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Amyloid-β-induced occludin down-regulation and increased permeability in human brain endothelial cells is mediated by MAPK activation
Amyloid-β-induced occludin down-regulation and increased permeability in human brain endothelial cells is mediated by MAPK activation

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Abstract

Vascular dysfunction is emerging as a key pathological hallmark in Alzheimer’s disease (AD). A leaky blood-brain barrier (BBB) has been described in AD patient tissue and in vivo AD mouse models. Brain endothelial cells (BECs) are linked together by tight junctional (TJ) proteins, which are a key determinant in restricting the permeability of the BBB. The amyloid beta (Aβ) peptides of 1-40 and 1-42 amino acids are believed to be pivotal in AD pathogenesis. We therefore decided to investigate the effect of Aβ 1-40, the Aβ variant found at the highest concentration in human plasma, on the permeability of an immortalised human BEC line, hCMEC/D3. Aβ 1-40 induced a marked increase in hCMEC/D3 cell permeability to the paracellular tracer 70 kDa FITC-dextran when compared to cells incubated with the scrambled Aβ 1-40 peptide. Increased permeability was associated with a specific decrease, both at the protein and mRNA level, in the TJ protein occludin, whilst claudin-5 and ZO-1 were unaffected. JNK and p38MAPK inhibition prevented both Aβ 1-40-mediated down-regulation of occludin and the increase in paracellular permeability in hCMEC/D3 cells. Our findings suggest that the JNK and p38MAPK pathways might represent attractive therapeutic targets for preventing BBB dysfunction in AD.

Keywords: Alzheimer’s disease, amyloid beta, blood-brain barrier, brain endothelial cells, occludin, JNK, p38MAPK.
Introduction

The blood-brain barrier (BBB) restricts the movement of hydrophilic molecules across brain capillaries [1]. Cerebral capillaries are lined with brain endothelial cells (BECs), which are sparingly covered by pericytes on the abluminal membrane. BECs and pericytes are anchored within a basal lamina, which is surrounded by astrocytic end feet. The intimate association between astrocytes, pericytes and BECs is often referred to as the neurovascular unit as each cell type is important for the physiological function of the BBB [2]. BECs are interconnected by adherens and tight junctions (TJs), which confer the low permeability of the BBB. Whereas vascular adherens junctions are ubiquitous, continuous TJ are not found in endothelial cells in most other tissues. TJs consist of the transmembrane proteins junctional adhesion molecule 1 (JAM-1), occludin and claudin (cldn) family members, and of intracellular proteins, namely zona occludens family members (ZO-1, 2), cingulin, AF-6, and 7H6 [2], which serve to anchor the TJ to the actin cytoskeleton. Increased BBB permeability is correlated with disruption of TJ protein organization in many diseases including multiple sclerosis and cerebral ischemia [2]. More recently, BBB dysfunction is emerging as a key pathological hallmark in Alzheimer’s disease (AD).

AD is characterised by the accumulation of amyloid beta (Aβ) plaques [3] and oligomers [4], intraneuronal neurofibrillary tangles of tau [5], neuronal atrophy/dysfunction [6] and activated astrocytes and microglia [7]. Increased Aβ levels, in particular Aβ 1-40 and 1-42, are believed to play a major role in AD pathologies, inducing neuronal apoptosis and inflammation [8]. Recently, several features of vascular dysfunction in AD patients have been demonstrated, including
brain hypoperfusion [9], decreased Aβ clearance [10], reduced Glucose transporter 1 expression [11], BEC morphological alterations [12] and increased BBB permeability [13,14].

An increase in the permeability of the BBB could have potentially disastrous consequences for the neuronal homeostatic environment, and has been demonstrated in AD patients by the abnormal presence of plasma proteins in the CSF or brain parenchyma, although some contrasting results exist [15-17]. Most recent studies have demonstrated a correlation between BBB leakage and advanced stage AD [14] as well as medial temporal atrophy [13]. Animals receiving a peripheral Aβ injection [18] and transgenic AD mouse models [19] display increased BBB permeability. Aβ peptides have also been demonstrated to increase the permeability of non-human endothelial cell cultures [20,21]. Changes in TJ organization may in part be responsible for the increased BBB permeability found in AD. Indeed, morphological alterations of BEC TJs, suggestive of a leaky BBB, have been observed in AD patients brain biopsies [22] and occludin down-regulation is found in APP/PS1 transgenic mice [23]. Aβ 1-42 has also been noted to transiently decrease occludin expression and cause cldn-5 translocation from TJs in rat primary BECs [24].

In this study, we have investigated the effects of Aβ on the permeability of human BECs in vitro. To do so, we used a well established immortalised human BEC line, hCMEC/D3 cells [25], to determine whether Aβ peptides could induce increased BEC permeability and to explore the signalling pathways that could mediate such changes.

**Materials and methods**
Materials

Aβ 1-40 and 1-42 were obtained from Bachem (Weil am Rhein, Germany) whilst scrambled Aβ (sAβ) 1-40 and 1-42 were purchased from Covalence (California, USA). SP600125, TAT-TI-JIP153-163, PD98059 and SB203580 were purchased from Calbiochem (New Jersey, USA). All other reagents were from Sigma-Aldrich (Dorset, UK), unless stated.

