An in vitro bioengineered model of the human arterial neurovascular unit to study neurodegenerative diseases

Jerome Robert¹²,⁷* (jerome.robert@usz.ch), Nicholas L. Weilinger² (nlw15@mail.ubc.ca), Liping Zao¹²,³ (liping@can.ubc.ca), Stefano Cataldi³ (Stefano.Cataldi@nyspi.columbia.edu), Emily B. Button¹² (ebbutton@mail.ubc.ca), Sophie Stukas¹² (sophie.stukas@ubc.ca), Emma M. Martin¹² (emmmarie@mail.ubc.ca), Philip Seibler⁴ (philip.seibler@neuro.uni-luebeck.de), Megan Gilmour¹² (gilmour@mail.ubc.ca), Tara M. Caffrey¹² (tara.caffrey@ubc.ca), Elyn M. Rowe¹² (elyn.rowe@ubc.ca), Brian MacVicar² (bmacvicar@brain.ubc.ca), Matthew Farrer³ (Matthew.Farrer@neurology.ufl.edu), Cheryl L. Wellington¹²,⁴,⁶,⁷* (cheryl.wellington@ubc.ca)

¹ Department of Pathology and Laboratory Medicine, University of British Columbia, Vancouver. British Columbia, Canada, V6T 1Z3
² Djavad Mowafaghian Centre for Brain Health, University of British Columbia, Vancouver. British Columbia, Canada, V6T 1Z3
³ Centre for Applied Neurogenetics, University of British Columbia, Vancouver, British Columbia, Canada, V6T 1Z3
⁴ Institute of Neurogenetics, University of Luebeck, 23562, Luebeck, Germany
⁵ School of Biomedical Engineering, University of British Columbia, Vancouver. British Columbia, Canada, V6T 1Z3
⁶ International Collaboration on Repair Discoveries, University of British Columbia, Vancouver, British Columbia, Canada, V5Z 1M9
⁷ New address: Institute of Clinical Chemistry, University hospital Zurich, 8000 Zurich, Switzerland
* Corresponding authors: Jerome Robert, Institute of Clinical Chemistry, University Hospital Zurich, Wagistrasse 14, CH-8952 Schlieren, Switzerland. Jerome.robert@usz.ch or Cheryl Wellington, Djavad Mowafaghian Centre for Brain Health, University of British Columbia, 2215 Wesbrook Mall, Vancouver, British Columbia, Canada, V6T 1Z3, Cheryl.wellington@ubc.ca
Abstract

Introduction: The neurovascular unit (NVU) – the interaction between the neurons and the cerebrovasculature – is increasingly important to interrogate through human-based experimental models. Although advanced models of cerebral capillaries have been developed in the last decade, there is currently no in vitro 3-dimensional (3D) perfusible model of the human cortical arterial NVU.

Method: We used a tissue-engineering technique to develop a scaffold-directed, perfusible, 3D human NVU that is cultured in native-like flow conditions that mimics the anatomy and physiology of cortical penetrating arteries.

Results: This system, composed of primary human vascular cells (endothelial cells, smooth muscle cells and astrocytes) and induced pluripotent stem cell (iPSC) derived neurons, demonstrates a physiological multilayer organization of the involved cell types. It also reproduces key characteristics of cortical neurons and astrocytes, as well as the formation of a selective and functional endothelial barrier. We further provide proof-of-principle that our in vitro human arterial NVU may be suitable to study neurodegenerative diseases such as Alzheimer’s disease (AD), as we report both phosphorylated tau and beta-amyloid accumulation in our model over time. Finally, we show that our arterial NVU model enables the study of neuronal and glial fluid biomarkers.

