The amyloid peptide β disrupts intercellular junctions and increases endothelial permeability in a NADPH oxidase 1-dependent manner

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ABSTRACT

Alzheimer’s disease is the most common form of dementia and is associated with the accumulation of amyloid peptide β in the brain parenchyma. Vascular damage and microvascular thrombosis contribute to the neuronal degeneration and the loss of brain function typical of this disease. In this study, we utilised a murine model of Alzheimer’s disease to evaluate the neurovascular effects of this disease. Upon detection of an increase in the phosphorylation of the endothelial surface receptor VE-cadherin, we focused our attention on endothelial cells and utilised two types of human endothelial cells cultured in vitro: 1) human umbilical vein endothelial cells (HUVECs) and 2) human brain microvascular endothelial cells (hBMECs). Using an electrical current impedance system (ECIS) and FITC-albumin permeability assays, we discovered that the treatment of human endothelial cells with amyloid peptide β causes a loss in their barrier function, which is oxidative stress-dependent and similarly to our observation in mouse brain associates with VE-cadherin phosphorylation. The activation of the superoxide anion-generating enzyme NADPH oxidase 1 is responsible for the oxidative stress that leads to the disruption of barrier function in human endothelial cells in vitro. In summary, we have identified a novel molecular mechanism explaining how the accumulation of amyloid peptide β in the brain parenchyma may induce the loss of neurovascular barrier function, which has been observed in patients. Neurovascular leakiness plays an important role in brain inflammation and neuronal degeneration driving the progression of the Alzheimer’s disease. Therefore, this study provides a novel and promising target for the development of a pharmacological treatment to protect neurovascular function and reduce the progression of the neurodegeneration in Alzheimer’s patients.

1. Introduction

Dementia is one of the most common ageing-related pathologies and it is used as an umbrella term to define a cluster of symptoms involving progressive impairment in brain cognitive functions that can eventually lead to decreased quality of life, physical disability and

Abbreviations: AJs, Adherens junctions; AD, Alzheimer’s disease; APP, Amyloid precursor protein; BBB, Blood brain barrier; COX2, Cyclooxygenase 2; ECIS, Electrical current impedance system; EPR, Electron paramagnetic resonance; FITC, Fluorescein isothiocyanate; HO-1, Heme oxygenase-1; HUVECs, Human umbilical vein endothelial cells; hBMECs, Human brain microvascular endothelial cells; IFN-γ, Interferon γ; IL-1α, Interleukin-1α; IP-10, IFN-γ-induced protein 10; NADPH, Nicotinamide adenine dinucleotide phosphate; Nrf-2, Nuclear factor erythroid 2-related factor 2; PS1, Presenilin 1; ROS, Reactive oxygen species; RAGE, Receptor for advanced glycation end products; RANTES, Regulated upon activation, normal T cell expressed and presumably secreted; SEM, Standard error; siRNA, Small interfering RNA; TJs, Tight junctions; TNF α, Tumour necrosis factor α; VCAM-1, Vascular cell adhesion molecule 1.

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institutionalization [1]. There are an estimated 46.8 million people living with dementia worldwide and the numbers of patients will double every 20 years, rising to 115.4 million in 2050. The most dominant form of dementia is the Alzheimer’s disease (AD), which is responsible for 60–80% of dementia cases [2]. AD is a multifactorial and progressive neurodegenerative disease of the brain that was first described by the German psychiatrist Alois Alzheimer in 1906 [3]. AD is associated with the deposition of plaques formed by amyloid peptides β and the accumulation of hyperphosphorylated tau proteins in the brain. In turn, this causes inflammation, blood flow impairment (or hypoperfusion), and neuronal death, ultimately resulting in the progressive cognitive deterioration [4]. Cerebral blood hypoperfusion is particularly important in the development of AD, as it contributes to accumulation of toxic metabolites, ineffective clearance of amyloid peptides and reduced oxygenation of the brain [5,6]. The study of the effect of AD and especially amyloid peptide β accumulation on neurovascular cells is a growing and promising research area for the discovery of novel ways to treat this disease and control the dementia pandemic.

In AD brain, substantial morphological and functional cerebrovascular abnormalities are observed, including microvasculature irregularities and atrophy, basement membrane disruption and deposition of heparin sulphate proteoglycans, collagen IV and laminin, decreased cerebrovascular network density, endothelial cell alteration i.e. increased pinocytosis, decreased levels of mitochondria and detection of elevated endothelial cell markers VCAM-1 and E-selectin [7-10]. Cerebrovascular abnormalities associated with AD can result in hypoperfusion, hypoxia, inflammation and disruption of the blood brain barrier (BBB). These vascular alterations are referred to as cerebral amyloid angiopathy or CAA, which is associated with ischemic lesions, micro- and macro-haemorrhages, and impaired cerebral blood flow. Ultimately, impaired blood flow and resulting ischemia exacerbate neurodegeneration and accelerate the progression of AD [11-13]. In addition, initial inflammation and endothelial damage are likely to contribute to platelet stimulation in AD patients [14]. Several studies indicated elevated levels of inflammatory mediators in AD cerebral microcirculation, where endothelial cells overexpress cyclooxygenase 2 (COX2) [15], adhesion molecules (MCP-1, ICAM-1, CAP37), and inflammatory and stress markers, such as TNFα, TGF-β, interleukins (IL-1β), IL-6, IL-8) and metalloproteinases (MMPs) [16-18]. The inflammatory mediators overexpressed in the AD cerebrovasculature are likely to have toxic effects on neurons, which could represent an important link between vascular inflammation and neuronal loss in AD [19].

Recent studies suggested a causative link between the accumulation of amyloid peptide β in the brain and the loss of barrier function of endothelial cells [20]. Oxidative stress has been shown to link amyloid peptide β accumulation and neurovascular damage [21,22]. NADPH oxidases (NOXs) are key sources of ROS in neurovascular cells [23] and have been shown to play an important role in neurodegenerative diseases [24]. Although previous studies suggested a role for NOX2 in the amyloid β-dependent changes in cerebrovascular permeability [25], in this study we show for the first time a key role for NOX1 in the loss of barrier function and motility by endothelial cells exposed to amyloid peptide β. The amyloid β-dependent increase in endothelial monolayer permeability was associated with the phosphorylation of VE-cadherin, an event shown to cause the loosening of adherens junctions (AJs) and the inhibition of the endothelial cell barrier function [26,27]. In our experiments, VE-cadherin was also hyperphosphorylated in the brain of the AD model 3xTG-AD [28]. Overall, this study indicates a prominent role of NOX1 in the amyloid β-dependent impairment of the normal endothelial function in AD brains. NOX1 is therefore a promising target for the development of neuroprotective agents in the fight against AD.

2. Material and methods

2.1. 3xTG-AD mice maintenance, perfusion fixation and brain sectioning

All animal experiments complied with the ARRIVE guidelines. Mouse maintenance and sacrifice was approved by the local ethics committee (University of Exeter and Home Office Project licence 3003348). Triple mutant mice were utilised as a model of AD (APP Swedish, MAPT P301L, and PSEN1 M146V) [28]. B6129S2 wild-type mice were used as controls. Intra-cardiac perfusions with 4% paraformaldehyde were performed on 6 and 12 month old female mice as previously described [29]. The brains were collected in 4% paraformaldehyde and transferred to PBS before embedding them in paraffin for sectioning (10 μm thickness).

