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BRIEF COMMUNICATION

Human brain endothelial cells are responsive to adenosine receptor activation

Jeffrey H. Mills · Leah Alabanza · Babette B. Weksler · Pierre-Olivier Couraud · Ignacio A. Romero · Margaret S. Bynoe

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Abstract The blood–brain barrier (BBB) of the central nervous system (CNS) consists of a unique subset of endothelial cells that possess tight junctions which form a relatively impervious physical barrier to a large variety of blood components. Until recently, there have been no good *in vitro* models for studying the human BBB without the co-culture of feeder cells. The hCMEC/D3 cell line is the first stable, well-differentiated human brain endothelial cell line that grows independently in culture with characteristics that closely resemble those of resident human brain endothelial cells. As our previously published findings demonstrated the importance of adenosine receptor (AR) signaling for lymphocyte entry into the CNS, we wanted to determine if human brain endothelial cells possess the capacity to generate and respond to extracellular adenosine. Utilizing the hCMEC/D3 cell line, we determined that these cells express CD73, the cell surface enzyme that converts extracellular AMP to adenosine. When grown under normal conditions, these cells also express the A_1, A_{2A}, and A_{2B} AR subtypes. Additionally, hCMEC/D3 cells are responsive to extracellular AR signaling, as cAMP levels increase following the addition of the broad spectrum AR agonist 5′-*N*-ethylcarboxamidoadenosine (NECA). Overall, these results indicate that human brain endothelial cells, and most likely the human BBB, have the capacity to synthesize and respond to extracellular adenosine.

Keywords Human brain endothelial cells · Blood brain barrier · hCMEC/D3 cells · Adenosine receptors · CD73

J. H. Mills · L. Alabanza · M. S. Bynoe (✉)
Department of Microbiology and Immunology,
College of Veterinary Medicine, Cornell University,
Ithaca, NY 14853, USA
e-mail: msb76@cornell.edu

B. B. Weksler
Department of Hematology and Oncology,
Weill Medical College of Cornell University,
New York, NY 10021, USA

P.-O. Couraud
Département de BiologieCellulaire, Institut Cochin,
Paris, France

I. A. Romero
Department of Biological Sciences, The Open University,
Milton Keynes, UK

Introduction

The endothelial cells that make up the vasculature of the human central nervous system (CNS) are structurally and functionally unique in comparison to other endothelial cells in the body. CNS endothelial cells function at the microvascular level as the blood–brain barrier (BBB), which serves to protect the brain from a variety of potentially harmful blood-borne components, including toxins, viruses, bacteria, and cells [1, 2]. This evolutionarily conserved protective function of brain endothelial cells is achieved through specialized tight junctions that virtually seal off the brain from the blood circulation [3–5]. Complementing their structural barrier function, brain endothelial cells possess various transporters for vitamins, nutrients and metabolic precursors, which help to maintain proper physiologic brain function [6]. Therefore, the BBB functions both to protect the brain and maintain CNS homeostasis.

While the highly restrictive properties of the BBB are beneficial under normal conditions, situations do arise within the CNS when increased permeability/metabolite transport is advantageous. For example, when trauma or injury occurs within the brain, increased nutrient and

metabolic transport and phagocytic cell entry are required to promote tissue repair [7–12]. In addition, when cerebral viral or bacterial infections occur, the entry of immune cells into the CNS is required to help control the infection [13–15]. Therefore, brain endothelial cells comprising the BBB must be able to sense and respond to brain trauma/injury in order to adapt their function to best benefit the brain.

It is well known that damaged cells release certain factors that communicate their trauma to neighboring cells. One such factor is ATP. When a cell membrane is compromised, ATP is released into the extracellular space [16, 17]. This ATP can be converted into AMP, and subsequently AMP to adenosine, by the enzymatic action of the membrane-bound enzymes CD39 and CD73, respectively [18]. This extracellular adenosine can be "sensed" by cells that express any of the four G-protein coupled adenosine receptor (AR) subtypes (A_1, A_{2A}, A_{2B}, or A_3) [19]. For example, in the context of immune-cell-mediated inflammation, lymphocytes that express the A_{2A} AR are functionally inhibited in the presence of high levels of extracellular adenosine to prevent excessive inflammatory damage [20, 21]. The responsiveness of human brain endothelial cells to extracellular adenosine has yet to be determined.