Conclusion: This model is a suitable tool to investigate arterial NVU functions such as neuronal electrophysiology in health and disease. Further the design of platform allows culture under native-like flow conditions for extended periods of time and yields sufficient tissue and media for downstream immunohistochemistry and biochemistry analyses.
The brain consumes ~20% of total body oxygen and glucose utilization despite representing only 2% of total body mass \(^1,2\). These high metabolic demands vary both temporally and spatially in the brain, and are met by the coordinated action of several cell types known collectively as the neurovascular unit (NVU) \(^3,4\). Neural activity increases local cerebral blood flow (CBF) through a process known as neurovascular coupling \(^5\). This process links neuronal glutamate release to astrocyte calcium waves, triggering the release of vasoactive molecules that modulate vascular tone of nearby pericytes in the capillaries, or smooth muscle cells (SMC) in the arterioles and arteries \(^6\)–\(^8\). Endothelial cells (EC) within the NVU form the blood-brain barrier (BBB) that restricts blood-brain exchange and regulates brain waste excretion \(^9\). Disease-associated changes in CBF and the BBB are observed in many neurodegenerative brain disorders including Alzheimer’s disease (AD) \(^3,10\). As such, there is tremendous interest in developing cell-based models that mimic the BBB and NVU. Such models would greatly facilitate gaining a better understanding of the interactions between neurons and the vasculature in both physiological and pathophysiological conditions. If made with human cells, they would also provide an invaluable translational platform for the development of neurodegenerative therapeutics.

Tissue engineering, organoid culture, and microfluidic technologies have emerged in the last decades as powerful tools to study how different cell types interact in the context of their native extracellular matrices (ECM), thus driving next-generation models of human disease \(^11\)–\(^13\). Among the tens of models relevant to the central nervous system that have been developed thus far, the most advanced include the Parker model that links a BBB microfluidic chip containing EC, pericytes and astrocytes to a brain microfluidic chip composed of neurons and astrocytes via artificial cerebrospinal fluid perfusion \(^12\), and the Svendsen model consisting of a single
microfluidic chip where the vascular chamber consisting of EC is separated from the brain chamber consisting of neurons, astrocytes and pericytes by a semi-permeable membrane. These groups, and many others, have focused their efforts on modeling the microvasculature, given the importance of brain capillaries in neuronal function and the need for better models to assess drug uptake across the BBB.

By contrast, we aimed here to develop a model of the large arterial NVU, as cerebral arteries underlie many physiological and pathophysiological processes important for brain function. We recently developed a human cerebrovascular model consisting of EC and SMC cultured with or without astrocytes, mimicking penetrating and leptomeningeal arteries, respectively. Using this model we demonstrated the possibility to study key pathovascular features of AD in vitro, namely the accumulation of beta amyloid (Aβ) in the vascular wall and subsequent vascular inflammation, which comprises cerebral amyloid angiopathy (CAA). However, this model lacks neurons and relied solely on exogenous recombinant Aβ, and thus, is limited in its ability to study the role of neuronal biology and function.

In the present study, we successfully expanded this platform to generate a model of the arterial NVU composed of primary human EC, SMC and astrocytes cultured in the presence of human induced pluripotent stem cells (iPSC)-derived glutamatergic cortical neurons. Under luminal native-like flow conditions, this method creates distinct vessels that can be sampled from both the “brain” and “blood” sides. Histological analyses confirmed a multi-layer structure similar to native human cerebrovascular tissues, and biochemical analysis confirmed the presence of a tight BBB separating a closed “brain” compartment from a separate “blood” compartment that
circulates through the vessel lumen. We further showed that iPSC-derived neurons cultured in our bioengineered arterial NVU were electrically excitable and could both secrete glutamate and had measurable α-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid receptor (AMPAR) currents, suggesting possible synapse formation. Finally, we confirmed that our model could be used to study key elements of arterial pathophysiology relevant to AD in vitro, as Aβ peptides were produced by neurons and transported from the “brain” compartment to the “blood” compartment, where they gradually accumulated in the vascular wall with a stronger deposition of Aβ40 than Aβ42. Phosphorylated tau was also confirmed to deposit in the vascular wall, further validating this system as a viable AD model. We thus demonstrate here that our model serves as a controlled platform that can be used to interrogate the physiology of the human arterial NVU, including the possibility of measuring tau, neurofilament light (NFL), glial fibrillary acidic protein (GFAP), and ubiquitin carboxyl-terminal hydrolase L1 (UCH-L1) as brain biomarkers.