2.2. Differential quantitative proteomics of mouse hippocampus

The hippocampus was excised from paraformaldehyde-fixed mouse brain sections (100 μm thickness) and lysed in 1% w/v sodium deoxycholate - 100 mM triethyl ammonium bicarbonate buffer. After boiling for 1 h at 95 °C for antigen retrieval, samples were sonicated and reduced in the presence of 10 mM dithiotreitol. Samples were alkylated in presence of 20 mM iodoacetamide and the tryptic digestion of 20 μg was performed at a 50:1 protein to enzyme ratio overnight. Samples were resuspended in 0.1% w/v formic acid and chromatographic separation was achieved on a Dionex Ultimate 3000 UPLC system (Thermo Fisher Scientific) with a two-buffer system (buffer A: 0.1% w/v formic acid in water, buffer B: 0.1% w/v formic acid in acetonitrile). Peptides were separated using a 60 min gradient with increasing ACN concentration from 2% to 30% v/v. The eluting peptides were analysed on a quadrupole orbitrap mass spectrometer (QExactive, Thermo Fisher Scientific). The QExactive was used to analyze the top 12 most intense ions per precursor scan (1 × 10 [6] ions, 70,000 Resolution, 120 ms fill time) and were analysed by MS/MS in the orbitrap (HCD at 25 normalized collision energy, 17,500 resolution, 1 × 10 [5] ions, 50 ms fill time) in a range of 400–1300 m/z. Acquired LC-MS/MS data were searched against the reviewed mouse protein data base Uniprot (October 2020, 17,053 protein entries, EMBL) using the Sequest algorithm integrated in the Proteome Discoverer software version 2.4 (Thermo Fisher Scientific). Only peptide with a high confidence (false discovery rate <1% using a decoy data base approach) were accepted as identified. Peptide precursor area under the curve values were summed to protein areas and used for quantitative analysis. Protein areas were imported into Perseus software version 1.5.8 for statistical analysis. Proteins with a T-test p values < 0.05 and expression difference±15% were considered differentially expressed. Pathway analysis was performed using the STRING software (https://string-db.org/, version 11.5, by Peer Bork, Lars Juhl Jensen, and Christian von Mering, © String Consortium 2022) and the Reactome Pathway Database (https://reactome.org/, © Reactome 2022).

2.3. Mouse hippocampus imaging

Brain sections were deparaffinized (3x wash xylene for 15 min each, 1x wash 50% xylene/50% ethanol, 1x wash ethanol, 1x wash methanol for 3 min each, 2x of 95% ethanol for 10 min each, 2x wash in ddH2O 5 min each), then heat-induced epitope retrieval was performed (10 mM sodium citrate pH 6, 10 min, 90 °C, then 2x wash in tap water). Afterwards the sections were stained with Thioflavim S (1% w/v solution in 50% ethanol, T1892, Sigma-Aldrich, St Louis, US) for 15 min, then destained with 50% ethanol for 5 min and washed extensively with PBS. For immunofluorescence staining, sections were blocked with 3% v/v donkey serum in PBS-TritonX-100 (0.5% v/v), then stained with antibodies against amyloid peptide β (BioLegend #803001, 1:100), VE-cadherin (R&D Systems, #AF1002, 1:100), and phosphorylated VE-cadherin (Abcam, #ab49785, 1:50) overnight at 4 °C, then washed 3x PBS-TritonX-100 (0.5% v/v), stained with secondary antibodies and
DAPI, and the autofluorescence was quenched with 0.1% w/v solution of Sudan Black. Coverslips were mounted with Fluoromount mounting medium. The imaging was performed with the Leica TCS SP5 confocal microscope (Leica, Wetzlar, Germany). Images were analysed using the FIJI software [30] and staining intensity is expressed as mean intensity ratio (phospho-VE-cadherin vs total VE-cadherin).

2.4. Endothelial cell culture

Mycoplasma free Human Umbilical Vein Endothelial Cells (HUVECs) were purchased from Lonza and were routinely cultured in EGM-2™ (Endothelial Cell Growth Medium-2) supplemented with SingleQuots™ Supplements. Human Brain Microvascular Endothelial Cells (hBMECs) were purchased from Science Cell Research Laboratories (Carlsbad, US) and were cultured in Endothelial Cell Medium (ECM) supplemented with Endothelial Cell Growth Supplement (ECGS), Foetal Bovine Serum (FBS) and penicillin/streptomycin solution were all from Science Cell Research Laboratories. Incubation with amloidipine β 1–42 (DAEFHDS-GYEVHQKLFFAEDVGSNKIGAIGLMYGGVVIA) or scrambled control (DEFAKNIGHHDGVAVHMYKGRQVEFSGIALVTEDEVGSAVL) was performed with a maximal DMSO concentration of 0.2% v/v. For gene silencing with siRNA, HUVECs were transfected with NOX1 (#sc-43939), NOX2 (#sc-335503), NOX4 (#sc-41586), NOX5 (#sc-45486) or scrambled control (#sc-37007) siRNAs (Santa Cruz Biotechnology) as per manufacturer’s instructions. In brief, the siRNAs were diluted to 100 nM in transfection medium (#sc-36686; Santa Cruz Biotechnology) containing transfection reagent (#sc-29528; Santa Cruz Biotechnology), incubated for 45 min at room temperature, and then further diluted to 20 nM in transfection medium. The cells were incubated with the transfection medium for 5 h at 37 °C, after which growth medium containing normal concentrations of serum and growth factors was added. Experiments were performed 48 h after transfection.

2.5. Quantitative reverse transcription PCR (RT-qPCR) analysis

2 × 10^5 [5] HUVECs were cultured in Nunc™ cell culture treated 6-well plates. The cells were treated with 25 μM Aj1-42, SCR peptide or DMSO as vehicle control for 8 h or 24 h. Where indicated, cells were pretreated with 10 μM VAS2870 before treatment with Aj1-42 or SCR peptide. For RT-qPCR, total RNA was extracted using TRIzol™ reagent (Thermo Fisher Scientific) according to manufacturer’s instructions. 1 μg of RNA was reverse transcribed to make cDNA using the Applied Biosystems™ High-Capacity cDNA Reverse Transcription Kit (Thermo Fisher Scientific) according to manufacturer’s instructions. Gene specific amplification for was performed on QuantStudio™ 7 Flex Real-Time PCR System and quantified using Power SYBR Green PCR Master Mix (Thermo Fisher Scientific). IL-1β, VCAM, RAGE, HMOX and NFR2 primers were purchased from Sigma, while NOX1, NOX2 NOX4 and NOX5 primers were designed (Nox1 Forward TTGAAAGGTTGGTTAGCT (21); Nox1 Reverse AAATGGAACCCCTTGAGCA (19); Nox2 Forward AAAGAAACT CCTCTGCTGTGAA (23); Nox2 Reverse GCCGACCAGAACCCTTGAGAA (23); Nox4 Forward: CAGATGTGGGCTTGAGATT (20) Nox4 Reverse: AGAAGTTGGAGGCTTACCG (20); Nox5 Forward CAGATCTCACTGCACT (20); Nox5 Reverse GCCGATGAAGCAGT (20)). The 2^∆ΔCT method was used to calculate relative expression levels for each gene with GAPDH and actin as endogenous controls as previously described [31].

2.6. Immunoblotting, ELISA and immunocytochemistry of endothelial cells

2 × 10^5 [5] HUVECs were cultured in Nunc™ cell culture treated 6-well plates. The cells were treated with 25 μM Aj1-42, SCR peptide or DMSO as vehicle control for 24 h. To harvest proteins, the cells were lysed in radioimmunoprecipitation assay (RIPA) buffer in the presence of protease and protein phosphatase inhibitor cocktails 2 and 3 (Merck-Sigma). Sodium dodecyl sulphate polyacrylamide gel electrophoresis (SDS-PAGE) was used to separate proteins as described previously [32]. Antibodies for phospho-VE-cadherin (Y658) (Invitrogen, #44-1144G) and total VE-cadherin (Santa Cruz, #sc-9989) were purchased from commercial suppliers. The NOX1 antibody was from Novus Biologicals (#NB1P31546). Actin from Merck-Sigma was used as loading control. Immunoblot densitometry was performed using Image studio Version 5.2 (LI-COR Biosciences). For ELISA, the culture medium from HUVECs treated as described above was analysed using a commercial inflammation ELISA kit following manufacturer instructions (Signosis, #EA-1031). For HUVEC immunocytochemistry, cells were cultured on cell culture-compatible coverslips for 48 h to reach 95% confluence. Following fixation in 4% w/v paraformaldehyde, cells were stained with anti-VE-cadherin antibody (#ab33168 from Abcam), fluorescein isothiocyanate (FITC)-labelled secondary antibodies and 4’, 6-diamidino-2-phenylindole (DAPI). The imaging was performed with the Leica TCS SP5 confocal microscope (Leica, Germany) and densitometry was performed using the FIJI software [30].