In this study, we sought to determine if human brain endothelial cells (i.e. the human BBB) have the capacity to produce and respond to changes in extracellular adenosine. Therefore, we utilized the recently established hCMEC/D3 cell line, which is the first stable, well-differentiated, highly characterized human brain endothelial cell line that represents a reliable model system to study the function of the human BBB [22]. The hCMEC/D3 cells possess many of the same characteristics of primary human brain endothelial cells without the support of glial cells, such as astrocytes and pericytes [22]. For these reasons, hCMEC/D3 cells have been utilized in a variety of studies aimed at characterizing the properties of the human BBB [23–29]. Here, we show that hCMEC/D3 cells express the membrane-bound nucleosidic enzyme required for converting AMP to adenosine. We also show that hCMEC/D3 cells express AR subtypes, which are expressed at varying levels depending on the conditions in which the cells are cultured. Finally, we show that these human brain endothelial cells are responsive to AR agonist induced stimulation based on measurements of intracellular cAMP levels. Overall, our results suggest that human brain endothelial cells, and subsequently the human BBB, have the capacity to produce and respond to extracellular adenosine.

Materials and methods

hCMEC/D3 cells and culture conditions The hCMEC/D3 human brain endothelial cell line was established from endothelial cells (isolated from human brain temporal lobe of an adult female with epilepsy) that were subsequently transduced with hTERT and SV40 large T antigen for immortalization as previously described [22]. The hCMEC/D3 cells were grown in EBM-2 (Lonza) media supplemented with human fibroblast growth factor, epidermal growth factor, vascular endothelial growth factor, insulin-like growth factor, gentamycin, ascorbic acid, and 2.5% FBS at 37°C with 5.0% CO_2. Cells were used in most studies following the establishment of a confluent monolayer (as observed by light microscopy). In studies which called for growing/subconfluent conditions, cells were harvested at approximately 60–70% confluency, as observed by light microscopy. In studies which called for starved/stressed conditions, confluent cells (initially cultured in complete media) were cultured in EBM-2 media lacking FBS and growth factors for 18 h prior to harvest. For all experiments, cells were given fresh media (either complete or lacking FBS/growth factors) 18 h before harvesting.

Flow cytometry The hCMEC/D3 cells were grown to a confluent monolayer in T-75 flasks (BD Biosciences) and then mechanically scraped from the surface. The released cells were washed in PBS containing 5.0% BSA and 0.5% sodium azide and stained with primary fluorochrome-labeled antibodies specific for CD73 (BD Biosciences), CD39 (eBioscience), or an appropriate isotype control. Stained cells were run on the BD FACS Canto II and the results were analyzed utilizing the FlowJo flow cytometry analysis software (Tree Star).

Immunoblotting Confluent hCMEC/D3 cells were isolated using trypsin digestion and then lysed in reducing SDS-PAGE sample buffer (1×10^6 cells/200 μl buffer). Samples were forced through a 27-gauge needle to shear the DNA and then incubated at 100°C for 5 min. Samples were separated by 10% SDS-PAGE and proteins were electro-transferred onto nitrocellulose membranes. These membranes were blocked in 0.1% Tween 20 in Tris-buffered saline (TBST) containing 5% non-fat dry milk for 1 h at room temperature. The membranes were then incubated overnight at 4°C with primary monoclonal antibodies for human ARs (A_1, sc-7500; A_{2A}, sc-7502; A_{2B}, sc-7505; A_3, sc-7508 – Santa Cruz Biotechnology) in 5% BSA/TBST. After washing blots in TBST, antibody binding was detected with a horseradish peroxidase-conjugated secondary anti-goat antibody (Jackson Immunoresearch) in TBST containing 5% non-fat dry milk. Following 1 h incubation, membranes were washed in TBST and developed using a chemiluminescence-based detection system (SuperSignal; Thermo Scientific). To confirm equal loading of samples, membranes were stripped with Restore Western Blot

Stripping Buffer (Thermo Scientific) and subsequently reprobed with an anti-human β-actin monoclonal antibody (Rockland) and detected as described above.