Cell culture and treatments

hCMEC/D3 cells [25], were cultured on collagen-coated tissue culture plastic or collagen-coated glass coverslips (0.005 % (w/v) collagen, 1 h). Cultures were maintained in a humidified atmosphere at 37°C in 5 % CO₂ in EGM-2 medium (Lonza, Switzerland), which was supplemented with VEGF, IGF, bFGF, hydrocortisone, ascorbate, gentamycin and 2.5 % fetal bovine serum (FBS). The concentrations of the supplements are not made available by the supplier; however ¼ of the supplied volume of VEGF, IGF, bFGF and the total volume of hydrocortisone, ascorbate, gentamycin was added. For all experiments, hCMEC/D3 cells were grown to confluence (~ 1 x 10⁵ cells / cm²) with the EGM-2 media changed every 2-3 days. At confluence, hCMEC/D3 cells were left for 2-3 days without media change and then incubated with Aβ or sAβ peptides at the concentrations and times indicated. For inhibition studies, hCMEC/D3 cells were pre-treated for 30 min prior to Aβ or sAβ treatment with the inhibitors shown in Table 1. Soluble monomeric Aβ form was applied to the culture medium. Following 48 h incubation, no Aβ oligomers could be detected as assessed by western blotting (data not shown). In addition to Aβ treatment
as described above, for some experiments, hCMEC/D3 cells were incubated under UV light for 30 min as a positive control of MAPK activation.

**MTT assay**

hCMEC/D3 cells were incubated with 500 µg/ml of MTT for 4 h at 37°C, washed twice in PBS and the reaction stopped by the addition of isopropanol. The colour intensity of the dissolved formazan salts was then measured at a wavelength of 550 nm and a background reading was taken at 695 nm using a BMG plate reader (Offenberg, Germany). After subtracting background readings, cell viability was expressed as a percentage of control vehicle-treated cells.

**Permeability studies**

hCMEC/D3 cells were grown to confluence on transwell polyester membrane inserts [Corning Costar, Buckingham, UK (0.4 µm pore, 12mm diameter)], which were first coated with collagen as before and then with fibronectin (5 µg/ml for 1 h). The paracellular permeability to 70 kDa FITC-dextran of the hCMEC/D3 cell monolayers was then investigated as previously described [25]. Briefly, 2 mg/ml of 70 kDa FITC-dextran was added to the apical chamber, and the fluorescence that crossed to the basolateral chamber was determined over 30 min, in successive 5 min periods, using a BMG plate reader (Offenberg, Germany). The volume cleared was plotted against time, and the slopes of the curves used to calculate the permeability coefficients (P_e, cm/min) of the endothelial cell monolayer:
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\( P_e = \text{PS}/s \), where PS (clearance) is the permeability surface area of the endothelial monolayer and \( s \) is the surface area of the filter (1.1 cm\(^2\)).

PS is given by: \( 1/\text{PS} = 1/m_e - 1/m_f \), where \( m_e \) and \( m_f \) are the slopes of the curves corresponding to endothelial cells on filters and to filters only, respectively. \( m_e \) and \( m_f \) were calculated by plotting the cleared volume against time.

The cleared volume was calculated by: \( (\text{AU}_a - \text{AU}_b)/\text{Fi} \), where \( \text{AU}_a \) is the total fluorescence (arbitrary units) in the basal compartment, \( \text{AU}_b \) is the background fluorescence and \( \text{Fi} \) is the fluorescence of the initial solution (AU/ml).

Western blotting

Confluent hCMEC/D3 cells were lysed in 500 µl of RIPA buffer [Sigma-aldrich (150 mM NaCl, (1.0 % (w/v) IGEOL® CA-630, 0.5 % (w/v) sodium deoxycholate, 0.1 % (w/v) SDS, 50 mM Tris, pH 8.0)] containing 1 mM PMSF and 10 ng/ml of aprotinin, leupeptin and pepstatin A. Protein levels were measured by a Biorad DC protein assay (Herfordshire, UK). Samples were denatured in 1 x Laemmli’s buffer and 20 µg of total protein in the whole cell lysates was loaded per lane, resolved on SDS-PAGE gels, appropriate for the molecular weight of each protein under investigation, and transferred to a nitrocellulose membrane. After incubation with one of the primary antibodies shown in Table 2 for 2 h at room temperature (RT), membranes were incubated for 1 h at RT with a species-specific secondary antibody conjugated to horseradish peroxidase (Pierce Biotechnology, Cheshire, UK) (1 in 1000 dilution), and were visualised by enhanced chemiluminescence detection (ECL, Amersham, Buckinghamshire, UK). To confirm equal loading, membranes were stripped in 2 % (w/v) SDS, 0.0625 M Tris pH 6.8,
0.008 % (v/v) β-mecaptoethanol for 40 min at 50°C and re-probed with one of the antibodies indicated in table 1. For the detection of MAPK activation, membranes were first probed with antibodies for the phosphorylated form of each MAPK member, then stripped as above and re-probed with antibodies for total MAPK levels. A measure of MAPK activation following Aβ treatment was calculated using the following formula:

\[
\frac{([\text{Active MAPK}]_{A\beta}/[\text{total MAPK}]_{A\beta})}{([\text{Active MAPK}]_{sA\beta}/[\text{total MAPK}]_{sA\beta})}
\]