2.7. Apoptosis assay by flow cytometry

After incubation, cells were harvested with the gentle dissociating buffer TrypLE®*, pelleted by centrifugation, washed with PBS, and resuspended in PBS. Cell suspensions were stained with Annexin V/FITC + propidium iodide (PI) according to the manufacturer’s instructions (Thermo Fisher Scientific, #88–8005). After 15 min of incubation in the dark on ice, cells were centrifuged at 2000 rpm for 10 min, resuspended in PBS, and analysed using a FACSCanto II (BD Biosciences). Annexin V/PI scatter plots divided in quadrants were utilised to assess early apoptotic (Annexin V+/PI−), late apoptotic (Annexin V+/PI+) and necrotic (Annexin V+/PI+) cells.

2.8. Electron paramagnetic resonance (EPR)

1 × 10^6 [4] HUVECs or hBMECs were cultured in triplicate in Nunc™ cell culture treated 96-well plates for 24 h. Aj1-42, scrambled control peptide DMSO or NOX inhibitors were added as indicated for 4 h 200 μM 1-hydroxy-3-methoxycarbonyl-2,2,5,5-tetramethylpyrrolidine (CMH), 5 μM diethyldithiocarbamate (DETC) and 25 μM deferoxamine were then added to the cultures for 45 min 50 μL of supernatant were transferred into the Hirschmann precision micropipettes and read using an e-scan (Noxygen, Germany), as previously described [33]. EPR spectra were recorded using the following EPR settings: centre field 3492.5 G, field sweep 60 G, modulation amplitude 2 G, sweep time 10 s, number of scans 10, microwave frequency 9.39 GHz, microwave power 20 mW, conversion time 327.68 ms, time constant 5242.88 ms. A calibration curve was obtained from standard CM• diluted to concentrations of 0, 0.3, 1, 3, 10, and 30 μM and utilised to estimate the CM• concentration in the samples as described in Supplementary Fig. 1. The CMH oxidation rate was obtained using the formula below: CMH oxidation rate = [CM•] x Volume / (cell density x Volume x Time)

2.9. Electrical cell-substrate impedance sensing (ECIS)

The 8W1E PET arrays (AppliedBioPhysics) were electrically stabilised and collagen coated (#354233, Fisher Scientific) prior to the addition of cells as per manufacturer’s protocol. 1 × 10^5 [5] cells/well were dispensed in 400 μL and allowed to settle in the wells at RT for 30 min before placing them in the incubator. Electrical impedance was measured at frequency 4000 Hz using a Model Z Theta from Applied-BioPhysics (NY, US), which estimates current leakage “between cells” and therefore informs about cell barrier function [34]. ECIS recordings reached a steady-state corresponding to the formation of a compact
endothelial cell monolayer within 24 h from cell seeding. The Aβ1-42, scrambled peptide or the drugs as indicated were prepared at 2X concentration in the culture medium and kept at 37 °C for 30 min before adding to the cells to minimize any unrelated impedance changes. 200 μl of media was removed very gently from the wells and replaced with 2X solutions containing treatment to reach the desired final concentration. In order to monitor the effect of Aβ1-42 on barrier function, the electrical impedance measurements were recorded for further 24 h (up to t = 48 h). Then, in order to study the effect Aβ1-42 on endothelial cell migration and reparative potential, electrical wounding was performed (time 1 s, current 1400 μA) and electrical impedance was measured for a further 24 h (up to t = 72 h).

2.10. FITC-albumin permeability assays

HUVECs or hBMECs were grown to confluence in the inserts of transwell plates (Costar #3413, pore size 0.4 μm). At the beginning of the experiments, the treatments were administered at the indicated concentrations (e.g. Aβ1-42, NoxA1 ds, etc.) and 1 mg/ml FITC-albumin (Thermo Scientifics, Albumin from Bovine Serum (BSA), FITC conjugate, cat. no. #A23015) was added. The filtration of FITC-albumin from the transwell insert to the bottom of the well was monitored over time (0–48 h) by fluorescence microplate reading (λexc = 490 nm) using a Spark 10 M microplate reader (Tecan, Männedorf, Switzerland).

2.11. Statistical analysis

Dual comparisons were analysed by non-parametric Mann-Whitney test. One-way ANOVA with Tukey post-test was used for multiple comparison tests after testing that data are normal and homoscedastic. For time courses (e.g. permeability assays), two-way ANOVA with Bonferroni post-test for different time points was utilised. The software
3. Results

3.1. Amyloid peptide beta deposition and gene expression changes in the hippocampus of 3xTG-AD mice

The AD model 3xTG-AD [28] and the wild type control B6129SF2 were maintained for 3, 6 and 12 months, which represent preclinical, early and advanced stages of the disease. The hippocampal region of the brain of the mice was analysed by LC-MS/MS based proteomics as described in the material and methods section (Fig. 1A). As shown in Supplementary Tables 1, 2, 7,979 proteins were identified in the hippocampus of 3xTG-AD mice was analysed by LC-MS/MS based proteomics as described in the material and methods section (Fig. 1A). As shown in Supplementary Tables 1, 2, 7,979 proteins were identified in the hippocampal sections. The proteomics analysis at age 6 and 12 months identified clear and consistent differences between 3xTG-AD and wild type mice (Fig. 1B and C, respectively). The comparison of 6 animals per group led to the identification of 224 and 228 differentially expressed proteins in the hippocampus of 3xTG-AD mice compared to wild type mice at 6 and 12 months of age, respectively. The complete list of proteins with differential expression is shown in Supplementary Tables 2 and 3, ordered by p value. The proteome analysis indicated statistically significant changes in the expression of proteins involved in important physiological processes, such as mitochondrial and cytoskeletal function, neurotransmission, intracellular signal transduction, protein synthesis and turnover, and redox homeostasis (Tables 1 and 2, for 6 and 12 months of age, respectively). Automated pathway analysis using the STRING software and the Reactome Pathway Database is shown in Fig. 2 and Supplementary Table 4. It highlighted significant changes in a number of metabolic pathways (citric acid pathway and amino acid pathways), membrane trafficking (including clathrin-mediated endocytosis), biological oxidations, and opioid signalling. At month 12, cell degradative pathways such as apoptosis and mitophagy were also significantly affected. The entire proteomics dataset is available in PRIDE (Project accession: PXD030300, Project DOI: 10.6019/PXD030300). Amyloid β deposits in the hippocampus of 3xTG-AD mice (versus B6129SF2 wild type) became evident at 6 and 12 months of age, as shown by Thioflavin-T staining (Fig. 3A). In addition, the hippocampal region of 3xTG-AD mice displayed significantly higher levels of VE-cadherin phosphorylation at the residue Tyr658 at 6 and 12 months of age, compared to wild type controls (representative examples and densitometry analysis in Fig. 3 B).

