sodium salt; Sigma-Aldrich, cat# C3912) was dissolved in distilled water and used at 250 µM, in combination with the phosphodiesterase inhibitor RO201-1724 (dissolved in DMSO) at 17.5-µM concentration. To open brain endothelial junctions, EGTA (dissolved in distilled water) was used at a 500-µM concentration.

2.12 | Nomenclature of targets and ligands

Key protein targets and ligands in this article are hyperlinked to corresponding entries in <http://www.guidetopharmacology.org> and are permanently archived in the Concise Guide to PHARMACOLOGY 2025/2026 (Alexander, Davenport et al., 2025; Alexander, Fabbro et al., 2025; Alexander, Gibb et al., 2025).

3 | RESULTS

3.1 | Adenosine receptor expression in cells of the BBB

Primary rat brain endothelial cells in culture expressed predominantly the A_{2A} adenosine receptor gene, A_{2B} receptor mRNA was also present at low levels, and the genes of A₁ and A₃ receptors were not detected in our exploratory experiment (Figures 1 and S1). Primary rat brain pericytes expressed all adenosine receptor genes, except for A₃ receptors, with the highest mRNA level for A_{2B} receptors. All four types of adenosine receptor genes were expressed in cultured rat primary glial cells and rat brain cortex samples (Figures 1 and S1). Brain cortex samples, expressing all four *Adora* genes, served as reference tissue in the assay.

3.2 | Adenosine increases the impedance of brain endothelial cells

All four concentrations of adenosine increased the impedance of brain endothelial cell layers, expressed as cell index, as early as 10 min after treatment compared to the control group receiving only culture medium (Figure 2a,c). The adenosine effect was still present at the 1-h time point (Figure S2). The adenosine receptor agonist NECA showed a similar effect to adenosine (Figure 2b). The impedance values of all treatment groups receiving A_{2A} adenosine receptor inhibitor SCH-58261 remained at the level of the control group (Figure 2c).

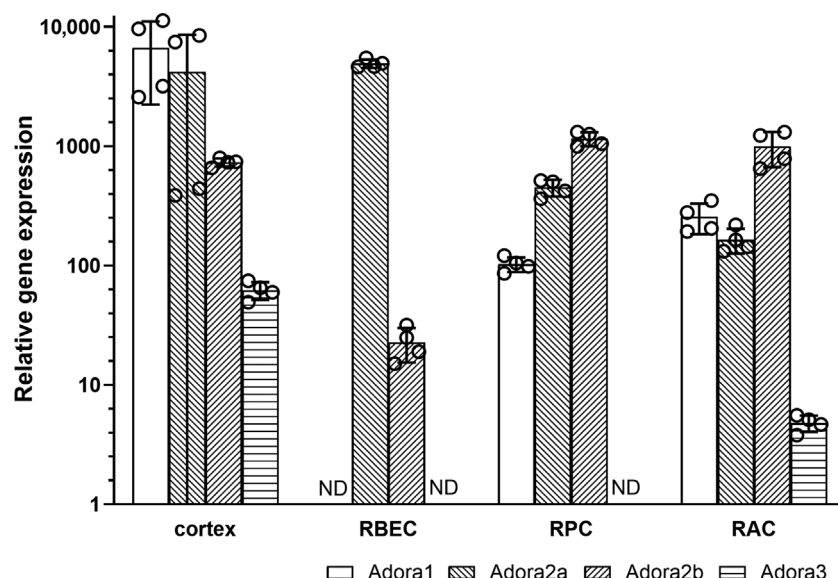


FIGURE 1 The expression of adenosine receptor genes *Adora1*, *Adora2a*, *Adora2b* and *Adora3* in the cell types of the blood–brain barrier grouped by cell and tissue types. The relative gene expression in rat brain cortex, primary rat brain endothelial cells (RBEC), brain pericytes (RPC) and astroglial cells (RAC) were normalized to an endogenous control (18S rRNA) gene. ND, not detected. Data are presented as mean $\pm$ SD, $n = 4$.

Caffeine, which is a non-selective antagonist of adenosine receptors, at the concentration of 50 μ M blocked the impedance elevating effects of NECA on brain endothelial cells at the 30 min time point, while it did not show an effect when given alone (Figure S3). Elevation of intracellular cAMP, a well-known treatment to induce the paracellular tightness of BBB models (Deli et al., 2005), significantly increased the impedance at all time points (Figure 1b–c) (Figure 1b–c). Treatment with the calcium chelator EGTA, used as a reference molecule to open the cell-cell adhesions, resulted in impedance values below the control level at the 30-minute and 1-hour time-points (Figure S2–S3).

We found a statistically significant increase in impedance values for both adenosine and NECA treatment groups at early time points (Figure 2), but the effects lasted for 2 and 3 h, respectively (Figures 3a–c and S2). The impedance of all groups treated with SCH-58261, alone or with adenosine receptor agonists, remained at the level of the control group (Figure 3c). The effects of cAMP and EGTA, the reference molecules to tighten and open the paracellular barrier, were long-lasting (Figure 3a–c, Figure S2–S3).

3.3 | Adenosine exerts polarized effects on the barrier integrity of a co-culture model of the BBB

The effects of adenosine on the tightness of the paracellular barrier were measured in three types of BBB models, namely brain endothelial cell layers in monoculture (E00), and co-culture models of brain endothelial cells with glial cells (EOA), and with both brain pericytes and glial cells (EPA; Figure 4). As described in our previous study (Nakagawa et al., 2009), the paracellular tightness of the co-culture BBB models measured by TEER (Figure S5) and fluorescein permeability (Figure 4) was significantly higher than that of the monoculture (Figure S4).

Adenosine decreased the permeability of fluorescein in all three types of BBB models, but this effect could be inhibited by SCH-

58261 only in the E0A model, although a trend was seen for the other two models, too (Figure 4a). To elucidate whether the effect is polarized, adenosine was given only luminally, mimicking treatment from the blood side. The fluorescein permeability of E00 and E0A models was reduced by adenosine, but these changes were not affected by the inhibitor. No significant effect was seen in the EPA model with the tightest barrier (Figure 4b). When adenosine was given in the abluminal compartment, representing the brain parenchyma side of the BBB models, a slight decrease was seen in the paracellular permeability for fluorescein in the E00 and E0A models, while in the EPA model containing brain pericytes, an elevation was measured, which could be inhibited by SCH-58261 (Figure 4c). The *F* and the *P* values of the group comparisons for Figure 4 are shown in Table S6. No change was seen in the TEER values of the BBB models treated with adenosine for 30 min given to either the luminal or abluminal compartments, or both (Figure S5).

Morphological experiments were also performed on the BBB culture models. Immunostaining for claudin-5, the major transmembrane tight junction protein at the BBB, revealed elongated, fusiform cell shape, tight intercellular adhesions without gaps and predominantly junctional distribution at the cell borders in brain endothelial cells (Figure 5a). The intensity of the claudin-5 staining in brain endothelial cells was elevated by luminally administered adenosine in the EPA model, while no change in either cell morphology or claudin-5 staining intensity was seen in the abluminally treated groups (Figure 5b).