**RNA extraction and qPCR**

RNA was extracted from hCMEC/D3 cells using Trizol reagent (Invitrogen, Paisley UK), and 1 µg of RNA was then converted to cDNA using a reaction ready first strand synthesis kit (Superarray, Maryland, USA) according to the manufacturer’s instructions. Quantitative PCR (qPCR) was then carried out using SYBR green and primers for cldn-5, occludin, actin and ZO-1 according to the manufacturer’s protocol (Superarray, Maryland, USA). Amplification was performed in a thermal cycler (DNA engine Opticon2, Bio-Rad, California, USA) using a 40 cycle program (95°C for 30 sec, 55°C for 30 sec and 72°C for 30 sec.). The expression of each target mRNA in hCMEC/D3 cells treated with Aβ or sAβ was compared to that of the housekeeping gene, actin, using the following formula:

\[
\text{CT}_{\text{target}} - \text{CT}_{\text{actin}} = \Delta \text{CT},
\]

\[
\Delta \text{CT}_{A\beta} - \Delta \text{CT}_{sA\beta} = \Delta \Delta \text{CT}
\]

Comparative levels = \(2^{-\Delta \Delta \text{CT}}\).

**Immunocytochemistry**
hCMEC/D3 cells were fixed and permeabilised in ice-cold methanol for 10 min at -20 °C, followed by incubation in blocking buffer [3 % (w/v) normal goat serum (NGS) in phosphate buffered saline (PBS)] for 30 min. Cells were then incubated with antibodies for cldn-5, ZO-1, occludin (Table 1) or rabbit IgG as an isotype-matched control at a concentration of 2.5 µg/ml, overnight at 4°C, washed three times in PBS and incubated with Alexa Fluor-488-anti-rabbit polyclonal IgG [10 µg/ml, 1 h, RT (Invitrogen, Paisley, UK)]. After three further washes in PBS, the slides were mounted and viewed with an Olympus BX61 fluorescent microscope (Hertfordshire, UK).

**Flow cytometry**

hCMEC/D3 cells were washed in HBSS without Ca\textsuperscript{2+} and Mg\textsuperscript{2+} and then detached with 0.25% w/v porcine trypsin and 0.2 % w/v EDTA. EGM-2 (containing supplements as described above) was then added to neutralise trypsin activity and the cells were fixed in 4% p-formaldehyde (PAF) for 10 min. After one wash in PBS, cells were blocked and permeabilised for 30 min (0.1 % (v/v) triton X-100, 3 % (w/v) NGS in PBS), then incubated for 2 h at RT with occludin or cldn-5 antibodies (2.5 µg/ml in PBS). hCMEC/D3 cells were then washed three times in PBS and incubated with a species-specific Alexa-Fluor-488 or FITC-conjugated secondary antibody (10 µg/ml). After two final washes with PBS, the cells were suspended in 500 µl PBS and analysed using a FACSCalibur flow cytometer with Cellquest software [488 nm λ\text{excitation}, 530 nm λ\text{emission}, (Becton Dickinson, Oxfordshire, UK)]. For each sample the median fluorescence of 10,000 cells was determined. After background subtraction
the data was represented as percentage median fluorescence of cells treated with the sAβ peptide.

**Statistical analysis**

All data are represented as means +/- SEM and the number of experiments, n, indicated. Statistical significance was calculated using ANOVA followed by Student’s t test, comparing each treatment to the sAβ control (* P < 0.05, ** P < 0.01). For the western blotting, qPCR and permeability experiments a paired t test was employed due to variability in control values between experiments. For qPCR, analysis was carried out using ∆CT values as the $2^{-\Delta\Delta CT}$ method standardised all data so that the sAβ value is always 1.

**Results**

**High concentrations of Aβ peptides reduce hCMEC/D3 cell viability**

An aim of this study was to investigate the effect of non-cytotoxic concentrations of Aβ peptides on hCMEC/D3 cell permeability. We therefore measured hCMEC/D3 cell viability in the presence of Aβ 1-42 or Aβ 1-40 or their scrambled counterparts using an MTT assay. As shown in Fig. 1, hCMEC/D3 cell viability was unaffected by 48 h incubation with Aβ 1-42 or Aβ 1-40 at concentrations up to 5 µM. However, at 10 µM, both Aβ 1-40 and Aβ 1-42, but not sAβ peptides, reduced cell viability compared to vehicle treated cells (70 % and 74 %, respectively). Therefore, for subsequent investigations, to avoid cytotoxic effects, treatments with Aβ peptides were at 5 µM for 48 h. It should be noted that the 4 kDa
form of Aβ was added initially to the culture medium, and no soluble oligomers were detected by western blotting over 48 h incubation (data not shown).