Quantitative real-time PCR Using the Trizol (Invitrogen) extraction method, RNA was isolated from hCMEC/D3 cell monolayers. The cDNA was synthesized using the High-Capacity cDNA Reverse Transcription Kit (Applied Biosystems) and subsequent qPCR was performed using Kapa Sybr Fast (Kapa Biosystems) run on a BioRad CFX96 real-time qPCR machine. Primers specific for human adenosine receptors were used to determine gene expression levels, which were normalized to the averaged GapDH and β-actin housekeeping gene levels. The primers utilized are as follows: human A_1AR forward: 5′-GGA CCG CTA CCT CCG G-3′, human A_1AR reverse: 5′-GAA GGA GAG GAT CCA GCA-3′, human A_{2A}AR forward: 5′-CAT CGT CCT CTC CCA CAC CAA-3′, human A_{2A}AR reverse: 5′-GTG GCT GCG AAT GAT CTT GC-3′, human A_{2B}AR forward: 5′-TTC TGG CCG TGG CAG TC-3′, human A_{2B}AR reverse: 5′-AGG ACA GCA ATG ACC CCT-3′, human A_3AR forward: 5′-TCG CTG TGG ACC GAT ACT-3′, human A_3AR reverse: 5′-ACC AGC CAG CAA AGG CC-3′, human GapDH forward: 5′-GGT GGT CTC CTC TGA CTT CAA CA-3′, human GapDH reverse: 5′-GTT GCT GTA GCC AAA TTC GTT GT-3′, human β-actin forward: 5′-CGC TGC CCT GAG GCA C-3′, human β-actin reverse: 5′-TGT CCA CGT CAC ACT TCA T-3′. Melt curve analyses were performed to determine the specificity for each qPCR reaction.

cAMP assay The hCMEC/D3 cells were grown on 24-well plates (BD Falcon) to a confluent monolayer, which was maintained for at least 24 h. Cells were then treated for 15 min alone, or in combination with the following drugs: the broad spectrum AR agonist 5′-*N*-ethylcarboxamidoadenosine (NECA; Tocris), the A_{2A} AR selective antagonist SCH58261 (Tocris), the A_{2B} AR selective antagonist PSB 603 (Tocris), the A_{2A} AR selective agonist CGS 21680 (Tocris), the A_1 AR selective agonist 2-chloro-N6-cyclopentyladenosine (CCPA; Tocris), vehicle (DMSO/Media), or forskolin (Tocris) plus IBMX (Tocris), which served as a positive control for inducing increases in cAMP in hCMEC/D3 cells. The medium was then aspirated off and 100 μl of cold Assay/Lysis buffer (Applied Biosystems) was added to each well to stop the reaction. Utilizing the Applied BiosystemsTropix cAMP Screen Immunoassay System according to manufacturer instructions, cAMP levels were determined by chemiluminescence measured using the Veritas Microplate Luminometer (Turner BioSystems). Absolute cAMP concentrations were derived from a standard curve.

Statistical analysis The determination of significant differences was established through the use of the Student *T* test, when $p<0.05$. Error bars within graphs are displayed at the standard error of the mean.

Results

hCMEC/D3 human brain endothelial cells express CD73, but not CD39

Following our recent findings in mice that suggest that AR signaling influences the ability of lymphocytes to overcome the BBB to gain entry into the CNS in a mouse model of multiple sclerosis (MS) [30], we wanted to determine if the human BBB has the ability to produce and respond to extracellular adenosine. Therefore, we utilized the hCMEC/D3 human brain endothelial cell line, which is well-characterized and reported to very closely mimic the phenotype and properties of *in situ* human brain endothelial cells [22]. However, it is not known whether these cells possess the membrane-bound enzymes necessary to catalyze the formation of adenosine from extracellular ATP. To assess this, the hCMEC/D3 cells were grown under normal conditions (see "Materials and methods") until a confluent monolayer was formed. To preserve membrane protein expression, a cell scraper (instead of trypsin digestion) was utilized to harvest the cells. The isolated hCMEC/DE cells were then stained with fluorescently labeled antibodies for CD39, which hydrolyzes ATP to ADP and ADP to AMP, and CD73, which hydrolyzes AMP to adenosine, or isotype control antibodies (Fig. 1). Based on flow-cytometric analysis, hCMEC/D3 cells express CD73 (Fig. 1a), but not CD39 (Fig. 1b). These results suggest that human brain endothelial cells possess the capacity to synthesize adenosine from extracellular AMP.