3.4 | Adenosine modifies BBB permeability depending on the administration route in vivo

To investigate if adenosine modifies the paracellular permeability of the BBB in vivo in a polarized way, we measured permeability to

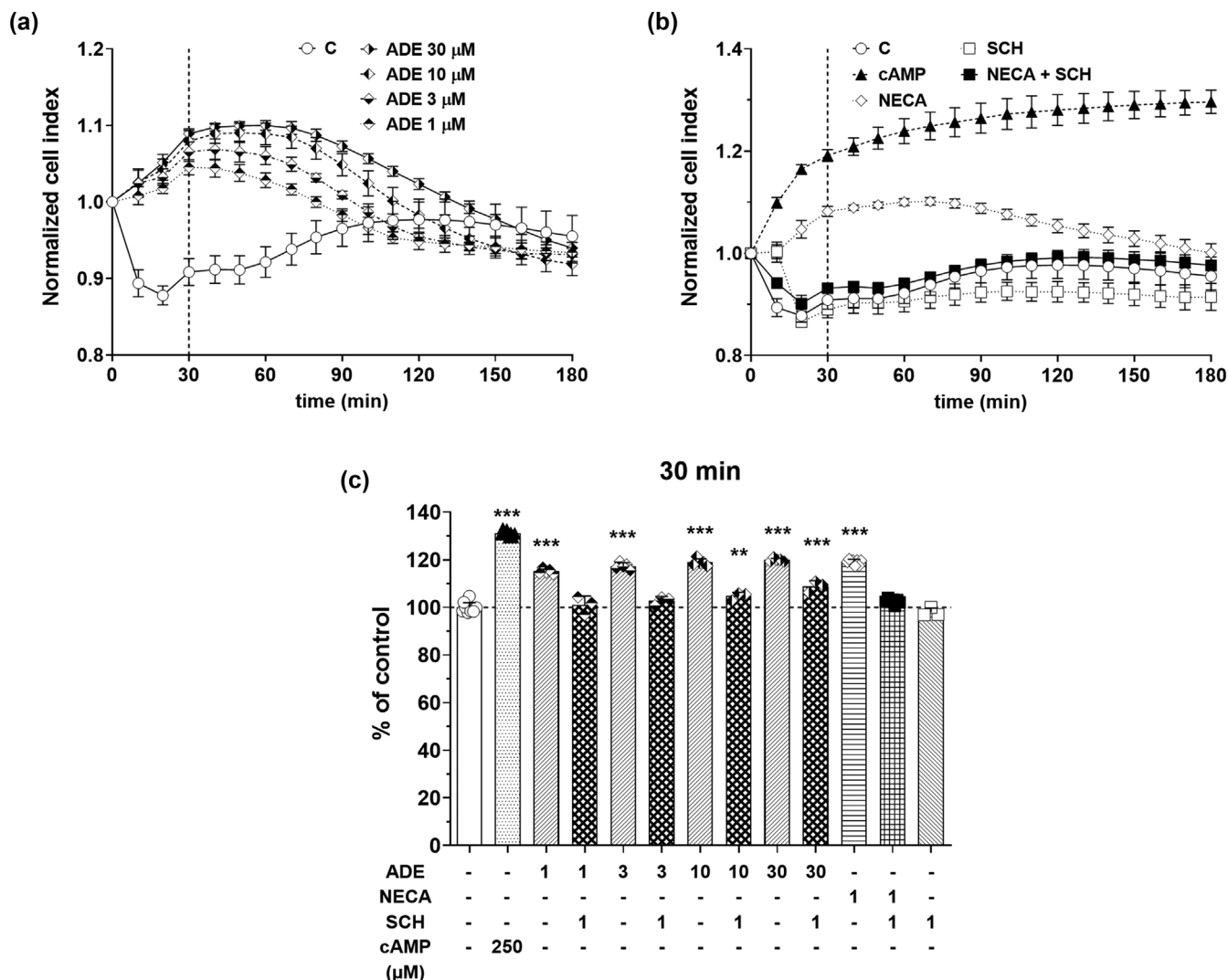


FIGURE 2 The effect of adenosine, adenosine receptor agonist NECA and A_{2A} adenosine receptor inhibitor SCH-58261 on the short-term impedance kinetics of rat brain endothelial cell monolayers. Cultured brain endothelial cells were treated (a) with different concentrations of adenosine (ADE, 1–30 μ M) and (b) with NECA (1 μ M) and/or SCH-58261 (SCH, 1 μ M) for 3 h. The control group (C) received culture medium. To tighten brain endothelial cell junctions, cyclic AMP (cAMP, 250 μ M) was used. (c) When normalized to the control group at the 30 min time point, the impedance of rat brain endothelial cells significantly increased due to stimulation of adenosine receptors, which was blocked by co-treatment with SCH-58261. Data are presented as mean $\pm$ SD. Control, cAMP and all NECA groups, $n = 8$; all ADE groups, $n = 5$; ADE+SCH groups, $n = 3$. *** $P < 0.001$, ** $P < 0.01$, significantly different from the control group; one-way ANOVA followed by Tukey's test.

sodium fluorescein (SF) in rodents injected with adenosine i.c. or i.c.v. In rats, we found that i.c. administration of adenosine decreased the extravasation of SF in the cortex and the hippocampus, compared to the control group that received only vehicle (Figure 6a).

The antagonist of the A_{2A} receptor showed a trend to inhibit the decrease in BBB permeability to SF induced by adenosine in both the cortex and the hippocampus (Figure 6a). SCH-58261 alone also decreased SF extravasation (Figure 6a), indicating that adenosine action on luminal A_{2A} receptors in physiological conditions may maintain BBB integrity. Similar effects were seen in mice: i.c. adenosine also decreased SF extravasation in the cortex and the hippocampus (Figure 6b). The co-administration of adenosine and the A_{2A}

antagonist blocked the SF extravasation in both brain regions. The i.c. administration of SCH-58261 did not affect the SF permeability in the cortex, while in the hippocampus, a small decrease was measured (Figure 6b).

The i.c.v. administration of adenosine increased SF extravasation into the cortex and the hippocampus in both rats and mice (Figure 6c–d). In rats, the i.c.v. co-administration of adenosine with SCH-58261 did not change the increased BBB permeability to SF in the cortex and the hippocampus (Figure 6c). In mice, the i.c.v. co-administration of adenosine and SCH-58261 blocked the increase in SF extravasation in the cortex, but not in the hippocampus (Figure 6d). In line with the results obtained in rats, i.c.v. injection of SCH-58261 increased the BBB permeability to SF in the cortex.