**Aβ 1-40 increases hCMEC/D3 cell paracellular permeability**

Increased BBB permeability has been demonstrated in AD patient brain tissue and *in vivo* AD models [13,14,19]. We therefore investigated the effect of Aβ peptides on the paracellular permeability of hCMEC/D3 cells to 70 kDa FITC-dextran. As shown in Fig. 2, Aβ 1-40 or Aβ 1-42 incubation increased the paracellular permeability of hCMEC/D3 cells by 50 % and 27 % respectively, compared to cells treated with sAβ peptides. However, only Aβ 1-40-mediated increased BEC permeability was statistically significant. For subsequent studies, we focused our attention on the increased paracellular permeability induced by Aβ 1-40, since, compared to Aβ 1-42, this peptide is found at higher concentrations in the plasma [26] and in cerebrovascular deposits [27].

**Aβ 1-40 specifically decreases levels of occludin at the protein and mRNA levels**

The mechanisms (s) behind Aβ 1-40 induced increases in hCMEC/D3 cell paracellular permeability were investigated next. TJ proteins mediate BEC cell-cell contacts at the BBB, and changes in their expression are associated with increased BEC permeability. We therefore investigated the expression of occludin, cldn-5 and ZO-1, the most extensively investigated TJ proteins expressed by BECs, following Aβ 1-40 or sAβ 1-40 treatment. As shown in Fig. 3A and B, 48 h incubation with Aβ 1-40 caused a decrease in occludin levels, but not in cldn-5 or ZO-1 levels, as assessed
by western blotting. After normalising for equal loading with actin, occludin expression was 50% in cells treated with Aβ 1-40 compared to those treated with sAβ 1-40. Alterations in occludin protein expression were preceded by a decrease in occludin mRNA levels in hCMEC/D3 cells. As can be seen in Fig. 3C, after 24 h incubation, Aβ 1-40 induced a significant decrease in mRNA levels for occludin but not for cldn-5 or ZO-1. Interestingly, after 48 h incubation, the mRNA levels of ZO-1 were increased with Aβ 1-40 compared to treatment with sAβ 1-40.

Occludin expression is often found at TJ, where it acts to reduce BEC paracellular permeability. As demonstrated in Fig. 4E, after sAβ 1-40 incubation, occludin expression was strongly detected at the cell-cell borders between confluent hCMEC/D3 cells, indicative of TJ localisation, with a lower level of intracellular staining. In comparison, in hCMEC/D3 cells incubated with Aβ 1-40 (Fig. 4F), staining was reduced both at the cell borders and intracellularly, and in some instances, a complete loss of occludin at the cell junctions was observed. In contrast, there were no changes in either overall expression levels or in the sub-cellular localisation of ZO-1 (Fig. 4C, D) or cldn-5 (Fig. 4A, B) in hCMEC/D3 cells after Aβ 1-40 incubation, compared to sAβ 1-40 incubation. These data suggest that the Aβ 1-40-induced increase in hCMEC/D3 cell paracellular permeability may be a consequence of reduced occludin levels at cell junctions.

**Occludin down-regulation is mediated by the JNK and p38MAPK signalling pathways**

We next sought to identify the contribution of various signalling pathways in Aβ-mediated occludin down-regulation, using flow cytometry as a quantitative
measure of protein expression analysis. We found that inhibitors for pathways that
have been previously shown to regulate TJ expression (ROCK, Src, PKC, PI3K) [28]
and those known to be activated by Aβ in neurones (NF-κβ, GSK-3β) [29] did not
prevent occludin down-regulation in hCMEC/D3 cells mediated by Aβ 1-40 (data not
shown).

MAPK family members ERK [30], JNK [31] and p38MAPK [32] have been
reported to be up-regulated in neurones from AD patients. Aβ 1-40 treatment induced
a time-dependent activation of JNK (46 kDa form), ERK (42 kDa form) and
p38MAPK (42 kDa) in hCMEC/D3 cells (Fig. 5A, B). JNK activation was detected as
early as 6 h post incubation with Aβ 1-40 and increased with time up to 48 h, whereas
ERK2 and p38MAPK were also activated by Aβ 1-40, but from 24 h onwards.

Inhibitors for each MAPK family member were used in order to investigate
the signalling pathways mediating Aβ 1-40-induced occludin down-regulation (Fig
6.A). In this set of experiments, hCMEC/D3 occludin levels were decreased to 71 %
in Aβ 1-40 treated cells compared to those treated with sAβ 1-40 and pre-incubation
with 50 µM of the MEK inhibitor PD98059, which inhibits ERK, did not prevent this
down-regulation. In contrast, 20 µM SB203580 (a p38MAPK inhibitor), and 50 µM
SP600125 or 10 µM TAT-TI-JIP153-163, (both JNK inhibitors) prevented Aβ-induced
occludin down-regulation in hCMEC/D3 cells. In addition, pre-treatment of
hCMEC/D3 cells with SB203580, SP600125 or TAT-TI-JIP153-163, prevented the Aβ
1-40-mediated decrease in occludin mRNA levels after 24 h incubation (Fig. 6.B).
These results suggest that Aβ 1-40 induces brain endothelial occludin down-
regulation in a p38MAPK- and JNK-dependent manner.
JNK and p38MAPK inhibition attenuates Aβ 1-40 induced increased paracellular hCMEC/D3 cell permeability