Methods

Culture of iPSC-derived neurons

Glutamatergic cortical neurons were derived from human induced pluripotent stem cells (iPSC) using a modified protocol from Shi et. al. Briefly, iPSC (line L2131) were maintained in mTesRTM1 medium (StemCell). Seven days after the last passage, iPSC were groomed by removing any colonies having an appearance of differentiated cells, irregular borders or a transparent-center. iPSC were washed with Dulbecco’s Modified Eagle Media (DMEM)/F12 (Invitrogen) and dissociated into single cells using accutase (Invitrogen) and filtered through a 0.45 µm cell strainer. After 2 washes with IPS media (4:5 DMEM/F12, 1:5 knockout serum
replacement [KOSR], 15 mM HEPES, 1% glutamine, 1% MEM-non essential amino acids
[NEAA], 0.1 mM β-mercaptoethanol, 10 ng/mL human fibroblast growth factor 2 [hFGF2]),
IPSC were plated at a density < 200,000 cells/cm² on gelatin-coated plates in IPS medium
containing 10 μM ROCK-Inhibitor (Y-27632). After 1 h at 37 °C, non-adherent cells were
collected and suspended in murine embryonic fibroblast (MEF) conditioned media containing 10
μM Y-27632 and 20 ng/mL of human FGF and plated on matrigel-coated plates at a density of 1-
1.5x10⁶ per 6-well plate. MEF medium was changed daily until cells were 95% confluent, which
was usually after one day. To initiate neuronal differentiation, 2 mL of KSR (Knockout DMEM
with 15% KOSR, 1% glutamine, 1% MEM-NEAA, 0.1 mM β-mercaptoethanol) were added to
the cells for 4 days. On day 5, KSR media was gradually replaced by neural maintenance medium
(NMM: 1:2 DMEM/F12, 1:2 neurobasal medium, 0.25% N2 supplement, 0.25 μg/mL insulin,
0.5% MEM-NEAA, 50 μM M2-ME, 1% neuroCult SM1 Neuronal supplement, 1% glutamine,
1% Pen/strep) medium at a ratio of 3:1 KSR:NMM on day 5, 2:2 on day 7, 1:3 on day 9, and
100% NMM containing 1 μM dorsomorphine, 10 μM transforming growth factor-β (TGF-β)
inhibitor SB 431542, 10 μM Y-27632 on day 11. On day 12 of differentiation, medium was
removed and tissue was dissociated into clumps using a pipette. Cells were plated onto 6 cm
dishes coated with poly-D-lysine/laminin in NMM. On day 13-17 of differentiation, media was
removed and NMM supplemented with 20 ng/mL human FGF and 20 ng/mL of human brain
derived neurotrophic factor (BDNF) was added. On day 18 of differentiation, rosettes were
manually picked with a sterile pipette and plated in NMM supplemented with 20 ng/mL of
BDNF, 20 ng/mL of glial derived neurotrophic factor (GDNF), and 0.2 mM ascorbic acid on
poly-D-lysine/laminin-coated 6-well plates. From day 19-22 of differentiation, media was fully
refreshed every other day. On day 23 of differentiation, rosettes were manually picked with a
sterile pipette and plated in NMM supplemented with 20 ng/mL of BDNF, 20 ng/mL of GDNF, and 0.2 mM ascorbic acid on poly-D-lysine/laminin-coated 6-well dishes. From day 24-27 of differentiation, medium was refreshed every other day. On day 28 of differentiation, medium was removed, cells were washed with PBS and dissociated using accutase. After 10-15 min, cell were lifted by pipetting up and down before collecting and centrifuging at 160 g for 5 min. Cells were suspended in complete NMM and plated on poly-D-lysine/laminin coated 6-well dishes at a density of 1x10^6 cells per well. From day 29 of differentiation on, medium was refreshed with full NMM every three days.