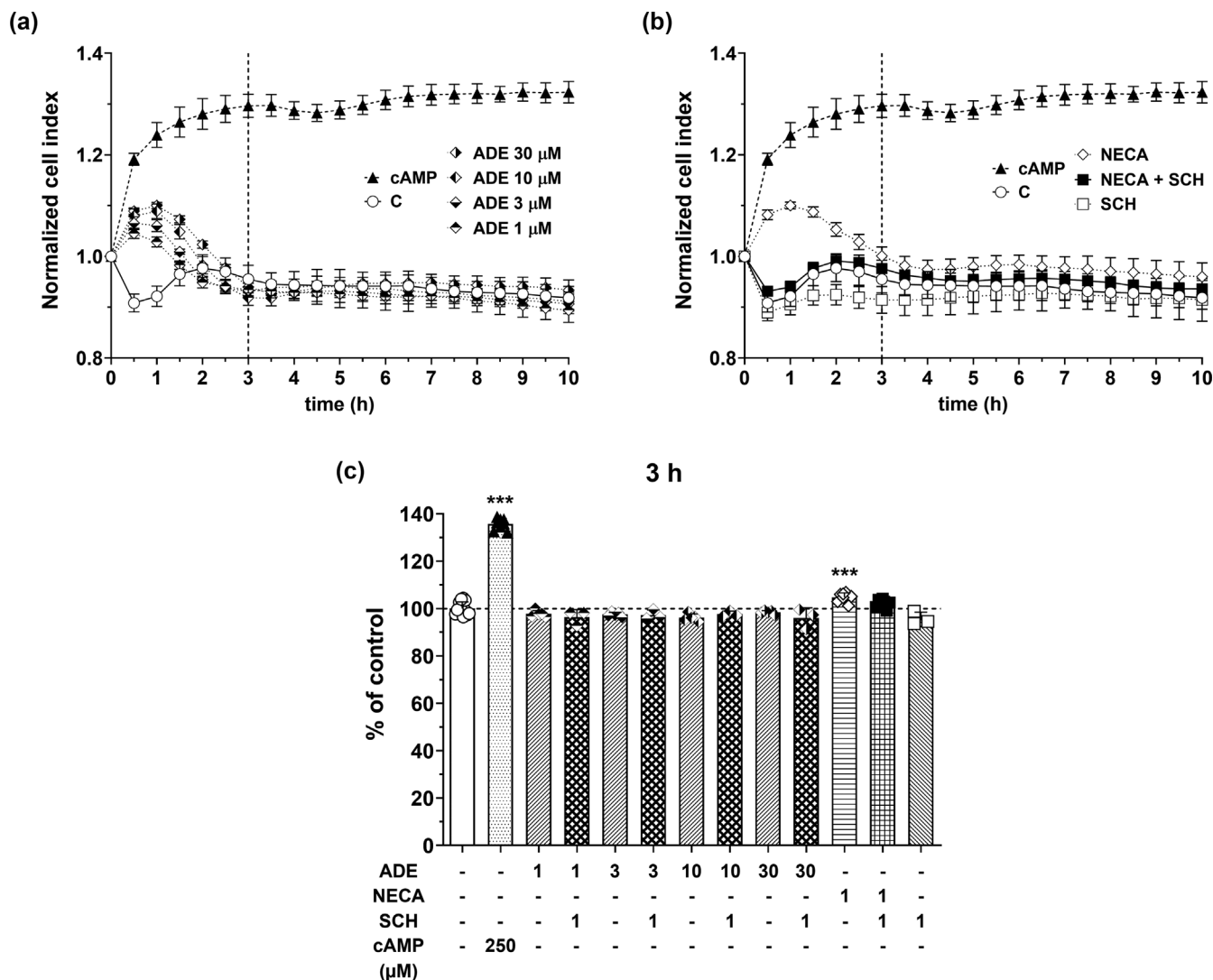


FIGURE 3 The effect of adenosine, adenosine receptor agonist NECA and A_{2A} adenosine receptor inhibitor SCH-58261 on the long-term impedance kinetics of rat brain endothelial cell monolayers. Cultured brain endothelial cells were treated (a) with different concentrations of adenosine (ADE, 1–30 μ M) and (b) with NECA (1 μ M) and/or SCH-58261 (SCH, 1 μ M) for 10 h. The control group (C) received culture medium. To tighten brain endothelial cell junctions, cyclic AMP (cAMP, 250 μ M) was used. (c) When normalized to the control group at the 3-h time point, the impedance of rat brain endothelial cells did not change significantly, except for NECA and the reference molecule cAMP. Data shown are means $\pm$ SD. Control, cAMP and all NECA groups $n = 8$, all ADE groups $n = 5$, ADE+SCH groups $n = 3$. *** $P < 0.001$, significantly different from the control group. One-way ANOVA followed by Tukey's test.

The antagonist had no effect in the hippocampus in mice (Figure 6d).

In the cerebellum of rats, a similar effect to the other brain regions was measured; the SF permeability was decreased when adenosine was given i.c. and increased after i.c.v. injection (Figure S4a). SCH-58261 inhibited the effect of adenosine injected i.c. but not when i.c.v. injections were given. SCH-58261 alone increased the BBB permeability for SF in the cerebellum for both administration routes. In mice, i.c. adenosine without or with SCH-58261 had no effect on BBB permeability in this brain region, whereas SCH-58261 alone increased it significantly (Figure S4b). In all three i.c.v. groups, a slight but significant BBB permeability elevation was measured in the cerebellum as compared to the vehicle group.

3.5 | Effects of adenosine on BBB permeability in sleep-deprived mice

Because of the key role of adenosine in sleep deprivation-induced BBB changes, we measured the effect of adenosine on BBB permeability in mice during the active and sleep periods and under sleep deprivation. We evaluated changes in the hippocampus, a sensitive brain region to sleep loss, and A_{2A} adenosine receptor expression is also increased in this region in sleep deprivation (Hurtado-Alvarado et al., 2016). Data show that i.c. adenosine decreased the BBB extravasation of SF only in the active phase, which was partly blocked by SCH-58261. We did not find differences in SF extravasation for the i.c. groups in the sleep period or after 12 h of sleep deprivation in the hippocampus in mice (Figure 7a).