3.2. Amyloid peptide beta induces pro-inflammatory changes in endothelial cells in vitro

Cerebrovascular dysfunction has been suggested to cause extensive abnormalities of cerebral capillaries, which result in reduced cerebral blood flow in AD patients [35]. Therefore, we have investigated the effects of Aβ1-42 on endothelial cells in vitro. Quantitative reverse transcription PCR (RT-qPCR) was utilised to study the effect of Aβ1-42 on HUVECs. Four genes associated with pro-inflammatory responses of endothelial cells were found to be significantly increased by 8 h or 24 h of incubation with 25 μM of Aβ1-42 versus scrambled control peptide: receptor for advanced glycation end products (RAGE), vascular cell adhesion protein 1 also known as vascular cell adhesion molecule 1 (VCAM-1), Heme oxygenase-1 (HO-1) and nuclear factor erythroid 2-related factor 2 (Nrf2) (Fig. 4A). In addition, the secretion of pro-inflammatory cytokines tumour necrosis factor α (TNF α), interleukin γ (IFN-γ), interleukin-1α (IL-1α), IFN-γ-induced protein 10 (IP-10) and “regulated upon activation, normal T cell expressed and presumably secreted” (RANTES) resulted increased by 24 h incubation with 25 μM of

Table 1

| Accession | Protein Name | Gene Name | P value |
|-----------|--------------|-----------|---------|
| Q61696    | Heat shock 70 kDa protein 1A | Hspa1a | 0.000746776 |
| OS5125    | Protein Nipssnap homolog 1 | Nipsnap1 | 0.0005605161 |
| Q9C64     | Biliverdin reductase A | Bvra | 0.002327897 |
| Q61165    | Sodium/hydrogen exchanger 1 | Slc9a1 | 0.0051395039 |
| P09671    | Superoxide dismutase (Mn), mitochondrial | Sod2 | 0.09113985 |
| Q9CR61    | NADH dehydrogenase 1 beta subcomplex subunit 7 | NdufB7 | 0.027246813 |
| Q8BMPF3   | NADP-dependent malic enzyme, mitochondrial | Me3 | 0.035158694 |
| Q8C4U9    | PE3S-related protein | Pex5l | 0.03598034 |
| Q91JK2    | Lamc-like protein 2 | Lenc2 | 0.04370087 |
| Q9RPF3    | S-formylglutathione hydrolase | End | 0.04954031 |
| Q9R0N7    | Synaptotagmin-7 | Syt7 | 0.00140352 |
| Q08S99    | Syntaxin-binding protein 1 | Sxtp1 | 0.01035179 |
| Q99NE8    | Regulating synaptic membrane exocytosis protein 1 | Rims1 | 0.01166644 |
| Q9WV18    | Gamma-amino butyric acid type B receptor subunit 1 | Gabb1 | 0.016828527 |
| Q9RIS2    | Tumor protein p63-regulated gene 1-like protein | Tprg1l | 0.020684394 |
| Q90TJ1    | Calcium-dependent secretion activator 1 | Cadps | 0.023937329 |
| Q61016    | Guanine nucleotide-binding protein subunit gamma-7 | Gng7 | 0.031149121 |
| Q62443    | Neuronal pentraxin 1 | Nptx1 | 0.04907128 |
| Q9PID8    | Mitochondrial function | Ppid | 0.0507128 |
| P97807    | Fumarate hydratase, mitochondrial | Fh | 1.89049-05 |
| Q8BB29    | Pyruvate dehydrogenase protein X component | Pdhx | 0.000182343 |
| Q9BF85    | Elongation factor Tu, mitochondrial | Tufm | 0.000219998 |
| Q8BIS9    | Calcium-binding mitochondrial carrier protein Aralar1 | Slc25a12 | 0.000343059 |
| Q9B749    | Dihydrolipoamide dehydrogenase, mitochondrial | Dld | 0.001056969 |
| Q9KBB8    | Hydroxyacylglutathione hydrolase, mitochondrial | Hagh | 0.001154019 |
| Q60930    | Voltage-dependent anion-selective channel protein 2 | Vadc2 | 0.003519833 |
| Q9UH5     | Isovaleryl-CoA dehydrogenase, mitochondrial | Ivd | 0.003828896 |
| Q6141     | NAD(P) transhydrogenase, mitochondrial | Ndt | 0.004292233 |
| P70404    | Isocitrate dehydrogenase (NAD) subunit gamma 1 | Idc3g | 0.005859061 |
| Q51413    | ATPase inhibitor, mitochondrial | Atp5f1 | 0.006163506 |
| Q5NC8E    | Magnesium transporter MRS2 homolog, mitochondrial | Mrx2 | 0.009684623 |
| Q90D51    | Pyruvate dehydrogenase E1 component subunit beta | Pdhb | 0.01119372 |
| Q5ILZ3    | Methyglutaryl-CoA hydratase, mitochondrial | Aah | 0.01150073 |
| Q9PC92    | COX assembly mitochondrial protein homolog | Cmc1 | 0.011930271 |
| Q9DCC8    | Mitochondrial import receptor subunit TOM20 homolog | Tmm20 | 0.013691041 |
| P51174    | Long-chain specific acyl-CoA dehydrogenase | Acadl | 0.01469291 |
| Q70417    | Short-chain specific acyl-CoA dehydrogenase | Acads | 0.014588163 |
| Q5IV5     | Serine protease HTRA2, mitochondrial | Htra2 | 0.01537337 |
| Q8BGKX2   | Mitochondrial import membrane translocase (Tim29) | Tim29 | 0.017629493 |
| Q9WVA2    | Mitochondrial import membrane translocase (Tim29A) | Timms1a | 0.020593563 |
| Q9D7B6    | Isobutyryl-CoA dehydrogenase, mitochondrial | Acd8 | 0.02848468 |
| Q08600    | Endonuclease G, mitochondrial | Endog | 0.02435898 |
| P47738    | | Aldh2 | 0.025395545 |

(continued on next page)
Table 1 (continued)

| Accession | Protein Name | Gene Name | P value |
|-----------|--------------|-----------|---------|
| O08587    | Aldehyde dehydrogenase, mitochondrial | Timm44 | 0.025846245 |
| Q6PE15    | Mycoplasmophilic acid acyl-glucaronidase esterase | Abhd10 | 0.028842325 |
| Q3UMR5    | Calcium unipporter protein, mitochondrial | Mcu | 0.033233774 |
| Q92299    | Succinate-CoA ligase [ADP-forming] subunit beta | Sula2 | 0.040013037 |
| P30275    | Creatine kinase U-type, mitochondrial | Ckm1 | 0.041798187 |
| Q8GCG3    | Lon protease homolog, mitochondrial | Lonp1 | 0.047715831 |