Since JNK and p38MAPK inhibitors all attenuated occludin down-regulation mediated by Aβ 1-40, we investigated whether they could also prevent Aβ 1-40 increased hCMEC/D3 cell permeability (Fig. 7). Aβ 1-40 treatment increased the $P_e$ values of hCMEC/D3 cells to 70 kDa FITC-dextran by 70 % compared to sAβ 1-40-treated cells. Pre-incubation with SB203580, TAT-TI-JIP$_{153-163}$, and SP600125 prevented this increase in brain endothelial permeability. However, it should be noted that pre-incubation with SB203580 slightly increased the $P_e$ values for hCMEC/D3 cells when incubated with both sAβ 1-40 and Aβ 1-40 (by 32 % and 25 % ± 12.6 respectively, when expressed as a percentage of vehicle-pre-incubated sAβ 1-40 controls). Our results show that JNK and p38MAPK inhibition can prevent Aβ 1-40-mediated increase in hCMEC/D3 permeability to 70 kDa FITC-dextran, most likely by preventing occludin down-regulation.

Discussion

In this study, we initially determined the cytotoxicity of Aβ peptides to hCMEC/D3 cells. We then selected non-cytotoxic concentrations to further investigate specific Aβ effects on BBB permeability, rather than Aβ-induced gross permeability changes due to BEC death. The lowest dose of Aβ 1-40 or Aβ 1-42 to elicit cytotoxic effects to human BECs after 48 h incubation was, in both cases, 10 µM. Results from other research groups have found a large variation in Aβ concentrations needed to elicit toxic effects, ranging from 100 nM to greater than 20 µM in studies using different EC types and culture conditions. Multiple factors may
affect the cytotoxicity of Aβ to ECs in vitro, including whether cultured ECs are primary or immortalised cell lines, their confluent state, tissue and species of origin and Aβ aggregation in the culture medium. Whether Aβ oligomers or aggregates were responsible for Aβ toxicity to hCMEC/D3 cells at high concentrations remains to be determined. In the current study, we observed that incubation with non-cytotoxic concentrations of Aβ 1-40, but not of Aβ 1-42, increased the permeability of human BECs. Aβ 1-40 was added in soluble monomeric form and, at the concentrations used here, no aggregate and/or oligomer formation was detected by western blotting over the duration of the experiment (data not shown). Although Aβ 1-40 aggregates and/or oligomers may still induce EC death at high concentrations, as previously shown in neurons [4], the soluble monomeric form of Aβ 1-40 may be responsible for subtler changes in BBB function that do not involve cell death.

Our in vitro findings are consistent with observations in AD patients, where a leaky BBB could potentially disrupt the CNS homeostatic environment and lead to neuronal degeneration. Increased CSF/plasma protein ratio of albumin and IgG has been noted in AD patients, suggestive of a leaky BBB [13,14,33-36]. However, other studies have not confirmed this finding [15-17]. The most compelling evidence for increased BBB permeability in AD patients was provided by Zipser et al [14], who found that prothrombin leakage from the blood into the CNS was significant in advanced stage AD. In addition, increased cerebrospinal fluid levels of albumin are associated with medial temporal atrophy as assessed by MRI measurements in AD patients [13]. Aβ could play a central role in mediating increased BBB permeability. Indeed, Tg2576 mice, which harbour the human Swedish mutation of APP, display increased BBB permeability after 4 months of age, prior to plaque deposition and disease onset, and these effects can be reversed via Aβ peptide immunisation [19,37].

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Further evidence comes from studies showing that intravascular jugular vein injections of Aβ 1-40 can induce a leaky BBB in rats [18]. *In vitro* endothelial cells of non-human origin also display increased permeability after Aβ treatment [20,21]. In the present study, Aβ 1-40 was added to the luminal compartment, which corresponds to the blood side *in vivo*. Strazielle *et al.* compared the effects of luminal and abluminal application of 5 µM Aβ 25-35 on the permeability of bovine BECs to the paracellular tracer PEG [21]. They observed that, although both luminal and abluminal Aβ increased BEC permeability, the rise in PEG efflux was not observed until much later when Aβ 25-35 was applied abluminally. These authors speculated that the lag period might be explained as the time required for the peptide to diffuse from the lower compartment through the BEC monolayer and subsequently reach high enough levels on the luminal interface to induce barrier changes. Due to the polarized nature of BECs, it is possible that the receptor(s) activated by Aβ (e.g. RAGE, megalin, P-gp or an unknown receptor) mediating permeability effects might be differentially expressed between the luminal and abluminal membranes. Whether Aβ acts on the luminal or abluminal membranes or on both to increase BEC permeability remains to be determined.