hCMEC/D3 human brain endothelial cells express adenosine receptors, which depend on growth and culture conditions

Since hCMEC/D3 cells have the capacity to catalyze the formation of extracellular adenosine, we next determined if these cells also express ARs. The hCMEC/D3 cells were grown under normal conditions to confluency, and the total protein was harvested. Based on western blot analysis, hCMECD/D3 cells express the A_1, A_{2A}, and A_{2B} ARs, but lack the A_3 AR (Fig. 2). These results were confirmed by quantitative real-time PCR (qPCR) analysis as hCMEC/D3 cells harvested at confluency also only express the A_1, A_{2A}, and A_{2B} ARs (Fig. 3). However, based on our qPCR analysis, we determined that growth conditions can influ-

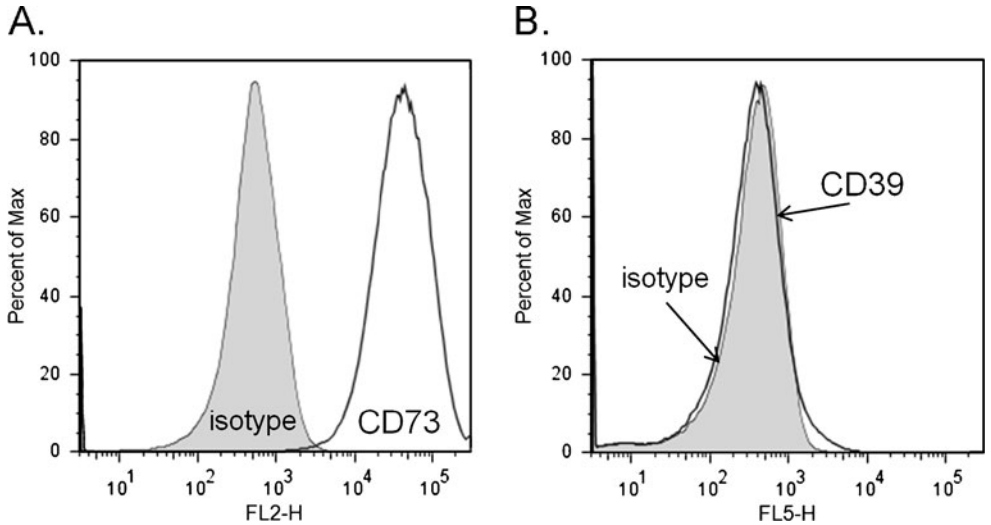

Fig. 1 The hCMEC/D3 human brain endothelial cell line expresses the enzyme capable producing extracellular adenosine. **a**–**b** The hCMEC/D3 cells were grown in T-75 flasks and harvested by mechanical dissociation. The cells were stained with fluorescent antibodies specific for **a** human CD73, **b** human CD39, or an isotype control. The stained cells were analyzed by flow cytometry and displayed as histograms as determined by forward versus side scatter plots (*shaded histogram* isotype control staining, *open histogram* CD73 or CD39 staining). These results are representative of two separate studies

ence the expression of ARs in hCMEC/D3 cells. For example in growing cells (harvested prior to confluency), the human brain endothelial cells expressed only the A_{2A} and A_{2B} ARs (Fig. 3). When the hCMEC/D3 cells reached a confluent state, both the A_{2A} and A_{2B} genetic expression increased (Fig. 3b and c), while A_1 AR expression was induced (Fig. 3a). Additionally, when the hCMEC/D3 cells were grown in media that lacked serum and growth factors, the stressful conditions led to increases in the expression of the A_1, A_{2A}, and A_{2B} ARs (Fig. 3a–c). The A_3 AR was not expressed in hCMEC/D3 cells grown in any condition (Fig. 3d). Therefore, the expression of ARs on hCMEC/D3 cells suggests that human brain endothelial cells have the potential to respond to extracellular adenosine.