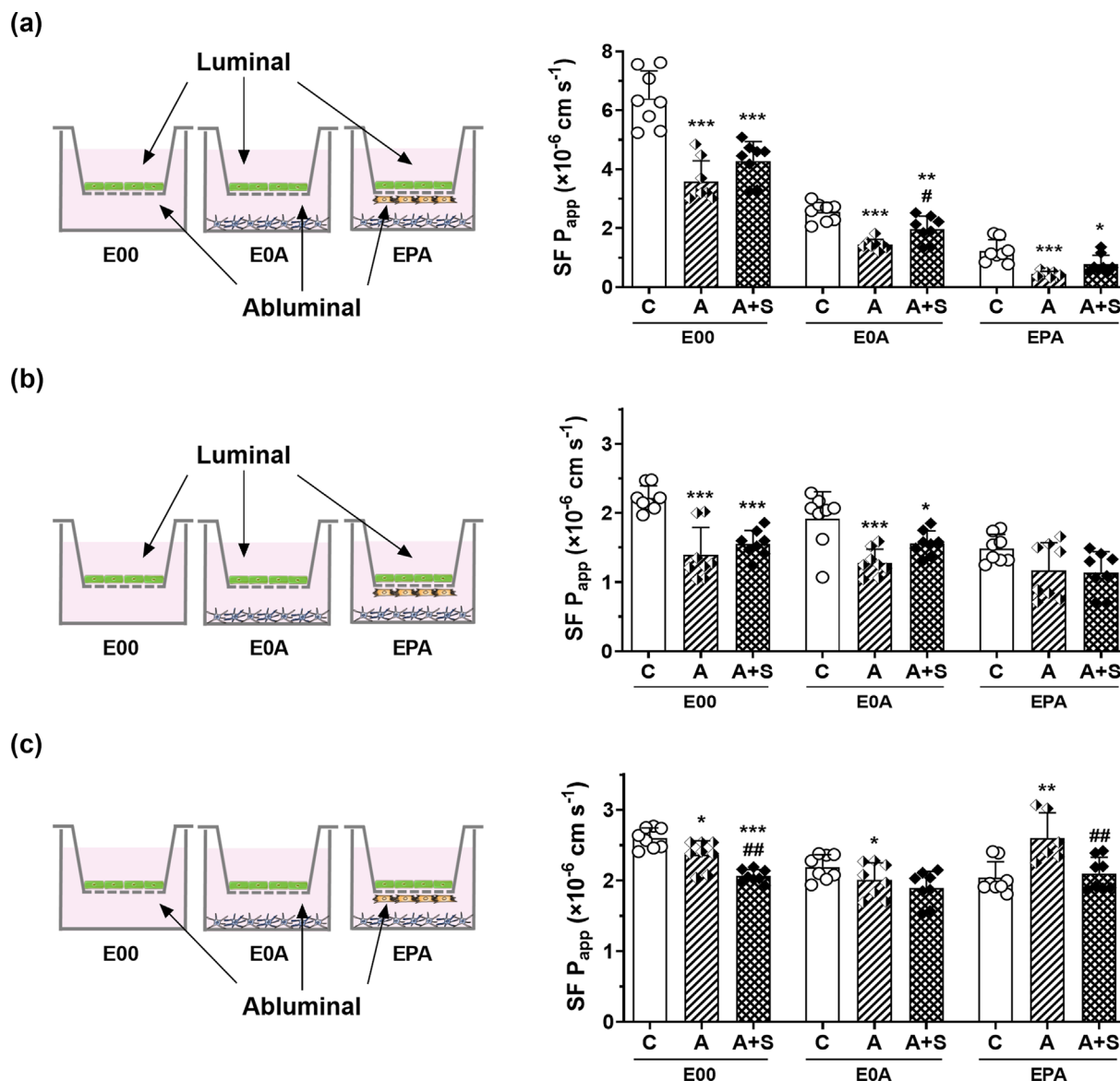


FIGURE 4 The effect of adenosine and A_{2A} adenosine receptor inhibitor SCH-58261 on the barrier function of rat BBB culture models measured by permeability (P_{app}) for small marker molecule sodium fluorescein (SF). Diagrams show rat brain endothelial cell monoculture (E00), double co-culture with astrocytes (E0A) and triple co-culture with brain pericytes and astrocytes (EPA). BBB models were treated with 30- μM adenosine (A) or co-treated with 1- μM SCH-58261 inhibitor (A + S) for 30 min in (a) both luminal and abluminal, (b) luminal or (c) abluminal fluid compartments. The control group (C) received culture medium. Data are presented as mean $\pm$ SD, $n = 8$. *** $P < 0.001$, ** $P < 0.01$, * $P < 0.05$, significantly different from the control group. ## $P < 0.01$, # $P < 0.05$, significantly different from the adenosine-treated group; one-way ANOVA followed by Tukey's test.

Notably, the administration of i.c.v. adenosine increased SF extravasation in the hippocampus in all three conditions, namely, in wakefulness, sleep and sleep deprivation groups (Figure 7b). Co-administration of adenosine and A_{2A} adenosine antagonist i.c.v. prevented the increase in BBB permeability for SF in sleep and sleep deprivation groups but not in wakefulness.

The rise in SF extravasation after i.c.v. administration of SCH-58261 was only observed in the wakefulness group (Figure 7b). For the in vivo permeability measurements, the F and the P values of the group comparisons for Figures 6, S3 and 7 are shown in Tables S1–S5.

4 | DISCUSSION

The focus of this work was to elucidate the role of the signalling molecule adenosine on BBB permeability. We used mono- and co-culture models of BBB to investigate the contribution of brain endothelial cells, astrocytes and pericytes. We measured the acute effects of adenosine injected systemically or directly to the CNS on the BBB permeability in the hippocampus, cerebral cortex and cerebellum in rats and mice. We found that adenosine exerts a polarized effect on both in vitro and in vivo models; it tightens the

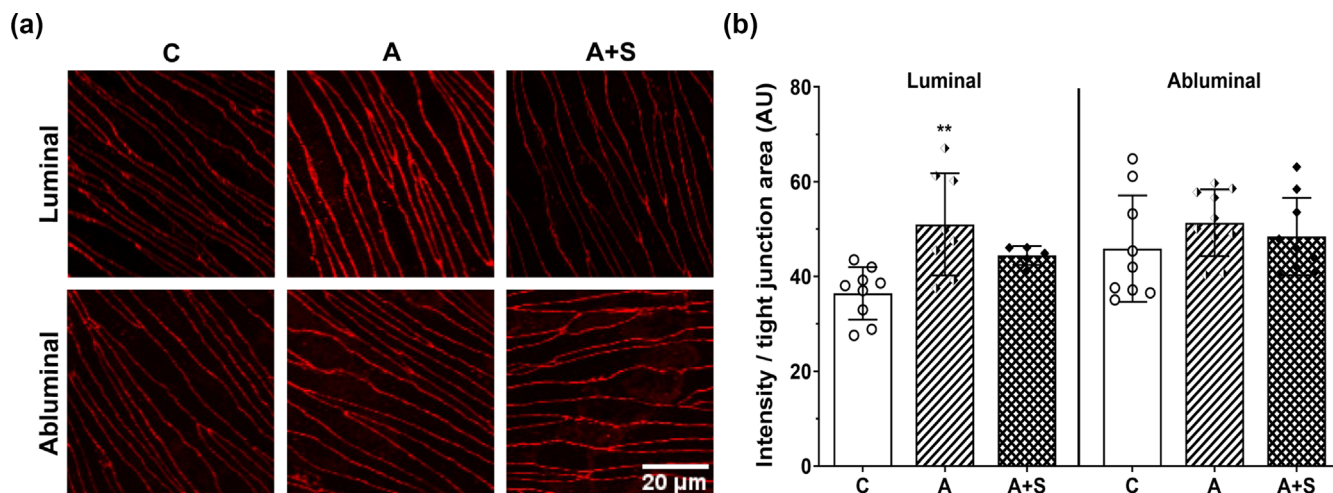


FIGURE 5 The effect of adenosine and A_{2A} adenosine receptor inhibitor SCH-58261 on the immunostaining of tight junction protein claudin-5 in rat brain endothelial cells (EPA model). Brain endothelial cells co-cultured with pericytes and astrocytes were treated with 30- μM adenosine (A) or co-treated with 1- μM SCH-58261 inhibitor (A + S) from the upper, luminal or lower, abluminal side of the culture filters for 30 min. The control group (C) received culture medium. (a) Representative confocal microscopy images of claudin-5 immunostaining. Scale bar: 20 μm . (b) The ratio (AU, arbitrary unit) of the intensity and the area of claudin-5 immunostaining calculated by MATLAB software. Data shown are means $\pm$ SD; all groups $n = 10$, except luminal A+S, which is $n=5$. ** $P < 0.01$, compared with the control group; one-way ANOVA followed by Tukey's test.