Isolation and culture of vascular cells

All experiments were conducted under an approved clinical protocol (UBC Clinical Ethics Research Board H13-02719) after obtaining written informed consent from all subjects. Human umbilical vein endothelial cells (EC) and human umbilical cord myofibroblasts (SMC) were isolated as described 21. Briefly, EC were isolated using the instillation method, where the vein lumen was filled with a solution of collagenase (2 mg/mL, Collagenase A, Roche) in serum-free DMEM (Invitrogen) before clamping both ends. After 20 min at 37°C, Advanced DMEM (Gibco) supplemented with 1% L-glutamine, 0.05% penicillin/streptavidin (pen/strep) and 10% fetal bovine serum (FBS) (Invitrogen) was flushed through the lumen and the cell suspension was centrifuged at 1,200 rpm for 5 min. EC were expanded in full endothelial growth medium (EGM™-2) (LONZA Inc, supplemented with vascular endothelial growth factor (VEGF), human recombinant insulin-like growth factor-1 (hrIGF-1), human epidermal growth factor (hEGF), amphotericin-B, hydrocortisone, ascorbic acid, heparin, and 2% (FBS) up to passage 10 with media changed every 3-4 days. SMC were isolated by mincing the vessel wall into small pieces (~2-3 mm) and incubating at room temperature for 20 min without medium under sterile laminar
flow to ensure physical attachment of the pieces. Advanced DMEM (Invitrogen) supplemented with 1% L-glutamine, 0.05% pen/strep and 10% FBS was subsequently added to the minced vessels and adherent cells were expanded up to passage 10 with media changed every 3-4 days. Mature human primary astrocytes (Sciencell) were cultivated in astrocyte media (Sciencell) supplemented with astrocyte growth factor (Sciencell), 0.05% pen/strep and 2% FBS up to passage 5 with media changed every 3-4 days.

**Bioengineering of an in vitro arterial NVU**

Bioengineered constructs were fabricated using a dynamic, semi-pulsatile flow bioreactor system. Tubular biodegradable scaffolds (length 1.5 cm and inner diameter 2 mm) were produced as previously described. Briefly, non-woven polyglycolic acid (PGA, Biomedical Structure) meshes (thickness: 1 mm and density: 70 mg/cc) were dip-coated with polycaprolactone (PCL) and polylactate (PLA) by dipping PGA mesh in a solution of 1.75% (w/w) PCL/PLA/tetrahydrofuran (THF) solution (Sigma Aldrich), shaped into tubes using heat, and externally coated with a 10% PCL/THF (w/w) solution. Scaffolds were sterilized by immersion in 70% ethanol for 30 min, followed by three PBS washes and finally immersion in advanced DMEM supplemented with 10% FBS for at least 12 h. SMC were seeded at density of 2-3x10^6 cells/cm^2 on the inner surface of the scaffold using fibrin (15 μL fibrinogen 10 mg clottable protein/mL PBS and 15 μL thrombin 100 μU/mL PBS) as a cell carrier that was added directly to the scaffold, then incubated under static conditions in advanced DMEM supplemented with 10% FBS, 1% L-glutamine and 0.05% pen/strep and ascorbic acid. After 3 to 5 days, advanced DMEM supplemented with 10% FBS, 1% L-glutamine and 0.05% pen/strep and ascorbic acid was flowed through the lumen of the vessel using a peristaltic pump to mimic blood flow for 7
days. Vascular intermediates were then seeded with EC (1x10^6 cells/cm²) on the luminal side and cultivated first in static conditions in full EGM™-2 supplemented as above. After 3 days, primary astrocytes were seeded (1x10^6 cells/cm²) using fibrin as a cell carrier as above (10 µL thrombin and 10 µL fibrinogen) on the antelumen side of the tissue. After 5 min at RT, tissue constructs were placed in complete astrocyte media under static conditions. After 24 h, iPSC-derived neurons (age 60 to 80 days) were seeded on the antelumen at a density of 2x10^6 cells/cm² using 10 µL matrigel as a cell carrier. Tissues were maintained at RT for 5 minutes before mounting in the bioreactor with completed NMM media both in the tissue and circulation chamber. Tissues were maintained under flow condition for a maximum of 21 days before experiments.

**Green fluorescent protein (GFP) electroporation**

Neurons were transfected with the pmaxGFP vector (Lonza) using the Nucleofector 2b (Lonza, Switzerland) device. Briefly, neurons were washed twice with PBS and detached by adding accutase to the wells for 5 to 15 minutes. Neurons were collected in NMM+ as described above and centrifuged at 250 g for 3 minutes. They were then suspended in Mouse NSC Nucleofector Solution (Lonza) at a density of 4 x 10^6 neurons/100 µL with 4 µg of pmaxGFP, followed by transfection in the Nucleofector 2b using the program B-016. 500 µL warm NMM+ was then directly added to the transfected cells. After 5 min, cells were centrifuged at 250 x g at room temperature for 3 min, suspended in 10 µL matrigel and 10 µL NMM with Y-27632 and seeded on the antelumen of the tissues as above.