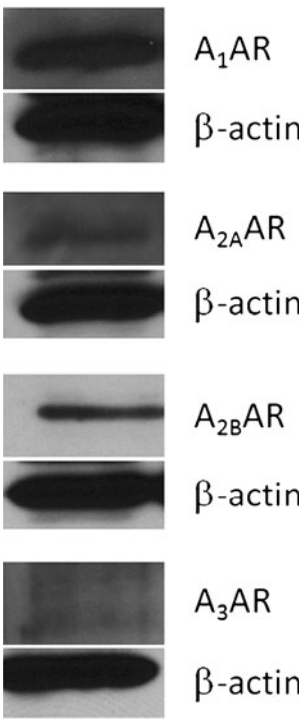

Fig. 2 The hCMEC/D3 human brain endothelial cell line expresses adenosine receptors. The hCMEC/D3 cells were grown to confluency, isolated, and then lysed for subsequent western blot analysis utilizing antibodies specific to the four human AR subtypes (A_1, A_{2A}, A_{2B}, and A_3). To confirm equal loading of samples, membranes were stripped and reprobed with an anti-human β-actin monoclonal antibody

Adenosine receptor signaling in hCMEC/D3 cells can modulate cAMP levels

As we have shown that hCMEC/D3 cells express the enzyme necessary to produce extracellular adenosine (Fig. 1) and membrane receptors that can bind adenosine (Figs. 2 and 3), we next determined if hCMEC/D3 cells are responsive to AR stimulation. As AR activation can differentially regulate intracellular cAMP levels (A_{2A} and/or A_{2B} ARs stimulation increases cAMP levels while A_1 and/or A_3AR stimulation decreases cAMP levels) [19, 31], cAMP was measured in hCMEC/D3 cells following treatments with various AR agonists and antagonists. Treatment with NECA, a broad spectrum AR agonist, significantly increased cAMP levels in hCMEC/D3 cells compared to a DMSO vehicle control (Fig. 4a). The NECA induced increases in cAMP levels could be inhibited in hCMEC/D3 cells if selective antagonists for the A_{2A} AR (SCH 58261) and/or the A_{2B} AR (PSB 603) were given along with NECA treatment (Fig. 4a). These results suggest that both A_{2A} and A_{2B} receptor activation are involved in NECA induced cAMP increases in hCMEC/D3 cells. Forskolin treatment was used as a positive control for cAMP induction (Fig. 4a). To determine if the A_1 AR