**Protein synthesis and degradation**

| Accession | Protein Name | Gene Name | P value |
|-----------|--------------|-----------|---------|
| Q8BP47    | Asparagine-tRNA ligase 1 | Nars1 | 0.000134741 |
| Q9D127    | D-aminomalonate-tRNA deacylase 1 | Scl7a5 | 0.003859166 |
| Q92282    | Aspartate-tRNA ligase | Sars1 | 0.004177611 |
| Q6P912    | COP9 signalosome complex subunit 2 | Cop2p | 0.013009051 |
| Q92B72    | Cysteine-tRNA ligase, cytoplasmic | Cysl1 | 0.015261735 |
| Q921X4    | Interleukin enhancer-binding factor 3 | Ile3 | 0.02768976 |
| Q70194    | Eukaryotic translation initiation factor 3 subunit D | Eif3d | 0.029492568 |
| Q3U0V1    | Far upstream element-binding protein 2 | Khsrp | 0.000094851 |
| Q8K0B2    | Lysosomal cobalamin transport escort protein LMBD1 | Lmbd1 | 0.003895409 |
| Q98099    | Ubiquitin carboxyl-terminal hydrolase isozyme L1 | Uch1 | 0.041488581 |
| Q98050    | Ubiquitin-associated protein 2-like | Ubap2l | 0.00494063 |
| Q30598    | Dihydroripidinimidine-related protein 4 | Dpy34 | 9.41366E-05 |
| Q8U1V4    | Vacuolar protein sorting-associated protein 51 homolog | Vps51 | 0.000664568 |
| Q9D898    | Actin-related protein 2/3 complex subunit 5-like protein | Arpc5l | 0.001142625 |
| P70206    | Flexin-A1 | Flexn4 | 0.00259489 |
| Q3U1HB8   | Coiled-coil domain-containing protein 277 | Cdc177 | 0.002273706 |
| Q6I059    | Src substrate cortactin | Ctn | 0.002443861 |
| Q9XK0T    | Protein canopy homolog 2 | Cnp2p | 0.002607573 |
| P61027    | Ras-related protein Rab-10 | Rab10 | 0.003105873 |
| Q8D1D4    | Transmembrane emp24 domain-containing protein 10 | Tem10 | 0.003931995 |
| Q7T0Q2    | Tubulin polymerization-promoting protein | Tppp | 0.003942978 |
| O3S382    | Exocyst complex component 4 | Exoc4 | 0.00170172 |
| Q8X4G5    | Actin-binding LIM protein 1 | Abml1 | 0.004307204 |
| A2A5R2    | Brefeldin A-inhibited guanine exchange protein 2 | Arfgel2 | 0.005885934 |
| Q8U049    | Centrosomal protein of 170 kDa protein B | Cep170b | 0.007734822 |
| Q8CHU3    | Epsin-2 | Eps2 | 0.009808381 |
| Q9OXY7    | Protein piccolo | Pclo | 0.008826 |
| Q55SM3    | Rho GTPase-activating protein 44 | Arbap44 | 0.012362 |
| Q62417    | Sorbin and SH3 domain-containing protein 1 | Sorbs1 | 0.01625649 |
| F45591    | Cofilin-2 | Cofi2 | 0.012924621 |
| P68373    | Tubulin alpha-1C chain | Tuba1c | 0.013120303 |
| D3Y2U1    | SH3 and multiple ankyrin repeat domains protein 1 | Shank1 | 0.017417396 |
| Q6I301    | Catenin alpha 2 | Cnn2 | 0.019615454 |
| Q8VHE6    | Dynamin heavy chain 5, axonemal | Dynb1 | 0.01927187 |
| Q92096    | Intersection-2 | Itm2 | 0.0231552 |
| Q9ET54    | Palladin | Palld | 0.02616493 |
| Q8HQC4    | Synaptopartin-1 | Synj1 | 0.027795015 |
| Q68638    | Myosin-11 | Myh11 | 0.027787667 |
| Q92E8     | PRA1 family protein 2 | Praf2 | 0.030172925 |
| P74777    | F-actin-capping protein subunit beta | Capzb | 0.030845069 |
| P61022    | Ca2+-calmodulin-dependent kinase A | Camk1a | 0.030891636 |
| Q61546    | Clathrin coat assembly protein 1 | Snap91 | 0.004752117 |
| Q9J7M6    | Actin-related protein 2/3 complex subunit 3 | Arpc3 | 0.03906293 |

Table 1 (continued)

| Accession | Protein Name | Gene Name | P value |
|-----------|--------------|-----------|---------|
| Q9JM96    | Cdc42 effector protein 4 | Cdc42ep4 | 0.041125024 |
| Q8C754    | Vascular protein sorting-associated protein 52 homolog | Vps52 | 0.041757532 |
| P63044    | Vesicle-associated membrane protein 2 | Vamp2 | 0.044359734 |
| Q91W86    | Vascular protein sorting-associated protein 11 homolog | Vps11 | 0.045630805 |
| Q69233    | Dynamin heavy chain 17, axonemal | Dynb1 | 0.047942193 |
| Q9EF39    | ADP-riboseylation factor GTPase-activating protein 1 | Arfgap1 | 0.049281009 |
| P74809    | Dual specificity mitogen-activated protein kinase 4 | Mapk2 | 0.000582444 |
| Q9JH4    | Diphosphoinositol phosphatase phosphohydrolase 5 | Pps5c | 0.000267554 |
| Q8R071    | Inositol-triphosphate 3-kinase A | Itpta | 0.003906467 |
| Q61306    | Serine/threonine-protein kinase PAK 3 | Pak3 | 0.005076213 |
| P97797    | Tyrosine-phosphatase non-receptor | Sirpa | 0.009320041 |
| Q9D7X3    | Dual specificity protein phosphatase 3 | Dusp3 | 0.009359351 |
| B9ER1K    | Receptor-type tyrosine-protein phosphatase | Ptprr1 | 0.00971021 |

**Aβ1-42** (Fig. 4B). In order to assess whether the pro-inflammatory effect of Aβ1-42 is associated with increased cell death, apoptosis and necrosis were tested by Annexin V/propidium iodide (PI) staining. These experiments showed no significant changes in cell apoptosis and necrosis as a consequence of Aβ1-42 treatment (Fig. 4C).

3.3. **Amyloid peptide beta causes NOX1-dependent oxidative stress in endothelial cells**

In order to understand the molecular nature of the inflammatory injury of endothelial cells by Aβ1-42, we used electron paramagnetic resonance spectroscopy. CMH was used as superoxide anion-specific spin probe (Supplementary Fig. 1) [35]. Using this technique, we were able to show that Aβ1-42 results in a dose-dependent increase in superoxide generation in HUVECs, reaching a significant increase at both 10 and 25 μM when compared to the scrambled peptide (Fig. 5A).

Next, we used the NOX-specific inhibitor VAS2870 [36] to identify NOX as the source of the oxidative burst induced by Aβ1-42 (Fig. 5B). The use of a highly selective NOX1 inhibitory peptide [37] allowed us to pinpoint NOX1 as the most relevant source of superoxide anion in response to Aβ1-42 (Fig. 5B). In order to confirm this finding, we used siRNA transfection to silence the genes of the four NOX enzymes.
Table 2

Selected differentially expressed proteins in the hippocampus of 3xTG-AD mice at 12 months of age divided by cellular function. The statistical significance of the difference was tested by Student t-test (n = 6).

| Accession | Protein Name | Gene Name | P value |
|-----------|--------------|-----------|---------|
| Q9DI0X | Redox Homeostasis | Nod3 | 0.0041000 |
| Q9HJ2K | Lectin-like protein 2 | Lnc12 | 0.0029787 |
| Q8RMBF | NADP-dependent malic enzyme, mitochondrial | Me3 | 0.0027963 |
| Q61578 | NADPH:adenodoxin oxidoreductase, mitochondrial | Fdxr | 0.0102717 |
| P51855 | Glutathione synthetase | Gsr | 0.0145300 |
| Q4KMM3 | Oxidation resistance protein 1 | Oxr1 | 0.0204449 |
| Q46133 | Amine oxidase [Flavin-containing] A | Maoa | 0.0224494 |
| Q8K097 | Protein lifeguard 2 | Faim2 | 0.0270026 |
| Q8R8B3 | Protein FAM177A1 | Fam177a1 | 0.0327368 |
| Q9R257 | Heme-binding protein 1 | Hepb1 | 0.0358492 |
| Q9DB73 | NADH-cytochrome b5 reductase 1 | Cyb5r1 | 0.0491211 |