**Electrophysiology**
Bioengineered tissues were carefully longitudinally cut in thirds and transferred to a recording chamber continually perfused (1-2 mL/min) with artificial cerebral spinal fluid (aCSF) consisting of: 126 nM NaCl, 26 mM NaHCO₃, 2.5 mM KCl, 1.25 mM NaH₂PO₄, 2 mM MgCl₂, 2 mM CaCl₂, and 10 mM glucose. aCSF was continuously bubbled with 95% O₂/5% CO₂ and warmed to 33°C using a stage heater (Luigs & Neumann). Whole-cell patch clamp recordings were obtained using thin-walled borosilicate glass microelectrodes (Warner) pulled to a tip resistance of 3-5 MΩ with a P-97 Flaming/Brown Micropipette Puller (Sutter Instrument). Electrodes were filled with an intracellular recording solution containing: 108 mM K-gluconate, 3 mM KCl, 2 mM MgCl₂, 8 mM Na-gluconate, 1 mM K₂-EGTA, 230 µM CaCl₂, 50 µM Alexa-594, 4 mM K₂-ATP, 200 µM Rhod-2 tripotassium salt (ThermoFisher), and 300 µM Na₃-GTP at pH 7.25 with 10 mM HEPES. Recordings were made using a MultiClamp 700B amplifier and a Digidata 1440A digitizer (Axon Instruments, Molecular Devices) controlled via Clampex 10.7 acquisition software. Cells were voltage clamped at -60 mV for glutamate puff experiments and passively current clamped (i.e. passive membrane potential monitoring) for Ca²⁺-imaging experiments. Stimulation trains (200 pA, 5 ms/pulse, 20 Hz, 5 s total) were applied for transient membrane depolarizations to trigger Ca²⁺ entry. Access resistance was always <20 MΩ. Glutamate (200 µM) was transiently applied (5 s puffs) by using a puff electrode connected to a Picospritzer II (General Valve Corporation). The relative magnitude of AMPAR currents were quantified as normalized charge (i.e. the area under the curve) to control for the variability of the peak current responses. CNQX (10 µM) was bath applied and was purchased from Tocris.

Two-photon microscopy
All experiments were performed on a LSM MP710 2-photon imaging system (Zeiss, Germany). Cells were identified for whole-cell patch clamp and imaging using either widefield infrared illumination captured with a DAGE IR-1000 camera (DAGE-MTI). This was preferable to patching GFP-labelled cells due to the sparse labeling and ease in identifying healthy neurons with transmitted illumination. GFP and/or Rhod-2 imaging was achieved by 2-photon excitation with a Ti:Sapphire Chameleon Ultra II 2-photon laser (Coherent) tuned to 850 nm. Images were acquired with a Zeiss 20X-W/1.0 NA objective at a pixel resolution of either 512 x 512 or 256 x 256 for fast Rhod-2 Ca\(^{2+}\)-imaging. Emission light was split with a 575 nm longpass filter, and green and red emissions were filtered with 535/50 nm and 630/75 nm bandpass filters, respectively (all from Chroma Tech). Emission light was collected with LSM BiG GaAsP detectors from Zeiss, and data were acquired using Zen software (Zeiss) and analyzed in Fiji.

**Glutamate quantification**

Media was removed and cultures received a treatment of 56 mM KCl or regular Hanks Buffered Salt Solution (HBSS) for 30 minutes. Glutamate was measured by high pressure liquid chromatography (HPLC) coupled to electrochemical detection (ALEXYS Neurotransmitter platform, Antec). 5 µL of sample was automatically injected (AS 110 Autosampler, Antec) onto an Acquity UPLC HSS T3 analytical column (1 mm inner diameter, 50 mm length; Waters) perfused at a flow rate of 200 µL/min (LC 110S pump, Antec) with a mobile phase containing 50 mM phosphoric acid, 50 mM citric acid, 0.1 mM EDTA, and 2% acetonitrile (pH 3.5). At the end of each sample, a solution of 50 mM phosphoric acid, 50 mM citric acid, 0.1mM EDTA, and 50% acetonitrile (pH 3.5) was run to flush the column before the next sample. Each sample was mixed with a solution of 0.025 g of ortho-phthalaldehyde (a derivatization agent) in 250 µL of methanol, 250µL of 1M sodium sulfite, and 4.5 mL of 0.1 M borate buffer (pH 10.4), for analytic
detection. Glutamate was detected by means of an electrochemical detector (Decade II, Antec) with the cell potential set at 0.85V vs. salt bridge. Retention times were 3.1 ± 0.4 min.