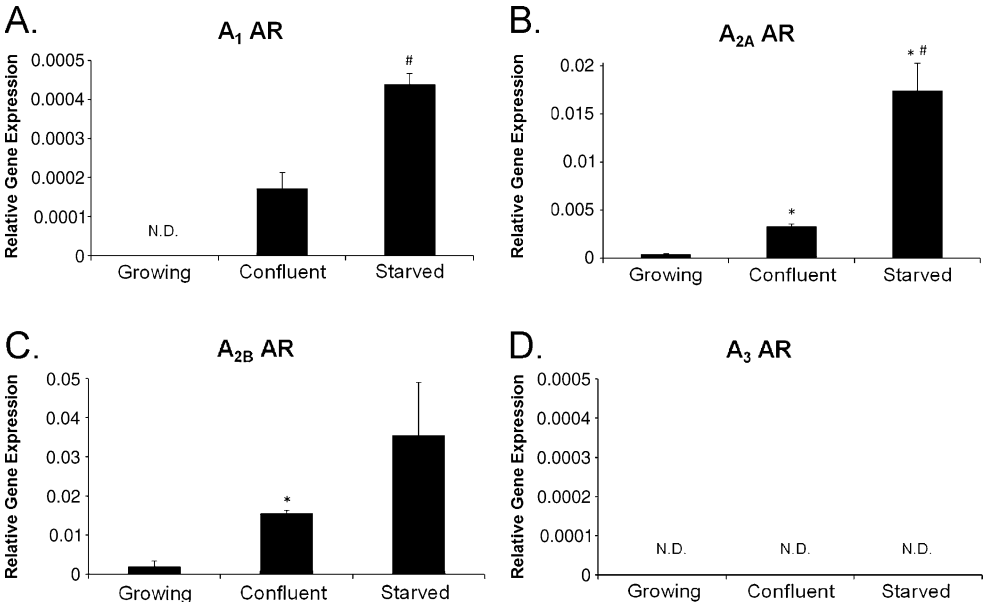

Fig. 3 The hCMEC/D3 human brain endothelial cell line genetic expression of adenosine receptors is dependent on growth conditions. **a**–**d** The hCMEC/D3 cells were grown in six-well plates and harvested at 60–70% confluency (*Growing*), at 18 h after fresh medium was added to confluent cells (*Confluent*), or at 18 h after medium lacking serum and growth factors was added to confluent cells (*Starved*). The RNA was extracted and cDNA was synthesized. Primers specific for the four human AR subtypes (A_1, A_{2A}, A_{2B}, and A_3) were used to determine gene expression levels. Expression results measured in triplicate were normalized to averaged GapDH and β-actin housekeeping gene levels (*N.D.* not detected). *Error bars* represent the standard error of the mean (n=4). Statistically significant differences compared to "Growing" condition ($^{*}p<0.05$) and compared to "Confluent" condition ($^{\#}p<0.05$) are displayed. The results are representative of two separate studies

expressed on hCMEC/D3 cells is functional, we utilized the A_1 AR agonist CPPA. While hCMEC/D3 cells treated with the A_{2A} AR selective agonist CGS 21680 induced increases in cAMP levels, A_1 AR activation in hCMEC/D3 cells given CCPA significantly prevented increases in cAMP levels when given with CGS 21680 (Fig. 4b). Overall, our results indicate that human brain endothelial cells not only have the capacity to generate extracellular adenosine, but also possess functional ARs that are able to alter intracellular cAMP levels. Therefore, this data suggests that the human BBB, which is made up of endothelial cells physiologically similar to the hCMEC/D3 cell line [22], is able produce and respond to extracellular adenosine.

Discussion

In this study, we show that the hCMEC/D3 human brain endothelial cell line has the capacity to synthesize (via expression of CD73) and respond (via expression of A_1, A_{2A}, and A_{2B} ARs) to extracellular adenosine. As the hCMEC/D3 cell line represents an *in vitro* model system for studying and characterizing the human BBB, our study suggests that the BBB is responsive to AR signaling. These results are not surprising as there have been several studies which associate AR modulation with the progression of human neurological disorders associated with a compromised BBB. For example, there is evidence that the pathogenesis of Alzheimer's disease (AD), Parkinson's disease (PD), and MS are associated with a dysfunctional BBB to varying degrees [1, 32–37]. Additionally, it has been shown that caffeine, which is a broad spectrum AR antagonist, has protective effects against the development of these diseases in various animal model systems [30, 38–42]. In an MPTP neurotoxin mouse model of PD, caffeine was shown to block leakage of Evan's blue dye across the BBB [42]. Caffeine has also been shown to protect against high-cholesterol induced disruptions of the BBB barrier in a rabbit AD model system [38]. Additionally, caffeine and specific AR antagonists have been shown to protect against the development of MS and its associated lymphocyte CNS infiltration in mouse and rat MS models [30, 40, 41]. As our results demonstrate that human brain endothelial cells are responsive to AR-mediated signaling, these findings support those utilizing animal models which suggest that AR modulation can be used as a potential therapy for neurological diseases associated with BBB dysfunction.

The brain endothelial cells that make up the BBB of the CNS are considered a specialized cellular subset that have unique functional properties (especially in regards to barrier function) as compared to endothelial cells located outside the CNS. While it has been shown that various peripheral endothelial cell subtypes express both ARs and adenosine-