**Neurotransmission**

| Accession | Protein Name | Gene Name | P value |
|-----------|--------------|-----------|---------|
| Q9CXP8 | Guanine nucleotide-binding protein subunit gamma-10 | Gng10 | 1.8577E-06 |
| Q61016 | Guanine nucleotide-binding protein subunit gamma-7 | Gng7 | 0.0033138 |
| Q08599 | Syntaxin-binding protein 1 | Stxbp1 | 0.0008032 |
| P61264 | Syntaxin-1B | Stxbp1 | 0.0001398 |
| Q62442 | Vesicle-associated membrane protein 1 | Vamp1 | 0.0034329 |
| Q8BL87 | Vesicular glutamate transporter 2 | Slc17a6 | 0.0049574 |
| Q9S595 | Potassium voltage-gated channel subfamily C member 3 | Kcnc3 | 0.0168435 |
| P12961 | Neuroendocrine protein 7B2 | Scg5 | 0.0348492 |
| P26443 | Glutamate dehydrogenase 1 | Glud1 | 0.0117157 |
| Q9L0Q8 | Guanine nucleotide-binding protein subunit gamma-6 | Gng6 | 0.0232695 |
| Q9CZT8 | Heterogeneous nuclear ribonucleoprotein A0 | Hnrnpa0 | 0.0244433 |
| P14206 | 40S ribosomal protein SA | Rpsa | 0.0045845 |
| Q9R9P9 | Ubiquitin carboxyl-terminal hydrolase isoyzme L1 | Uchl1 | 0.0005190 |
| Q8CHW4 | Translation initiation factor elf-2B subunit epsilon | Elf2b | 0.0009779 |
| Q9O8B6 | RNA-binding protein 3 | Rbm3 | 0.0009383 |
| Q61035 | Histidine-tRNA ligase, cytoplasmic | Hsrl | 0.0014955 |
| Q9CZB8 | Elongation factor T2, mitochondrial | Tsfm | 0.0019621 |
| Q9CB86 | Heterogeneous nuclear ribonucleoprotein | Hnrna0 | 0.0024443 |
| Q9Q1Z1 | Large neutral amino acids transporter | Slc32a1 | 0.0116174 |

**Protein synthesis and degradation**

| Accession | Protein Name | Gene Name | P value |
|-----------|--------------|-----------|---------|
| Q8BMK4 | Cytoskeleton-associated protein 4 | Ckap4 | 0.0001625 |
| Q91YR5 | Eukaryotic translation initiation factor | Eif2a | 0.0001625 |
| Q91VR7 | Map1lc3a | 0.0011598 |
| Q80X50 | Ubiquitin-associated protein 2-like | Ubap2l | 0.0026879 |
| Q8B5W6 | Ubiquitin carboxyl-terminal hydrolase | Uch1 | 0.0034329 |
| Q692R2 | E3 ubiquitin-protein ligase HECTD1 | Hect1 | 0.0011598 |
| Q8K0B2 | Ubiquitin-associated protein 2-like | Ubap2l | 0.0026879 |
| Q9I1YR | Eukaryotic translation initiation factor | Eif2a | 0.0034329 |

**Mitochondrial function**

| Accession | Protein Name | Gene Name | P value |
|-----------|--------------|-----------|---------|
| Q8QWT1 | 3-ketoseryl-CoA thiode, mitochondrial | Acan2 | 0.0012530 |
| Q9HJS5 | Isovaleryl-CoA dehydrogenase, mitochondrial | Ivd | 0.0013191 |
| Q9229 | Succinate-CoA ligase [ADP-forming] subunit beta | Sucl2 | 0.0016019 |
| Q9D0K2 | Succinyl-CoA:3-ketoacid coenzyme A transferase 1 | Oxt1 | 0.0005680 |
| Q9CQJ6 | NADH dehydrogenase [ubiquinone] 1 beta subunit 9 | Ndufb9 | 0.0062653 |
| Q64521 | Alanyl-tRNA editing protein Aarsd1 | Aars1 | 0.0068882 |
| Q9D0G2 | Dihydropolyamine-residue succinyltransferase, mit. | Dst | 0.0071909 |
| P99008 | Oxytocin | Oxt | 0.0086600 |
| Q9QYI2 | Mitochondrial import receptor subunit TOM40 homolog | Tmm9 | 0.0089307 |
| P51174 | Long-chain specific acyl-CoA dehydrogenase, mitochondrial | Acad1 | 0.0098817 |
| Q8OUG3 | Mitofusin-2 | Mfn2 | 0.0106111 |
| Q9Q0G1 | Mitochondrial-processing peptide subunit alpha | Pmpca | 0.0151678 |
| Q99JR1 | Sideroflexin-1 | Sfxn1 | 0.0170372 |
| Q9CPW3 | Prostate-specific membrane antigen 4 | Mrp54 | 0.0192249 |
| Q5NCE8 | Mitochondrial import protein | Mx2 | 0.0196724 |
| P58281 | Dynamin-like 120 kDa protein, mitochondrial | Opa1 | 0.0198544 |
| Q9CQ92 | Mitochondrial fission 1 protein | Fis1 | 0.0235869 |
| Q9Q6B2 | Stomatin-like protein 2, mitochondrial | Stom2 | 0.0245708 |
| Q9QX88 | Mitochondrial-processing peptide subunit beta | Pmpcb | 0.0250706 |
| Q8BGX2 | Mitochondrial import membrane translocate (Tim29) | Timm29 | 0.0259164 |

Table 2 (continued)

| Accession | Protein Name | Gene Name | P value |
|-----------|--------------|-----------|---------|
| Q9CMU2 | NADH dehydrogenase 1 beta subcomplex 2 | Ndufb2 | 0.0286871 |
| Q8BF55 | Elongation factor Tu, mitochondrial | Tufm | 0.0309189 |
| Q8BVF5 | ADP-ribose pyrophosphatase, mitochondrial | Ucgc1 | 0.0343119 |
| Q9CZ13 | Cytochrome b-c1 complex subunit 1, mitochondrial | Timm8a1 | 0.0378318 |
| Q9C7B5 | Calcium uptake protein 3, mitochondrial | Micu3 | 0.0350402 |
| Q9P6T8 | Fumurate hydratase, mitochondrial | Fh | 0.0374148 |
| Q9WVA2 | Mitochondrial import membrane translocate (Tim8a) | Timm8a1 | 0.0395071 |
| Q9C16 | NAD(P) transhydrogenase, mitochondrial | Exog | 0.0412028 |

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Table 2 (continued)

| Accession | Protein Name         | Gene Name                     | P value  |
|-----------|----------------------|--------------------------------|----------|
| P63328    | Serine/threonine-protein phosphatase 2B catalytic subunit alpha isoform | Ppp3ca                           | 0.0141343 |
| P92212    | Regulator of G-protein signaling 2A activator | Rgs6                              | 0.0274676 |
| P58389    | Serine/threonine-protein phosphatase 1-42 | Ptpa                             | 0.0274467 |
| Q92962    | Active breakdown cluster region-related protein 3.5 | Abbr                             | 0.0300060 |
| Q9CQV8    | 14-3-3 protein beta/alpha | Ywhab                            | 0.0306968 |
| Q99BC7    | cAMP-depend. protein kinase type 1-alpha reg. subunit | Pkrarl1a                        | 0.0316314 |
| Q60B29    | Protein phosphatase 1 regulatory subunit 18 | Ppp1r18b                        | 0.0336775 |
| Q5A665    | C-Jun-amo-terminal kinase-interacting protein 4 | Spag9                          | 0.0331436 |
| Q8TS3     | Adhesion G protein-coiled receptor L3 | Adgrl3                          | 0.0343670 |
| Q90967    | Magnesium-dependent phosphatase 1 | Mdpl1                         | 0.0364410 |
| P84075    | Neuron-specific calcium-binding protein hippocalcin | Hpcal                         | 0.0425279 |
| P05480    | Neuronal proto-oncogene tyrosine-protein kinase Src | Srpc                          | 0.0425946 |
| Q6NSS2    | Dicycglycerol kinase beta | Dgkb                          | 0.0429672 |
| Q8CG7     | Guanine nucleotide-binding protein G (olf) subunit alpha | Gnal                          | 0.0431162 |
| Q8CGA0    | Protein phosphatase 1F | Ppm1f                           | 0.0432068 |
| Q3UMT1    | Protein phosphatase 1 regulatory subunit 12C | Ppp1r12c                       | 0.0456733 |
| F41242    | Megakaryocyte-associated tyrosine-protein kinase | Matk                           | 0.0495276 |