**ApoE measurement**

Secreted apoE levels in both the tissue chamber and circulation media were quantified by an apoE ELISA protocol as described previously. 1 µM of the liver-X-receptor agonist GW3965 or dimethyl sulfoxide (DMSO) vehicle control were circulated through the lumen for 96 h before collecting media. Fluorescence was read at 325$_{Ex}$/420$_{Em}$ on an Infinite M200 Pro plate reader (Tecan Life Science, Switzerland).

**Histology and immunohistochemistry of bioengineered arterial NVU**

For immunohistochemistry, bioengineered arterial NVU were washed in PBS and fixed in 4 % paraformaldehyde. After 30 minutes, tissues were washed three times in PBS, cryopreserved in 20% sucrose PBS solution for a minimum of 60 min, embedded in 5% bovine skin gelatin (Sigma Aldrich) and 20% sucrose in PBS and stored at -80 °C until further processed. Samples were processed on a cryotome (chamber -30 °C and object -25 °C) to generate 20 µm sections that were stored at –80 °C until analysis. Sections were rehydrated in PBS for 2×10 min before blocking for 30 min in 5% goat serum and 1% BSA in PBS. Alternatively, after fixation and washing, bioengineered arterial NVU were cut in half longitudinally and directly process for staining.

For immunofluorescence, sections and arterial NVU were blocked in 5% donkey serum and 1% BSA in PBS for 30 min at RT, incubated overnight at 4 °C with specific antibodies against the endothelial markers CD31 (RRID: AB_31432, WM59 Biolegend, 1:50) and von Willebrand factor (RRID:AB_259543, SigmaAldrich, 1:200), the SMC marker α-SM-actin...
(RRID:AB_476856, 1A4 SigmaAldrich, 1:200), the astrocyte marker glial fibrillary acidic protein (GFAP) (RRID: AB_880203, Abcam, 1:200), s100-β (RRID:AB_306716, Abcam, 1:200), and neuronal markers MAP2 (RRID:AB_776174, Abcam, 1:200) and β-tubulin III (RRID:AB_2256751, Tuj1, 1:200) and synapsin I (RRID:AB_2200097, Abcam, 1:200). After three additional PBS washes, sections and arterial NVU were incubated for 45 min at RT with anti-rabbit or anti-mouse Alexa-488 or Alex-594 secondary antibodies (Invitrogen). Sections were finally washed three times in PBS and mounted in Prolong Diamond antifade containing DAPI (Invitrogen). Sections were imaged with an Axioscan inverted microscope (Zeiss, Germany) and arterial NVU were imaged with an Axioscan inverted confocal microscope (Zeiss, Germany).

**Aβ quantification**

Luminal media was collected from the circulation loop and abluminal media was collected from the tissue chamber. In addition, 5 mm cross-sectional rings of tissue were crushed and lysed in RIPA buffer (10 mM Tris pH 7.4, 150 mM NaCl, 1.0% NP-40, 1.0% sodium deoxycholate, 0.1% SDS and cOmplete protease inhibitor with EDTA (Roche)). After homogenization, tissue samples were centrifuged for 15 min at 14000 g at 4 °C and the RIPA soluble fraction was transferred to a new tube. 5 mM of guanidine (GuHCl, Sigma Aldrich) was added to the tissue pellet and incubated overnight at RT under constant agitation before centrifugation at 14000 g at 4 °C for 15 min. RIPA soluble and GuHCl soluble fractions were stored at -20 °C until quantification. RIPA (soluble), GuHCl (insoluble) and media fractions were quantified without dilution using Aβ40 (KHB3442, Life Tech) and Aβ42 (KHB3482, Life Tech) commercial ELISA kits and normalized to total protein concentration measured by BCA assay (Fisher).
Single molecule array for biomarker quantification

Total tau, GFAP, neurofilament light (NFL) and ubiquitin carboxyl-terminal hydrolase L1 (UCH-L1) were quantified in media from the tissue chamber (abluminal) and from the circulation loop using the Neurology 4-plex A assay (Quanterix, USA) using the Simoa HD-1 analyzer (Quanterix) following the manufacturer’s guidelines. Abluminal and circulating media were diluted off-board 2500 and 20 fold, respectively.