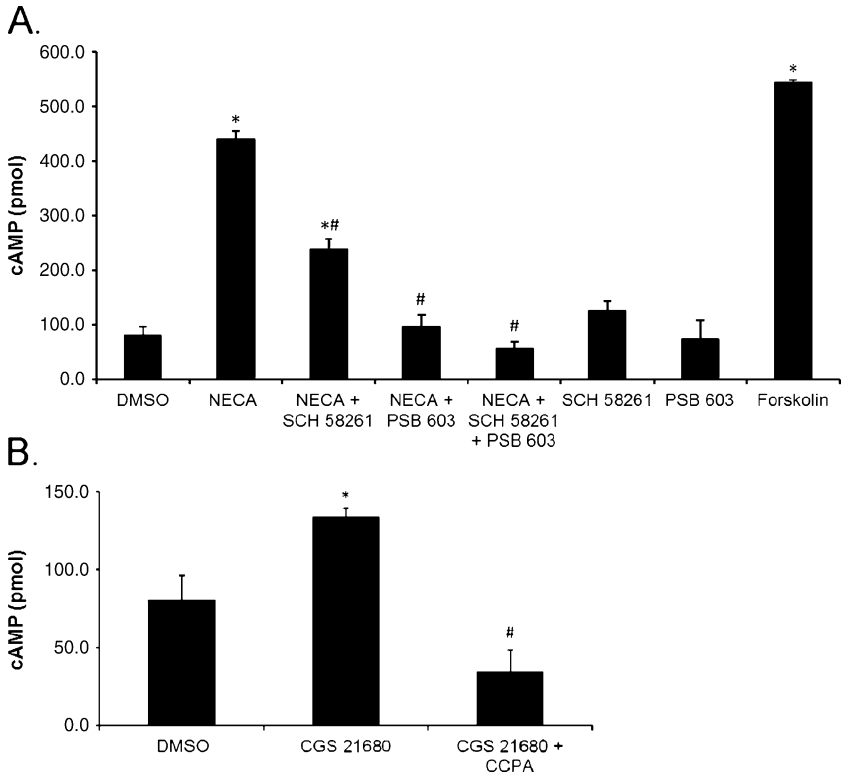

Fig. 4 The hCMEC/D3 human brain endothelial cell line produces cAMP following adenosine receptor activation. **a**–**b** The hCMEC/D3 cells were grown in 24-well plates and treated with **a** NECA (1 μM; broad spectrum AR agonist), NECA (1 μM) plus SCH 58261 (1 μM; selective A_{2A} AR antagonist), NECA (1 μM) plus PSB 603 (1 μM; selective A_{2B} AR antagonist), NECA (1 μM) plus SCH 58261 (1 μM) and PSB 603 (1 μM), SCH58261 (1 μM) alone, PSB 603 (1 μM) alone, Forskolin (1 μM; cAMP inducer/positive control) plus IBMX (500 μM; phosphodiesterase inhibitor), or DMSO (vehicle control) or **b** CGS 21680 (1 μM; selective A_{2A} agonist), CGS 21680 (1 μM) plus CCPA (1 μM; selective A_1 agonist), or DMSO. Fifteen minutes following treatment, cells were lysed and analyzed for cAMP levels. *Error bars* represent the standard error of the mean (n=4). Statistically significant differences compared to DMSO vehicle control (*p<0.05) and compared to NECA or CGS 21680 (#p<0.05) are displayed

associated enzymes, the responsiveness of brain endothelial cells to adenosine cannot be inferred from peripheral cells. For example, it has been reported that human microvascular endothelial cells (HMEC-1) primarily express the A_{2B} AR (with low levels of the A_{2A} AR, and no A_1 or A_3 AR expression) while human umbilical vein endothelial cells (HUVEC) highly express the A_{2A} AR (with low levels of the A_{2A} AR, and no A_1 or A_3 AR expression) [43]. Here, we show by mRNA and protein analysis that the A_1, A_{2A}, and A_{2B} AR subtypes are expressed on confluent human brain endothelial cells (Figs. 2 and 3). The lack of A_3 AR expression on hCMEC/D3 cells is interesting as this receptor is commonly found in a wide variety of cells [44–46], as low A_3 AR mRNA levels can still result in high A_3 AR protein expression [46]. The fact that we observe A_1, A_{2A}, and A_{2B} expression suggests that brain endothelial cells may have a more complex degree of regulation in response to extracellular adenosine signaling compared to peripheral endothelial cells. For instance, since the A_1 and A_3 ARs are coupled to $G_{\alpha i}$ subunits (inhibit adenylate cyclase), while the A_{2A} and A_{2B} ARs are coupled to $G_{\alpha s}$ subunits (stimulate adenylate cyclase), the presence of A_1/A_3 ARs along with A_{2A}/A_{2B} ARs on a cell indicate that the cellular response to extracellular adenosine will depend on a variety of factors. For example, since the A_1 and A_{2A} ARs have different affinities for adenosine [47], the concentration of extracellular adenosine may determine how a cell will respond. Low adenosine concentrations can activate the A_1 AR (which has a high affinity for adenosine) which will result in adenylate cyclase inhibition, while high concentrations of adenosine are required to activate the A_{2A} AR (which has a low affinity for adenosine) which will result in adenylate cyclase stimulation [48]. In the CNS of mice, there is supporting evidence that suggests that this A_1/A_{2A} AR interaction is relevant to neurological diseases, as mice that lack A_1 AR develop a severe form of MS [40], while mice that are given an A_{2A} AR antagonist are protected from MS-like disease development [30]. The potential for this complex interplay between differentially coupled AR G-protein subtypes is consistently observed in brain endothelial cells: human brain endothelial cells (express A_1 and A_{2A}/A_{2B} AR; Fig. 2), bovine brain endothelial