expressed in endothelial cells (NOX1, NOX2, NOX4 or NOX5) [38]. The silencing of NOX1 almost completely abolished the Aj1-42-dependent increase in superoxide anion generation (Fig. 5C), while the silencing of other NOXs had no significant effect. The efficiency of siRNA silencing was assessed by RT-qPCR, which showed all 4 siRNA treatment significantly reducing the expression of the targeted NOX enzymes by at least 70% (Supplementary Fig. 2). In order to test whether the Aj1-42 treatment upregulates the expression of NOX enzymes (which could explain the increase in superoxide anion generation), NOX1, NOX2, NOX4 and NOX5 were quantified by RT-qPCR following the treatment with Aj1-42. None of the above enzyme was significantly upregulated by Aj1-42 (Fig. 5D). The importance of NOX1 in the oxidative stress induced by Aj1-42 in HUVECs was also confirmed by experiments where the expression of the oxidative stress marker HO-1 was tested by RT-qPCR (Supplementary Fig. 3). In these experiments, the silencing of NOX1 abolished the HO-1 upregulation caused by Aj1-42.

3.4. Amyloid peptide beta impairs the barrier function of endothelial cells in endothelial cells

Using electric cell-substrate impedance sensing (ECIS) we tested the barrier functions of HUVECs treated with Aj peptide, which is proportional to the electric impedance of cell monolayers measured at frequency 4000 Hz. After the initial 24 h to allow the formation of a complete monolayer, treatment with the Aj1-42 peptide for further 24 h (up to t = 48 h) reduced electrical impedance as a consequence of intercellular junction loosening and loss barrier function (Fig. 6A). We then induced electrical damage of the monolayer by electrical wounding and assessed monolayer electrical impedance for further 24 h (up to t = 72 h), which measures the abilities of cells to migrate and restore a complete monolayer. The cells treated with Aj1-42 peptide were unable to heal after wounding, leading to persistent low impedance 24 h after wounding compared to cells treated with the scrambled control peptide. Using immunocytochemistry experiments, we showed that the impaired barrier function is associated with a reduction in the localisation of the intercellular junction protein VE-cadherin at the cellular edge (Fig. 6B). The assessment of the phosphorylation state of VE-cadherin by immunoblotting allowed us to detect a significantly increased level of VE-cadherin phosphorylation at tyrosine 658 (Y658) in response to Aj-42 peptide compared to scrambled control peptide (Fig. 6C). Next, we investigated the molecular mechanisms underlying the loss of endothelial barrier function induced by the Aj1-42 peptide. The NOX inhibition with the pan inhibitor VAS2870 (Fig. 7A) and the NOX1-selective abolishment of oxidative stress with the selective inhibitory peptide NoxA1ds (Fig. 7B) protected HUVECs from the injury caused by Aj1-42 peptide. Both pre-wounding barrier function and post-wounding barrier repair were preserved when NOX1 was inhibited, while the scrambled control peptide for NoxA1ds had no effect compared to untreated cells (shown in Fig. 7A). In order to confirm that the effect on endothelial monolayer impedance corresponded to a change in permeability, we performed experiments with FITC-albumin as a tracer. As shown in Fig. 7C, HUVEC treatment with Aj1-42 led to an increase in monolayer permeability (which was statistically significant at 24 h and 48 h after treatment). The selective inhibitory peptide NoxA1ds abolished the increase in HUVEC permeability caused by Aj1-42.

3.5. Aj peptide causes barrier function damage in hBMECs in a NOX1-dependent manner

In order to confirm the physiopathological relevance of our findings obtained with HUVECs, we repeated key experiments with the primary human cerebrovascular endothelial cells hBMECs. As for HUVEC experiments, also in primary human brain endothelial cells, we used NoxA1ds peptide to selectively inhibit NOX1. In Fig. 8A, similarly to what we observed in HUVECs, we present data showing that Aj1-42 induces VE-cadherin phosphorylation (Y658) in hBMECs. Moreover, Aj1-42 stimulates a significant increase in superoxide generation (compared to scrambled peptide) (Fig. 8B). The pre-treatment of hBMECs with the NoxA1ds peptide inhibited in a comprehensive and statistically significant manner this superoxide anion burst, suggesting that the Aj1-42 peptide treatment induces the activation of NOX1 also in this endothelial cell type. We were then able to confirm the loss in hBMEC barrier function following Aj1-42 treatment and its NOX1-dependence. In Fig. 8C, we show that Aj1-42 treatment causes a significant loss in impedance in ECIS experiments compared to scrambled control peptide both before and after wounding. When we inhibited NOX1 using the NoxA1ds, the Aj1-42-dependent loss in barrier function was abolished. The protective effect of NoxA1ds was more evident before monolayer wounding, but statistically significant after wounding as well. In order to confirm that the effect on hBMEC monolayer impedance corresponded to a change in permeability, we performed experiments with FITC-albumin as a tracer. As shown in Fig. 8D, hBMEC treatment with Aj1-42 led to an increase in monolayer permeability.
which was statistically significant at 24 h and 48 h after treatment). The selective inhibitory peptide NoxA1ds abolished the increase in hBMEC permeability caused by Aβ1-42.

4. Discussion

Despite some contrasting evidence [39], the impairment of the neurovasculature is suggested to contribute to the progression of neurodegeneration and dementia associated with AD [40, 41]. Autopsy studies have shown that vascular alterations are present in the majority of clinically diagnosed AD cases [42]. The perivascular accumulation of amyloid peptide β in the brain is referred to as cerebral amyloid angiopathy (or CAA) [43, 44]. CAA occurs in 85%–95% of AD patients, has a significant impact on neurovasculature function, and is considered an important contributor to AD [45]. Although some authors suggest that CAA only occurs at the later stages of the disease and leads to brain capillary rarefaction [46], the impairment of the neurovasculature is suggested to reduce clearance of amyloid peptide β and other toxic metabolites, and to limit oxygen and nutrient supply to the brain, which ultimately exacerbate neurodegeneration in AD [47]. Whether neurovascular degeneration plays a role in the early development of AD or its later stages of brain degeneration, its contribution to the disease is no longer disputed. It is therefore important to understand the effect of amyloid peptide β on vascular cells. In this study, following detection of markers of hippocampal oxidative stress by proteomics and the phosphorylation of the endothelial junctional protein VE-cadherin in a murine model of AD, we have focused on the oxidative damage of endothelial cell function caused by amyloid peptide β.