Measurements of plasma Aβ levels have been inconclusive as to whether Aβ levels are increased or unaltered in AD [26], though Van Oijen *et al.* [38] found that high Aβ 1-40 levels in plasma in early life corresponded with an increased risk of developing dementia in later life. Increased plasma levels of Aβ, acting at the luminal surface, might induce subtle changes in BBB permeability and lead to the leakage of plasma proteins observed in AD. On the abluminal side of the BBB, increased Aβ production by neurones, stiffening of arteries during aging, and/or increased plasma Aβ leakage (as a result of occludin down regulation in BECs) in AD, could potentially...
reduce the clearance of Aβ from the brain via drainage of interstitial fluid and lead to
cerebral amyloid angiopathy (CAA), a frequent occurrence in AD patients [39,40]. In
CAA, cerebrovascular deposits of Aβ (of both 1-40 and 1-42 amino acids in length)
are found aggregated with other proteins including apoE and alpha-2 macroglobulin.
CAA occurs in both capillaries and small arteries and, to a lesser extent, veins. With
respect to capillaries, Aβ deposition in CAA occurs on the BEC basement membrane
and protrudes into the neuropil, and can result in capillary occlusion. Whether luminal
or abluminal, soluble Aβ may induce occludin down-regulation in BECs prior to, or at
early stages of, CAA; thereby inducing an increased BBB permeability. Plasma
proteins that cross the endothelium could in turn bind to and exacerbate the deposition
of Aβ around cerebrovascular vessels.

Occludin is an important TJ protein and its down-regulation is associated with
increased permeability in BECs [2]. Indeed, the importance of occludin in conferring
restrictive TJs has been demonstrated in vitro and in vivo. Transfection of truncated
occludin or siRNA knockdown in MDCK cells [41] or Xenopus embryo cells [42]
results in increased paracellular permeability. In addition, occludin down-regulation
and increased permeability has been observed in models of inflammatory pain [43]
and oxidative stress [44], and CNS disorders associated with BBB breakdown such as
multiple sclerosis [45]. Here we found that occludin down-regulation was the most
likely cause of the Aβ-induced increase in hCMEC/D3 cell permeability. Marco and
Skaper [24] have observed that 20 µM Aβ 1-42 can induce a decrease in occludin
protein levels after 24 h, but not 48 h, incubation in rat BECs. In agreement with our
study, the authors did not detect any alterations in overall cldn-5 or ZO-1 levels,
although cldn-5 appeared to translocate to the cytoplasm. Cerebral extravasation of
IgG in APP/PS1 mice fed on a high saturated-fat diet has also been associated with
decreased occludin expression at the BBB [23]. A disruption in TJ organisation has also been noted in AD patient’s biopsy samples [22]. Intriguingly, neuronal occludin overexpression has been detected in brains of AD individuals [46], although the significance of this observation is unclear.

Changes in the levels of occludin protein at 48 h were preceded by a decrease in occludin mRNA at 24 h. At earlier time points, Aβ may still induce changes in BBB permeability by directly acting on TJ organization, either by post-translational modifications of TJ proteins such as phosphorylation or by inducing TJ protein degradation and/or subcellular reorganisation. Whether Aβ induces post-translational modifications of the occludin protein requires further investigation. Our observation that inhibition of JNK restores occludin mRNA levels to those of controls would indicate that Aβ acts on occludin expression either by suppression of occludin gene transcriptional activity or by decreasing occludin mRNA stability. Aβ-mediated occludin down-regulation in BECs could therefore represent an important mechanism of increased BBB permeability in AD, as suggested by the present study.

The observation that p38MAPK or JNK inhibition could prevent the Aβ-induced increase in BEC permeability suggests that these signalling pathways are potential targets for therapeutic treatments in AD. p38MAPK activation has been associated with occludin down-regulation in response to different stimuli including combined tumour necrosis factor-α (TNF-α) and interferon-γ (IFN-γ) incubation [47], transforming growth factor β (TGF-β) [48] and alcohol [49]. Indeed, Lui et al [48] have demonstrated that CdCl₂-induced TGF-β3 production, and occludin down-regulation, can be prevented by p38MAPK inhibition at the rat blood-testis barrier. Crucially, in a study by Munoz and co-workers [50], a novel p38MAPK inhibitor was observed to improve synaptic dysfunction and behavioural deficits after Aβ 1-42
intracerebroventricular injections into mice. Whilst they did not investigate the permeability of the BBB, the authors did find that p38MAPK inhibition suppressed an Aβ 1-42- induced increase in TNF-α and IL-1β levels in the hippocampus of these mice. Aβ-mediated activation of astrocytes and microglia can induce cytokine production in vitro [7,51-53] and astrocytic end feet swelling [54] and microglia activation [51] have been observed in AD tissue sections. IL-1β, TNF-α and IL-6 levels are found increased in the CSF or serum of AD patients [55] and cytokines can induce a leaky tight junctions in vitro [56-59]. p38MAPK inhibition could therefore act as a dual target in AD, preventing cytokine production and Aβ-induced increased BBB permeability.