cells (express A_3 and A_{2A} AR; [49]), and mouse brain endothelial cells (express A_1 and A_{2A} AR; unpublished observations).

An interesting observation from our study is that culturing/growth conditions have a strong influence on the expression of adenosine receptors on human brain endothelial cells. For instance, actively growing (subconfluent) hCMEC/D3 cells lack the gene expression of the A_1 AR, which is subsequently induced when cells form a monolayer (Fig. 2a). To our knowledge, this is the first reported observation of this phenomenon with AR expression on cells in culture. However, it is not novel for cell confluency to have an effect on protein levels in endothelial cells [50]. For example in corneal endothelial cells, the expression of KCNC3, which encodes for the potassium channel, voltage-gated, Shaw-related subfamily member 3, has been shown to increase as cell cultures become confluent [50]. Additionally, when brain endothelial cells are grown *in vitro* and allowed to form monolayers, they begin to take on the characteristics (i.e., specialized tight junctions) that resemble the *in vivo* BBB [22]. However, the fact that only confluent hCMEC/D3 cells express the A_1 AR suggest that BBB-like monolayers are subjected to more complex degree of regulation in regards to AR signaling (i.e., A_1 versus A_{2A}/A_{2B} AR modulation of adenylate cyclase activity) as compared to growing/subconfluent cells (A_{2A}/A_{2B} ARs both activate adenylate cyclase). These results suggest that growth conditions may dictate how and if cells will respond to AR stimulation.

Not surprisingly in our study, when hCMEC/D3 were subjected to stress conditions (grown in media without serum/growth factors), the genetic expression of the A_1, A_{2A}, and A_{2B} ARs were all increased. Previous studies have shown that various forms of cellular stress can induce AR expression in cells [51]. For example, oxidative stress has been shown to increase expression of A_1 AR in smooth muscle cells [52] and A_3 AR in epithelial cells [53]. Hypoxia has been shown to increase A_{2B} AR [54], while immune stressors, such as IL-1β and TNFα, upregulate A_{2A} and A_{2B} AR on smooth muscle and epithelial cells [55, 56]. It has been theorized that one function of ARs is to act as extracellular sensors of "danger." For example, adenosine levels found in the extracellular space are normally in the nanomolar range under physiological conditions, but rise significantly when cells are stressed or undergo damage [51]. When a cell membrane is damaged, intracellular ATP (normally in the millimolar range) spills out into the extracellular space, where it can be quickly converted to adenosine if both CD39 and CD73 are present. However, the half-life of extracellular adenosine is only a few seconds due to the activity of adenosine deaminase (quickly degrades adenosine to inosine) and the presence of equilibrative and concentrative nucleoside influx transporters on cells [57, 58]. Therefore, the presence of high levels of extracellular adenosine can only act as a local signal of cell damage/stress, which can be detected if neighboring cells express ATP metabolic enzymes (CD39 and CD73) and ARs. As for human brain endothelial cells, while we show that both CD73 and ARs are expressed on these cells, CD39 expression is absent (Fig. 1). However, it has been reported that CD39 is highly expressed in the brain in a variety of species [59–62], including humans [59], with the most likely sources being astrocytes, pericytes, and neurons. As astrocytes and pericytes are in close contact with the BBB, CD39 expression on these cells should be functionally sufficient to compensate for its absence on brain endothelial cells. Therefore, the presence of CD73 and ARs by human brain endothelial cells suggests that the BBB is equipped to respond cell damage through an AR-mediated mechanism.

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