The 3xTG-AD mice used as a model of AD in this study were previously described for the intracerebral accumulation of amyloid peptide plaques from 6 months of age and cognitive decline starting at 9 months of age [28]. Here, we confirmed the formation of amyloid plaques from 6 months of age. Extensive changes in the brain proteome of murine AD models as a consequence of disease progression have been previously reported [48–50]. In this study, we focused on the proteomic changes in the hippocampus of these animals at 6 and 12 months of age compared to wild type controls. 224 and 228 proteins appeared differentially expressed, respectively. Based on the classification of the differentially-expressed proteins, mitochondrial function, protein synthesis/degradation and signal transduction appear as the most heavily affected cell functions at both 6 and 12 months of age, while cytoskeletal integrity seems more heavily affected at 6 months of age. Other cellular phenomena affected by the accumulation of amyloid peptide β in the hippocampus are neurotransmission, cell adhesion and redox homeostasis. Automated pathway analysis indicated metabolism (citric acid and amino acid pathways), membrane trafficking (including clathrin-mediated endocytosis), biological oxidations, and opioid signalling significantly affected in the hippocampus of 3xTG-AD mice. Cell degradative pathways such as apoptosis and mitophagy were significantly affected only at 12 months of age. A number of previous studies have utilised proteomics to identify pathological changes of the hippocampus in animal models of AD. A related mouse model characterised by two of the three mutations of 3xTG-AD mice (APP/PS1) displayed 231 protein changes compared to wild type controls of similar age (7 months) [51]. Similarly to our study, cytoskeletal integrity, mitochondrial function, protein turnover and cell signalling were the cellular functions more heavily affected. Other recent proteomics studies on the hippocampus of the APP/PS1 mouse model of AD show similarities with our results, with changes in proteins involved in protein turnover pathways (both synthesis and degradation) [52, 53], neurotransmission [52], and, importantly, oxidative stress [53]. A key result of our proteomics study is in fact the identification of expression changes for proteins and enzymes associated with redox homeostasis, which suggests the possibility that the brain accumulation of amyloid peptide β leads to oxidative stress. This is in agreement with brain biochemistry studies suggesting that oxidative stress links amyloid peptide β accumulation
Fig. 3. Amyloid peptide β deposition and VE-cadherin hyperphosphorylation in the hippocampus of 3xTG-AD mice at 6 and 12 months of age. 3xTG-AD and B6129Sf2 (wild type) mice were maintained for 6 or 12 months before fixation/perfusion, brain collection and tissue section. The amyloid peptide β deposition was visualised by Thioflavin T staining of the hippocampal region (A), while the immunostaining of the same region was performed with VE-cadherin and phospho-VE-cadherin antibodies (B). The images are representative of 6 mice per experimental group. The intensity of the phospho-VE-cadherin staining has been normalised to the intensity of the VE-cadherin staining. The statistical significance of the difference between 3xTG-AD and wild type mice at age 6 and 12 months was assessed by one-way ANOVA with Tukey post-test (*, p < 0.05; **, p < 0.01; ***, p < 0.001; n = 6).
(caption on next page)
and neurovascular damage [21, 22]. The use of an alternative mouse model (i.e. 5XFAD) [54], also led to the discovery of the alteration of redox homeostasis pathways in the hippocampus in association with disease development (in particular, superoxide dismutases, which also appear amongst the changes on protein level associated to AD in our study) [55].

Because VE-cadherin is a protein expressed selectively by vascular endothelial cells [56], our data showing the phosphorylation of this protein in the brain of 3xTG-AD mice suggest an involvement of the vasculature in the changes associated with the onset or the progression of AD. It has previously been reported that tyrosine 658 phosphorylation of VE-cadherin controls vascular permeability and endothelial cell migration [57]. Our in vitro data confirm that amyloid peptide β impairs the barrier function of the endothelial monolayer and its repair after
damage. Although there is no existing consensus on the source of oxidative injury in endothelial cells exposed to amyloid peptide beta [58,59], the role of oxidative stress in the damage of the blood brain barrier and the progression of AD is well-established [60,61]. In this study, we identify NOX1 as the source of superoxide anion in endothelial cells exposed to amyloid peptide $\beta$. Superoxide anion is central to the chemistry of ROS, as it can directly modify biological molecules including proteins, nucleic acids and lipids, it can form highly reactive molecules such as peroxynitrite or hydroxyl radical, or it can be transformed hydrogen peroxide by superoxide dismutases [62]. Using pharmacological and genetic manipulations of endothelial cells combined with EPR detection of superoxide anions, we identified NOX1 as the main source of ROS in endothelial cells exposed to amyloid peptide $\beta$. The use of the EPR approach is particularly powerful as it allows the quantification of superoxide anion generation rates without the risk of artefacts [63]. NOX1 is indeed an important member of the NOX family with significant expression and physiological function in endothelial cells [64]. Interestingly a previous study on human endothelial cells by Carrano and colleagues reported a redox-dependent mechanism for the impairment of barrier function by amyloid peptide $\beta$ [25]. Differently to our results, the above study identified NOX2 as the key mediator of the effect of the amyloid peptide $\beta$ and the authors described the effect was on endothelial tight junctions (TJs). This discrepancy is likely to depend on the differential experimental approach utilised. Carrano and colleagues used Amplex Red for the detection of ROS (which is sensitive mainly to hydrogen peroxide) while we used EPR with a hydroxylamine-based probe (which selectively measures oxygen radicals such as superoxide anions). In addition, Carrano and colleagues

![Fig. 6. Aβ1-42 causes barrier function impairment and VE-cadherin phosphorylation in endothelial cells. HUVECs were cultured for 24 h to allow the consolidation of intercellular junctions and barrier function before incubation with 25 μM Aβ1-42 or scrambled Aβ1-42 (A). The barrier function was monitored by ECIS for 24 h (impedance measurement were performed at frequency 4000 Hz), after which the monolayer was damaged using a high intensity electrical injury (time 1 s, current 1400 μA). The impedance was measured for a further 24 h to assess cell monolayer and barrier function repair. A representative trace for the impedance within the 72 h of the experiments is shown (top panel) and the statistical analysis of data from 6 and 4 independent experiments (pre-wounding and post-wounding assessments, respectively) is shown in the bottom panel. The statistical significance of the difference was assessed by one-way ANOVA with Tukey post-test (*, p < 0.05; **, p < 0.01; ***, p < 0.001). The localisation of VE-cadherin following treatment with Aβ1-42 or scrambled control peptide was tested by immunocytochemistry (B). Following fixation with 4% w/v paraformaldehyde, VE-cadherin localisation was assessed by antibody staining (plus FITC-labelled secondary) and confocal imaging. VE-cadherin localisation at cell-cell junctions (magnified panel) was quantified by image analysis with FIJI software and the statistical significance of the difference was assessed (Mann Whitney non-parametric test; *, p < 0.05; **, p < 0.01; ***, p < 0.001; n = 4). Western blotting results show that the treatment of HUVECs with Aβ1-42 increases the phosphorylation levels of VE-cadherin$^{Y658}$ (C). Densitometry analysis of the results is expressed as ratio of phosho-VE-cadherin staining over total VE-cadherin staining (FIJI software). The statistical analysis was performed by Mann Whitney non-parametric test (*, p < 0.05; **, p < 0.01; ***, p < 0.001; n = 4).]
focused on the effect of amyloid peptide β on the expression of TJ proteins (e.g. claudins, occludins and ZO1), whereas we described endothelial barrier changes occurring by ECIS or FITC-albumin filtration [65]. Overall, our results and the study from Carrano and colleagues are likely to describe two different phenomena contributing in parallel to BBB alterations in vivo and in AD patients.

Importantly, although the signalling intermediates remains to be identified, we show that the generation of superoxide anion by NOX1 is absolutely essential for the impairment of endothelial cell barrier function. Because of the increasingly understood heterogeneity of endothelial cell types throughout the human body [66], we confirmed these findings in primary microvascular endothelial cells from human brain. Overall, our data describe a novel molecular mechanism linking amyloid peptide β with a loss of junctional stability and an impairment of barrier function of endothelial cells. Considering the central role of NOX1 in the deleterious effect of amyloid peptide β on the barrier function of endothelial cells in vitro, upon confirmation of our findings in vivo and in AD patients, NOX1-selective inhibitors may become important candidates for the development of novel drugs able to protect the cerebrovascular integrity of AD patients. By protecting the cerebrovascular function, NOX1 inhibitors may be able to control or slow down the progression of the neurodegeneration in AD patients.

**Author contribution statement**

AT and NW performed experiments and part of the data analysis for this manuscript. CK and HS performed and analysed the proteomics experiments. HS proofread the manuscript. GP designed the project, planned the experiments, and wrote the manuscript.
Data availability statement

Data in this article will be shared upon reasonable requests to the corresponding author by email.

Declaration of competing interest

The authors declare no conflict of interest.

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