The JNK pathway also represents a potential novel potential therapeutic target for AD, with its inhibition not only preventing Aβ-induced increase in BBB permeability, as demonstrated here, but also neuronal atrophy [60]. There are three genes that encode JNK in mammalian systems, jnk1, jnk2 and jnk3, and each gene is alternatively spliced to create 46 and 55 kDa forms of the proteins [61,62]. The significance of each variant is not fully understood. However, JNK1 and -2 are expressed ubiquitously whilst JNK3 is largely restricted to the brain (reviewed in [63]). Morishima et al [64] have demonstrated that Aβ 25-35 toxicity is significantly reduced in cortical neurones derived from JNK3 knock-out mice compared to the controls; however, they also state that JNK1-deficient neurones are also resistant to apoptosis. Using antisense technology, JNK1-depleted rat cortical neurones are found to be resistant to Aβ 1-40-induced apoptosis [65]. Aβ in this study, was found to activate the 46 kDa JNK isoform, which in some studies was found to correspond to JNK1 [65]. Regardless of the isoform, JNK inhibition protects SH-SY5Y neuroblastoma, rat cortical neurones and PC12 adrenoblastoma cells from Aβ-induced
apoptosis in vitro [66-68]. A further interesting study has demonstrated that JNK
inhibition can prevent H$_2$O$_2$- induced $\gamma$-secretase mediated APP cleavage and A$\beta$
production in SH-SY5Y cells [69]. The JNK pathway might therefore represent a very
attractive therapeutic target, to prevent A$\beta$ production, neuronal atrophy and BBB
dysfunction.

In summary, we have found that A$\beta$ 1-40 can increase the paracellular
permeability of human BECs and this correlates with occludin down-regulation.
p38MAPK and JNK inhibition prevented both occludin down-regulation and
increased paracellular permeability of BECs, as induced by A$\beta$. The p38MAPK and
JNK pathway therefore potentially represent interesting therapeutic targets to
ameliorate cerebrovascular dysfunction in AD.

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work was supported by the MRC UK.
Fig. 1 Cell viability of hCMEC/D3 cells incubated with Aβ 1-40 and Aβ 1-42 peptides for 48 h. hCMEC/D3 cells were incubated with (A) Aβ 1-40 or sAβ 1-40 and (B) Aβ 1-42 or sAβ 1-42 for 48 h at the concentrations indicated. hCMEC/D3 cell viability was measured using an MTT assay and expressed as a percentage of untreated cells. Data represents mean ± SEM, n = 3 experiments with quintuplet samples. * P < 0.05 compared to control.

Fig. 2 Permeability coefficient (P_e) of hCMEC/D3 cells to 70 kDa FITC-dextran after 48 h incubation with Aβ or sAβ peptides. hCMEC/D3 cells were incubated with 5 µM of Aβ 1-40, Aβ1-42, sAβ 1-40 or sAβ 1-42 for 48 h and the P_e of the hCMEC/D3 cell monolayer to 70 kDa FITC-dextran was investigated. Data represents mean ± SEM, n = 3 experiments with duplicate samples. * P < 0.05 comparing P_e values of cells treated with Aβ to those treated with sAβ.

Fig. 3 Tight junctional protein and mRNA expression by hCMEC/D3 cells after incubation with Aβ 1-40. hCMEC/D3 cells were incubated for 24 h or 48 h with 5 µM of Aβ 1-40 or sAβ 1-40. (A) The protein levels of tight junctional (TJ) proteins occludin, cldn-5 and ZO-1 were assessed by western blotting, (B) normalised to actin levels and expressed as a percentage of expression of cells treated with the sAβ 1-40 peptide. (C) mRNA levels of occludin, cldn-5 and ZO-1 were assessed by qPCR. Data represents mean ± SEM, n = at least three experiments with duplicate samples. * P < 0.05 comparing values for Aβ- treated vs sAβ- treated cells.
**Fig. 4** The expression of TJ proteins by hCMEC/D3 cells after 48 h Aβ 1-40 incubation as assessed by immunocytochemistry. hCMEC/D3 cells were incubated with 5 µM (A, C, E) sAβ 1-40 or (B, D, F) Aβ 1-40 for 48 h, fixed in methanol and the expression of cldn-5 (A, B), ZO-1, (C, D) or occludin (E, F) visualised by fluorescence microscopy. Image shown is of one experiment representative of three. Scale bar = 50 µM.

**Fig. 5** MAPK activation by Aβ 1-40 in hCMEC/D3 cells. (A) hCMEC/D3 cells were incubated with 5 µM sAβ 1-40 or Aβ 1-40 for 1, 3, 6, 24 and 48 h, or under UV light for 30 min as a positive control. The cells were then lysed in RIPA buffer and the protein levels of phosphorylated and non-phosphorylated MAPK family members were investigated by western blotting. (B) The optical densities of the active JNK (46 kDa), ERK2 and p38MAPK bands were normalised, respectively, to total JNK, ERK and p38MAPK levels. The normalised values for Aβ 1-40 were divided by those obtained for sAβ 1-40, as described in materials and methods. Data represents mean ± SEM, n = 3 experiments.