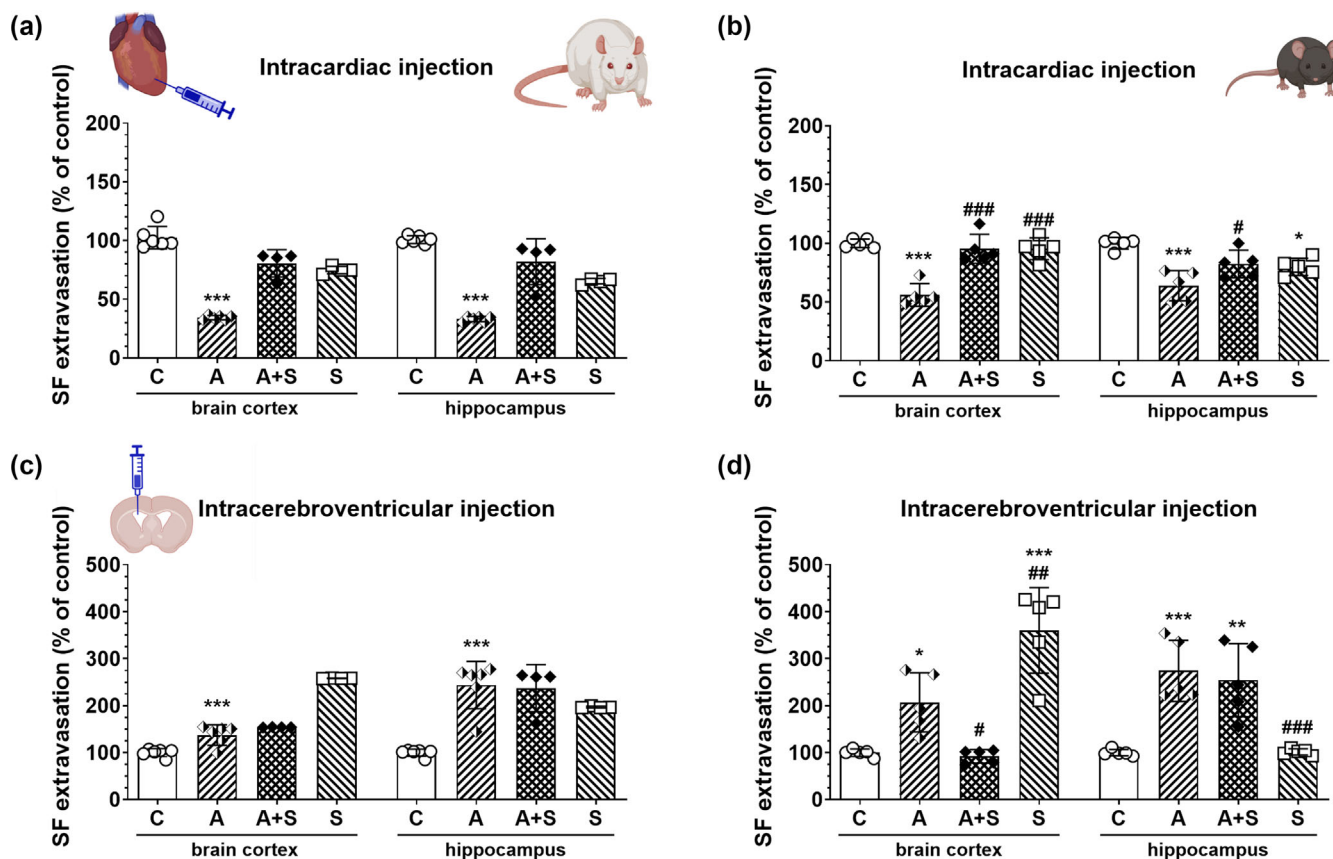


FIGURE 6 The effect of adenosine and A_{2A} adenosine receptor inhibitor SCH-58261 on BBB permeability in the brain cortex and the hippocampus. The permeability for sodium fluorescein (SF) was measured in rats (a,c) and mice (b,d) treated with adenosine (A) or SCH-58261 (S) alone or as co-treatment (A + S) administered (a,b) intracardially or (c,d) intracerebroventricularly at ZT3–ZT4. The control group (C) received vehicle. Data are presented as mean $\pm$ SD. In rats (a,c), C and A groups, $n = 6$; A+S and S groups, $n = 4$. In mice (b,d), all groups, $n = 5$. *** $P < 0.001$, ** $P < 0.01$, * $P < 0.05$, significantly different from the control group receiving vehicle. ### $P < 0.001$, ## $P < 0.01$, # $P < 0.05$, significantly different from the adenosine treated group; one-way ANOVA followed by Tukey's test.