SDS-PAGE and Immunoblotting

Tissues composed of EC, SMC and astrocytes without or with neurons were lysed in RIPA buffer with Phosphostop (Roche). After 20 min on ice, tissues were crushed using a manual pestle before centrifuging for 10 min at 12000 g at 4 °C. Total protein was quantified using BCA assay. Equal amounts of total protein (25 µg) were separated by 10% acrylamide SDS-PAGE, followed by electrophoretic transfer to polyvinylidene fluoride (PVDF) membranes (Millipore). Membranes were blocked for 1 h using 1% BSA in Tris-buffered saline (TBS) containing 0.5 % Triton X (TBST). Phosphorylated tau AT8 (RRID:AB_223648, ThermoFisher, 1:1000), CP13 (RRID:AB_2314223, kindly gifted by Dr. Peter Davies at Litwin-Zucker Research Center for The Study of Alzheimer’s Disease and Memory Disorders, 1:1000) and PHF1 (RRID:AB_2315150, Dr. Peter Davies, 1:1000) and total tau DA9 (RRID:AB_2716723, Dr. Peter Davies, 1:1000) were immunodetected by incubating overnight in blocking buffer at 4 °C. Membranes were washed extensively with TBST and incubated with anti-mouse, (1:1000, Jackson ImmunoResearch) secondary antibody in blocking buffer. After 1 h, membranes were washed extensively with TBST, developed using enhanced chemiluminescence (ECL,
Amersham) and imaged using ChemiDoc MP imager (Biorad). Band densitometry was quantified using ImageJ.

**Statistical analyses**

Comparisons between groups were performed using unpaired or paired Student t-test, or one-way ANOVA with Dunnett’s or Sidack’s multicomparison test. Dependence analyses were assessed through Pearson correlation analysis. Values below the detection limit of the ELISAs were considered as 0 for statistical analysis and plotted as gray points. Data were obtained from at least three independently seeded bioengineered arterial NVU and graphically represented as scatter or before-after plots with mean ± standard error of the mean (SEM). P-values of <0.05 were considered statistically significant. All statistical analyses were performed using GraphPad Prism-5 or SPSS software.

**Results**

*Production and anatomical characterization of bioengineered arterial NVU*

Human arterial NVU were fabricated by sequentially seeding in order, primary human SMC, primary human EC and primary human astrocytes into a tubular porous scaffold consisting of polyglycolic acid (PGA), and polycaprolactone (PCL), measuring 15 mm long and 2 mm in diameter as described previously. 24 h after seeding astrocytes, iPSC-derived neurons (aged 60 to 80 days) were seeded on the antelumen of the tissues using matrigel as a cell carrier. A schematic of the finished NVU and bioreactors system is provided in Fig. 1a. After 3 weeks in culture under native-like luminal flow condition, cryosections from bioengineered arterial NVU were prepare along the longitudinal axis and processes for immunofluorescence staining against the EC marker CD31, the SMC marker α-smooth muscle actin (αSMA), and the astrocyte marker
glial fibrillary acidic protein (GFAP). We observed a multilayer tissue organization resembling a cerebral artery; with a single layer of EC on the lumen, multiple layers of SMC surrounding the endothelium and astrocyte layers on the antelumen (Fig. 1b-d). Immunofluorescence staining also demonstrated the presence of the neuronal marker β–tubulin-III and microtubule associated protein (MAP2) positive cells in the most abluminal layers of the bioengineered vessels, supporting the presence of neurons in the culture (Fig. 1e). The abluminal structure was further characterized by immunofluorescence staining against GFAP and MAP2 by imaging a longitudinal cut of the bioengineered NVU using confocal microscopy. Co-staining demonstrated that neurons and astrocytes form an imbricated network of cells on the last abluminal layers of cells with astrocytes penetrating deeper in the tissue than neuron, whereas cells deeper in the tissue were negative for both markers (Fig. 1f). Interestingly, contrary to cells grown in 2D culture, iPSC-derived neurons onto the bioengineered vessels do not form colonies but are rather uniformly dispersed on the abluminal surface along astrocytes.