**Fig. 6** The effect of MAPK inhibitors on Aβ 1-40-mediated occludin down-regulation by hCMEC/D3 cells. hCMEC/D3 cells were preincubated for 30 min with 50 µM SP600125, 10 µM TAT-TI-JIP153-163, 20 µM SB203580, 50 µM PD98059 or a vehicle control, and then treated with Aβ 1-40 or sAβ 1-40 for 48 h. The expression of occludin was then measured (A) at the protein level by flow cytometry, or (B) at the mRNA level using qPCR. Data represents mean ± SEM, n = 3 experiments with duplicate samples. * P < 0.05 comparing cells treated with Aβ 1-40 to those of cells treated with sAβ 1-40.
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1. **Bechmann I, Galea I, Perry VH.** What is the blood-brain barrier (not)? *Trends in immunology.* 2007; 28: 5-11.
13. **Matsumoto Y, Yanase D, Noguchi-Shinohara M, Ono K, Yoshita M, Yamada M.** Blood-brain barrier permeability correlates with medial temporal lobe atrophy but not with amyloid-beta protein transport across the blood-

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Table 1. Inhibitors and concentrations used.

<table>
<thead>
<tr>
<th>Modulator</th>
<th>Pathway inhibited</th>
<th>Concentration</th>
<th>Source</th>
</tr>
</thead>
<tbody>
<tr>
<td>SP600125</td>
<td>JNK 1, 2 and 3</td>
<td>50 µM</td>
<td>Calbiochem NJ, USA.</td>
</tr>
<tr>
<td>TAT-TI-JIP&lt;sub&gt;153-163&lt;/sub&gt;</td>
<td>JNK 1, 2 and 3</td>
<td>10 µM</td>
<td>Calbiochem NJ, USA.</td>
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<tr>
<td>SB203580</td>
<td>p38MAPK</td>
<td>20 µM</td>
<td>Calbiochem NJ, USA</td>
</tr>
<tr>
<td>PD98059</td>
<td>MEK</td>
<td>50 µM</td>
<td>Calbiochem NJ, USA</td>
</tr>
<tr>
<td>pp2</td>
<td>Src family</td>
<td>10 µM</td>
<td>Calbiochem NJ, USA</td>
</tr>
<tr>
<td>Y-27632</td>
<td>ROCK inhibitor</td>
<td>700 nM</td>
<td>Calbiochem NJ, USA</td>
</tr>
<tr>
<td>Bisindolylmaleimide -1 (Bis-1)</td>
<td>PKC</td>
<td>10 µM</td>
<td>Calbiochem NJ, USA</td>
</tr>
<tr>
<td>LY294002</td>
<td>PI3-kinase</td>
<td>300 µM</td>
<td>Calbiochem NJ, USA</td>
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<tr>
<td>Lithium chloride (LiCl)</td>
<td>GSK-3β</td>
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<td>Sigma-Aldrich, Dorset, UK</td>
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<tr>
<td>sn50</td>
<td>NF-κB</td>
<td>200 µM</td>
<td>Calbiochem NJ, USA</td>
</tr>
<tr>
<td>sn50-m</td>
<td>Control sn-50</td>
<td>200 µM</td>
<td>Calbiochem NJ, USA</td>
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</tbody>
</table>
**Table 2.** List of antibodies used for immunocytochemistry, flow cytometry and western blotting. The concentrations indicated were those used for western blotting.

<table>
<thead>
<tr>
<th>Anti-</th>
<th>Species</th>
<th>Concentration</th>
<th>Source</th>
</tr>
</thead>
<tbody>
<tr>
<td>Phospho-Thr183/Tyr185 JNK</td>
<td>rabbit polyclonal</td>
<td>1 in 1000 dilution</td>
<td>Cell signalling, Massachusetts, USA</td>
</tr>
<tr>
<td>Phospho-Thr180/Tyr182 p38MAPK</td>
<td>rabbit polyclonal</td>
<td>1 in 1000 dilution</td>
<td>Cell signalling, Massachusetts, USA</td>
</tr>
<tr>
<td>Phospho-Thr185/Tyr187 ERK1/2</td>
<td>mouse IgG monoclonal</td>
<td>1 in 1000 dilution</td>
<td>Biosource, California, USA</td>
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<tr>
<td>ZO-1</td>
<td>Rabbit polyclonal</td>
<td>1 µg/ml</td>
<td>Zymed, California, USA</td>
</tr>
<tr>
<td>Cldn-5</td>
<td>mouse IgG monoclonal</td>
<td>1 µg/ml</td>
<td>Zymed, California, USA</td>
</tr>
<tr>
<td>Occludin</td>
<td>Rabbit polyclonal</td>
<td>1 µg/ml</td>
<td>Zymed, California, USA</td>
</tr>
<tr>
<td>JNK</td>
<td>rabbit polyclonal</td>
<td>1 in 1000 dilution</td>
<td>Cell signalling, Massachusetts, USA</td>
</tr>
<tr>
<td>p38MAPK</td>
<td>rabbit polyclonal</td>
<td>1 in 1000 dilution</td>
<td>Cell signalling, Massachusetts, USA</td>
</tr>
<tr>
<td>ERK2</td>
<td>mouse IgG monoclonal</td>
<td>1 in 1000 dilution</td>
<td>Upstate California, USA</td>
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<tr>
<td>β Actin</td>
<td>mouse IgG monoclonal</td>
<td>1 µg/ml</td>
<td>Sigma-Aldrich, Dorset, UK</td>
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</tbody>
</table>
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129x161mm (600 x 600 DPI)
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109x76mm (600 x 600 DPI)