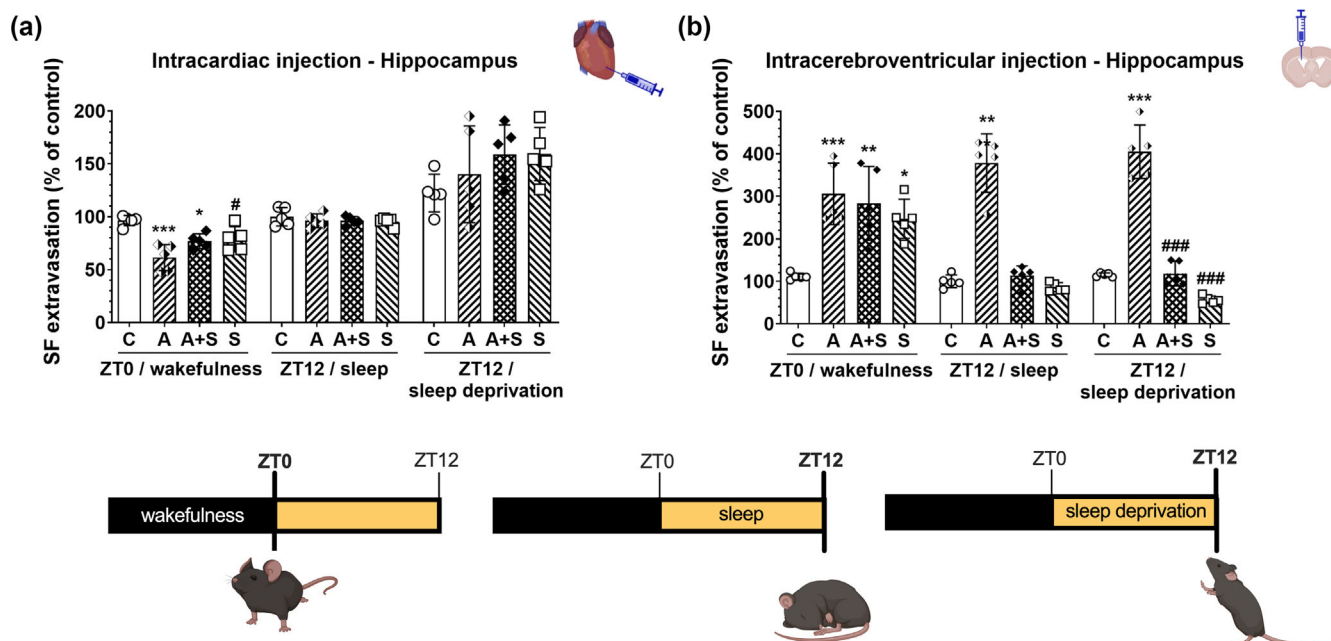


FIGURE 7 The effect of adenosine and A_{2A} adenosine receptor inhibitor SCH-58261 on BBB permeability in the hippocampus of mice with normal circadian rhythm or after sleep deprivation. The permeability for paracellular marker sodium fluorescein (SF) was measured in the hippocampus of mice treated with adenosine (A) or SCH-58261 in single (S) or co-treatment (A + S) administered (a) intracardially or (b) intracerebroventricularly at the end of the wakefulness period (ZT0), sleep period (ZT12/sleep) or after sleep deprivation (ZT12/sleep deprivation). The control groups (C) received vehicle. Data are represented as % of ZT12/sleep control group (mean $\pm$ SD), $n = 5$. *** $P < 0.001$, ** $P < 0.01$, * $P < 0.05$, significantly different from the respective control groups receiving vehicle. ### $P < 0.001$, # $P < 0.05$, significantly different from the adenosine treated groups; one-way ANOVA followed by Tukey's test.

paracellular barrier from the luminal side and increases fluorescein permeability from the abluminal or brain side. The abluminal, but not the luminal effect, was observed in sleep and in sleep deprivation. Based on the receptor profile, the results of co-culture experiments and the blocking effects of the A_{2A} receptor antagonist SCH-58261, the effect is mainly mediated luminally on brain endothelial, abluminally on brain endothelial and pericyte adenosine receptors.

Cells of the BBB actively synthesize and metabolize adenosine. As we showed in our previous study (Ceruti et al., 2011), brain endothelial cells, pericytes and astrocytes express ectonucleoside triphosphate diphosphohydrolase 1 and 2 enzymes (CD39/ENTPD1 and 2) producing adenosine. The CD73/NT5E ectonucleotidase is also expressed at the BBB (He et al., 2018; Vanlandewijck & Betsholtz, 2018). Adenosine is metabolized by deaminases (ADA1 and ADA2), expressed at the human BBB (Kutryb-Zajac et al., 2022; Yang et al., 2022), resulting in a short half-life in the extracellular space. In addition, adenosine (Murakami et al., 2005) and its analogues (Schaddelee, Voorwinden, Groenendaal, et al., 2003) are actively transported across the BBB by ENT/SLC29A1-4 and CNT/SLC28A1-3 nucleoside transporters (He et al., 2018; Vanlandewijck & Betsholtz, 2018; Yang et al., 2022). These mechanisms regulate local adenosine concentrations and signalling by adenosine receptors.

4.1 | Adenosine receptor profile in the cells of the BBB

The expression of adenosine receptors at the BBB is highly heterogeneous across cell types, species and contexts (Figure 8). In line with previous reports (Carman et al., 2011; He et al., 2018; Munji et al., 2019; Sabbagh et al., 2018; Schaffenrath et al., 2021; Song et al., 2020; Zhang et al., 2014), our study confirms that cultured brain endothelial cells from rodents show a high expression of *Adora2a*. Recent RNA-sequencing data show a high expression of *ADORA2A* in isolated human brain microvessels, as measured by bulk (Zhang et al., 2016), but not single-nucleus (Garcia et al., 2022; Yang et al., 2022) approaches, reflecting both species and methodological differences. *Adora2b* is expressed to a lesser extent, whereas *Adora1* and *Adora3* are not expressed in rat brain endothelial cells, similarly to cultured human brain endothelial cells (Feoktistov et al., 2002; Kim & Bynoe, 2015; Mills et al., 2011). Brain pericytes express *Adora1*, *Adora2a* and *Adora2b* in vitro and ex vivo, although the extent varies between studies. The most consistent expression of adenosine receptors is found in astrocytes, which highly express *Adora1* and *Adora2b* across studies and species.

At the BBB, *Adora3* is expressed only in astrocytes, and only in small amounts as reported by us and others (Bobermin et al., 2019; He et al., 2018; Vanlandewijck & Betsholtz, 2018; Yang et al., 2022;

Samples		Adora1	Adora2a	Adora2b	Adora3	Method	Reference
Brain endothelial cells							
rat	primary brain microvascular ECs	ND	+++	++	ND	qPCR	Present study
mouse	bEnd.3	+	+	NA	NA	WB	Carman et al., 2011
	primary brain microvascular ECs	+	+	NA	NA	WB	
	isolated brain microvessels	++	+	+	ND	bulk RNA-seq	Song et al., 2020
	sorted brain ECs	ND	+	ND	ND	bulk RNA-seq	Munji et al., 2019
		ND	+	ND	ND	bulk RNA-seq	Sabbagh et al., 2018
		ND	+	ND	ND	scRNA-seq	Vanlandewijck et al., 2018; He et al., 2018
		ND	+++	ND	ND	scRNA-seq	Zhang et al., 2014
human	HMEC-1	ND	+	+	ND	PCR	Feoktistov et al., 2002
	hCMEC/D3	+	+	+	+	qPCR	Mills et al., 2011
		NA	+	NA	NA	PCR, WB	Kim and Bynoe, 2015
	HBMVEC	NA	+	NA	NA	PCR, WB	
	isolated brain microvessels	+	++	+	ND	RNA-seq	Zhang et al., 2016
		+	ND	ND	ND	bulk RNA-seq	Song et al., 2020
	sorted brain ECs	+	++	+	++	bulk RNA-seq	Schaffnerath et al., 2021
		++	+	ND	+	scRNA-seq	Fekete et al., 2025
	isolated brain microvessels	ND	ND	ND	NA	snRNA-seq	Yang et al., 2022
		ND	NA	ND	NA	snRNA-seq	Garcia et al., 2022
Brain pericytes							
rat	primary brain PCs	++	++	+++	ND	qPCR	Present study
mouse	sorted brain PCs	ND	++	+	ND	scRNA-seq	Vanlandewijck et al., 2018; He et al., 2018
human	isolated brain microvessels	ND	+	+	NA	snRNA-seq	Yang et al., 2022
		+	NA	+	NA	snRNA-seq	Garcia et al., 2022
Brain astrocytes							
rat	primary brain Acs	++	++	+++	+	qPCR	Present study
		+	+	+	+	qPCR	Bobermin et al., 2019
mouse	sorted brain Acs	++	ND	++	ND	scRNA-seq	Vanlandewijck et al., 2018; He et al., 2018
		+	+	+++	ND	scRNA-seq	Zhang et al., 2014
human	isolated brain microvessels	++	+	+++	ND	RNA-seq	Zhang et al., 2016
		++	ND	+	NA	snRNA-seq	Yang et al., 2022
	sorted brain ACs	++	ND	+	ND	scRNA-seq	Fekete et al., 2025

FIGURE 8 Adenosine receptor expression in the cells of the BBB. Abbreviations: hCMEC/D3, human cerebral microvascular endothelial cells; HMEC-1, human microvascular endothelial cell line; MBMVEC, mouse brain microvascular endothelial cell line; NA, not applicable; ND, not detected; scRNA-seq, single-cell RNA sequencing; snRNA-seq, single-nucleus RNA sequencing. Red colour, expressed; blue colour, not detected.

Zhang et al., 2014, 2016), but is highly expressed in microglia (Fekete et al., 2025; Garcia et al., 2022; Vanlandewijck & Betsholtz, 2018; Yang et al., 2022). Overall, adenosine A_{2A} receptors are mainly expressed in brain endothelial cells and pericytes at the BBB. In pathological conditions, A_{2B} adenosine receptors may also play an important role, as hypoxia was shown to increase the expression of A_{2B} receptor on endothelial cells, which protected against vascular leakage in the mouse lung, colon, kidney and liver (Eckle et al., 2008). Although we cannot rule out a role for A_{2B} receptors, based on our present data, we propose that A_{2A} adenosine receptors are the key effectors on brain endothelial cells.

4.2 | Adenosine tightens the BBB from the luminal side

The polarized localization of transporters, channels, pumps and receptors is fundamental in the homeostatic function of the BBB

(Abbott, 2005). We have recently demonstrated that BBB maturation can be promoted by targeting three pathways that converge on Wnt/ β -catenin signalling and act via the tight junction protein claudin-5 (Porkoláb et al., 2024). The increase in paracellular barrier tightness, as the basis of morphological polarity, resulted in functional polarity demonstrated by the higher vectorial efflux pump activity.

Apicobasal polarized signalling effects at the BBB were observed for several vasoactive mediators including VEGFA (Dragoni & Turowski, 2018; Hudson et al., 2014) and transforming growth factor- $\beta 1$ (Raub, 1996) on brain endothelial cell monolayers, and histamine and lysophosphatidic acid on brain microvessels in vivo (Sarker et al., 1998, 2010). As adenosine acts on the cerebrovascular endothelium lumenally as a cardiovascular regulator and ablumenally as a neurotransmitter and sleep inducer, we hypothesized that its effects on the BBB barrier function are also asymmetrical.

When adenosine was administered lumenally in BBB permeability experiments in vitro and in vivo, the flux of paracellular small hydrophilic marker, fluorescein, decreased, indicating a tightening effect on

intercellular junctions. This direct effect on brain endothelial cells was brief, in agreement with the short half-life of adenosine. Adenosine induced a transient increase in brain endothelial cell layer impedance, which was the most pronounced in the first 2 h. Among the BBB models, the TEER was only increased in the brain endothelial cell and astrocyte co-culture model by adenosine, whereas the permeability was significantly reduced in all three types of BBB in vitro models. The luminal effect of adenosine was supported by morphological findings; the intensity of the junctional protein claudin-5 was increased in the BBB co-culture model.

The adenosine receptor agonist NECA, which has high affinity for A_{2A} adenosine receptors, exerted a similar short-term barrier-tightening effect. The actions of NECA were blocked by A_{2A} adenosine receptor antagonist SCH-58261, indicating a receptor-specific action. Caffeine, a general adenosine receptor blocker, had no effect alone on brain endothelial impedance but inhibited the actions of NECA. The effect of adenosine is mediated through cAMP signalling in many cell types (Chen et al., 2014). In our models, the action of cAMP on brain endothelial paracellular tightness was quick, similar to adenosine and NECA, but more pronounced and longer, in line with its well-known barrier-tightening effect on BBB models (Deli et al., 2005; Porkoláb et al., 2024).

The effect of adenosine on the barrier properties of brain endothelial cell cultures was not measured before. In previous studies, different adenosine receptor agonists, such as NECA, were used. On a bovine BBB co-culture model, adenosine agonists had no effects in the 1- to 5-h timescale on TEER and permeability (Schaddelee, Voorwinden, van Tilburg, et al., 2003). NECA and regadenosone reduced the relative TEER of human and mouse brain endothelial cell lines and mouse primary brain endothelial cells in monocultures (Carman et al., 2011; Kim & Bynoe, 2015) and increased the relative permeability of 10-kDa dextran (Kim & Bynoe, 2015). In bovine pulmonary endothelial cells, a peripheral endothelial model, luminal adenosine increased TEER by elevating pAkt and reorganizing the cytoskeleton (Verin et al., 2020) in line with our results on BBB models.

There might be several explanations for the diverse effects of adenosine or its analogues on BBB culture models. Despite published reviews and guidelines (Deli et al., 2005; Helms et al., 2016; Vigh et al., 2021), in most studies, baseline TEER and permeability values are given in different ways, making the direct comparison of the paracellular tightness of the BBB models difficult. The tightness of BBB models is the lowest in cell lines and monocultures; thus, co-cultures are used in physiological and pharmacological studies (Deli et al., 2005; Helms et al., 2016; Nakagawa et al., 2009). In BBB models with weak paracellular barriers, polarized signalling cannot be studied because of the paracellular leakage of the active molecule. The size of the paracellular marker molecules and the timescale were also different in the studies. For these reasons, it is challenging to assess the relationship between model tightness and the effects of signalling molecules on barrier properties and compare studies from different laboratories.

We confirmed the luminal effects of adenosine measured on BBB culture models in animal models, too. Adenosine given intracardially decreased the BBB permeability for fluorescein when measured at the

2-min time point. This short-term effect was measured in the brain cortex and hippocampal regions of Wistar rats and C57BL/6 mice and was antagonized by SCH-58261 in mice and showed a trend in rats, similar to the results from the BBB culture models.

In a previous study, adenosine given three times at every 20 s intravenously increased the extravasation of 10-kDa dextran in the whole brain of two C57BL/6 mice 1 min after treatment (Kim & Bynoe, 2015). NECA and regadenosone given to the retroorbital venous plexus increased BBB permeability for 10- and 70-kDa dextran in C57BL/6 mice and Sprague Dawley rats at the 15-min time point measured from whole homogenized brains (Carman et al., 2011). There are several important differences between these animal studies and our experiments. Firstly, the administration route in our study was intracardiac, whereas in all other in vivo experiments, adenosine analogues were given intravenously, resulting in a longer circulation time to reach the cerebrovasculature, which can be important considering the 2- to 4-min half-life of regadenosone. The other major difference is the tracer size: we used fluorescein (376 Da), which is 30- to 190-times smaller than 10- and 70-kDa dextran, respectively, that measures BBB paracellular permeability more sensitively. We investigated brain regions, whereas other studies used whole-brain homogenates. The paracellular permeability of the BBB for fluorescein is different in brain regions and reacts differently to pathological challenge as measured in newborn pigs (Ábrahám et al., 1999); therefore, small regional changes cannot be measured from whole brain homogenates. Pilot studies in humans do not support the results of animal experiments, suggesting that intravenously administered adenosine analogues increase BBB permeability. One study investigated the effects of intravenous regadenosone in FDA approved dose on the integrity of the BBB and found no change in brain extravasation of imaging agents measured by SPECT and CT in volunteers (Jackson et al., 2017). In a recent clinical trial, regadenosone did not increase the BBB permeability for gadolinium in the normal appearing white matter or in the brain adjacent to tumour in glioma patients in a consistent or significant way (Grossman et al., 2025). It is a limitation of our work that we focused only on paracellular permeability changes, and the activity or polarized membrane distribution of efflux pumps was not studied. For further discussion on adenosine and BBB transport of efflux pump ligands, please see the Supporting Information.

4.3 | Adenosine increases BBB permeability from the abluminal side

The abluminal adenosine effect depended on the complexity of the in vitro model. In the monolayer model, abluminal co-treatment with adenosine and SCH-58261 reduced permeability, indicating paracellular barrier tightening. In the EPA co-culture model, where pericytes were present in the abluminal compartment and the barrier was the tightest, paracellular permeability for fluorescein increased, which was blocked by SCH-58261, suggesting an indirect effect mediated by pericytes probably involving additional signalling pathways. Polarized

effects were already observed on brain endothelial monolayers, VEGFA (Dragoni & Turowski, 2018; Hudson et al., 2014) and transforming growth factor- β 1 (Raub, 1996) induced barrier leakage from the abluminal side, while the asymmetrical, abluminal effect of a histamine H₁ receptor agonist was demonstrated on brain microvessels in vivo (Sarker et al., 1998). In our study, the permeability-increasing effect of adenosine from the abluminal side was indirect and needed the presence of brain pericytes.

This asymmetrical effect of adenosine was measured in vivo, too. Adenosine given intracerebroventricularly increased BBB permeability both in the brain cortex and hippocampus in rats and mice. The BBB permeability elevation was ~3-fold, compared to the respective vehicle groups, in both rats and mice at all time points (ZT0, ZT3–4, ZT12) with no statistical difference between the groups (Figure S7). SCH-58261 blocked this action in the mouse brain cortex only and had itself a permeability-increasing effect, indicating an indirect abluminal adenosine effect via other perivascular cell types and mediators.

The effects of adenosine were not inhibited by SCH 58261 in all experimental conditions, but an interesting pattern was observed (Figure S8). The effects of intracardiac adenosine were inhibited by SCH 58261 at ZT3–4 but not in sleep or sleep deprivation, while the effects of intracerebroventricular adenosine were inhibited in ZT12/sleep and ZT12/sleep deprivation but not in ZT0. These observations indicate that circadian rhythm (wakefulness, sleep) and polarized luminal or abluminal mechanisms at the BBB are more important factors in the regulation of permeability than brain regional (cortex vs. hippocampus) or species differences.

Adenosine levels in the brain show a circadian change. At the end of the wakefulness period (ZT0), when endogenous adenosine levels are higher, fluorescein permeability in the hippocampus of mice was decreased luminally and increased abluminally by adenosine. The luminal effect was observed at ZT3–ZT4 but not at the end of sleep time or under acute, short-term sleep deprivation at ZT12. In contrast, abluminal adenosine increased hippocampal fluorescein permeability in all three conditions but could be inhibited by SCH-58261 only in sleep and sleep deprivation (Figure S8). These results support our previous data showing that increased BBB permeability in the hippocampus of chronic sleep-deprived rodents could be mediated by A_{2A} receptors and pericytes from the brain parenchymal or abluminal side (Avilez-Avilez et al., 2024; Hurtado-Alvarado et al., 2016 and 2018).

In conclusion, our data provide new knowledge regarding the polarized and circadian effects of adenosine on BBB paracellular permeability regulation under physiological conditions and help to understand how adenosine and its receptors are involved in barrier breakdown during pathophysiological conditions such as sleep restriction.

AUTHOR CONTRIBUTIONS

András Harazin: Writing—original draft; writing—review and editing; conceptualization; data curation; investigation; methodology; formal analysis; visualization. **Gabriela Hurtado-Alvarado:** Conceptualization; methodology; data curation; investigation; formal analysis; visualization; writing—original draft; writing—review and editing. **Lilla Barna:**

Methodology; data curation; investigation; formal analysis; writing—review and editing. **Arturo Contis-Montes de Oca:** Methodology; data curation; investigation; writing—review and editing. **Rocío Ruiz-Manzano:** Methodology; data curation; investigation; writing—review and editing. **Krisztián Laczi:** Methodology; data curation; investigation; writing—review and editing. **András Kincses:** Methodology; data curation; formal analysis; writing—review and editing. **Gergő Porkoláb:** Data curation; formal analysis; writing—original draft; writing—review and editing; methodology. **Gang Chen:** Supervision; conceptualization; writing—review and editing. **Beatriz Gómez-González:** Conceptualization; supervision; funding acquisition; writing—review and editing. **Mária A. Deli:** Conceptualization; supervision; funding acquisition; writing—original draft; writing—review and editing.

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ACKNOWLEDGEMENTS

We thank Dr. Enrique Becerril-Villanueva and the Vivarium of Facultad de Medicina, Universidad Nacional Autónoma de México, for donating the mice used for the experiments. We thank our late colleague Dr. Gábor Rákhely from the Institute of Biophysics, HUN-REN Biological Research Centre, for his help in the qRT-PCR by providing reagents and access to research infrastructure.

This work was funded by the National Research, Development and Innovation Office of Hungary (K143766, 2024-1.2.2-ERA_NET-2024-00018) and the Hungarian Academy of Sciences (NAP2022-I-6/2022). G.P. was supported by the National Academy of Scientist Education Program of the National Biomedical Foundation under the sponsorship of the Hungarian Ministry of Culture and Innovation, as

well as the European Union's Horizon 2020 research and innovation programme under the Marie Skłodowska-Curie grant agreement (HORIZON EUROPE Marie Skłodowska-Curie Actions) to G.P. (No. 101034252). G.H.A. was supported by the IBRO Rising Stars Award. The animal studies were partially funded by the Universidad Autónoma Metropolitana (UAM-14407033).

CONFLICT OF INTEREST STATEMENT

The authors declare no conflicts of interest.

DATA AVAILABILITY STATEMENT

The data that support the findings of this study are available from the corresponding author upon reasonable request. Some data may not be made available because of privacy or ethical restrictions.

DECLARATION OF TRANSPARENCY AND SCIENTIFIC RIGOUR

This Declaration acknowledges that this paper adheres to the principles for transparent reporting and scientific rigour of preclinical research as stated in the BJP guidelines for [Design and Analysis](#), and [Animal Experimentation](#), and as recommended by funding agencies, publishers and other organizations engaged with supporting research.

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How to cite this article: Harazin, A., Hurtado-Alvarado, G., Barna, L., de Oca, A. C.-M., Ruiz-Manzano, R., Laczi, K., Kincses, A., Porkoláb, G., Chen, G., Gómez-González, B., & Deli, M. A. (2026). Polarized effects of adenosine on blood-brain barrier integrity: Tightening from the luminal and opening from the abluminal side. *British Journal of Pharmacology*, 183(16), 4761–4777. <https://doi.org/10.1111/bph.70446>