Cell functionality in the bioengineered arterial NVU

Several approaches were used to evaluate neuronal function in the bioengineered arterial NUV. We first analysed synapsin expression using immunofluorescence staining of cryopreserved cross sections. Confocal imaging confirmed punctate synapsin staining in MAP2 positive cells (Fig. 2a, white arrows). Second, we measured glutamate release after KCl treatment using high performance liquid chromatography (HPLC) with electrochemical detection. After 3 weeks under flow conditions, tissues were treated with KCl. After 30 min, five of the six tissues with neurons tested demonstrated significant increased glutamate release (Fig. 2b-c). Next, we used two-photon microscopy analysis and electrophysiology to assess the morphology and electrical
properties of the neurons and to determine their sensitivity to glutamate stimulation. Neurons were identified by sparse (1/5) green fluorescent protein (GFP) labelling, and qualitative morphological analysis by two-photon microscopy revealed a typical neuronal phenotype with long processes extending from the soma (Fig. 2d). To assess the intrinsic properties of these cells, we first tested for depolarization-induced Ca\(^{2+}\)-entry by driving action potential firing. Neurons were whole-cell patch loaded with the membrane impermeant Ca\(^{2+}\)-sensor Rhod-2 for Ca\(^{2+}\) imaging with 2-photon microscopy (Fig. 2e). Stimulation trains (200 pA, 5 ms/pulse, 20 Hz, 5 s total) triggered action potentials (Fig. 2f-g) and temporally correlated with Ca\(^{2+}\) entry in the soma and dendrites as measured by an increase in Rhod-2 fluorescence (Fig. 2h), suggesting that the cells were both electrically excitable and expressed voltage-gated Ca\(^{2+}\) channels. Lastly, we tested for functional expression of AMPAR in the membrane by exogenous glutamate stimulation. Glutamate was locally applied via a puff electrode (200 µM, 5s) and elicited reliable inward currents in cells voltage clamped at $V_m = -60$ mV. These currents were reversibly inhibited by bath application of the AMPA receptor antagonist 6-cyano-7-nitroquinoxaline-2,3-dione (CNQX, 10 µM), confirming that these currents were mediated by AMPA receptor opening (Fig. 2i-j). We further characterized the arterial NVU function by measuring apoE secretion after stimulation with the brain-penetrant Liver-X-Receptor (LXR) agonist GW3965 added to the circulating media before collecting tissue chamber and circulation media. After 96 h, ELISA quantification confirmed a significant increase of apoE secretion in the chamber media of GW3965-treated tissues while the concentration of apoE in the circulation media was below the detection limit of the ELISA, and no different than control media (Fig. 2k), suggesting a lack of apoE transport across the BBB in our model. These observations provide evidence for both astrocyte functionality via apoE secretion and a tight BBB, as apoE does not cross the BBB in vivo. Together, these data confirm that our bioengineered arterial NVU possesses both
structural and functional characteristics of native neurons, and astrocytes surrounding a functional blood vessel.

Bioengineered arterial NVU might serve as model to study neurodegenerative diseases

In vitro models are used as research tools to address specific physiological or pathological questions that are not feasible to study in human subjects or are difficult to translate from animal models. Here we tested the hypothesis that our bioengineered arterial NVU could be used to study cerebral contributions to AD or other neurodegenerative diseases. AD is the leading cause of dementia affecting over 50 million people worldwide with a global economic burden of over one trillion USD\(^2\). While extracellular plaques composed of beta-amyloid (Aβ) peptides, and intracellular neurofibrillary tangles are the classical neuropathological hallmarks of AD, 90% of AD patients also have some form of cerebral vessel disease, including vascular Aβ deposition known as CAA\(^5\). We first tested if native, neuronally secreted Aβ was detectable in the tissue chamber and circulation media as well as in bioengineered tissue lysates. We first measured the level of Aβ40 and Aβ42 in the tissue chamber and circulation media by ELISA and found that Aβ40 levels were significantly enhanced both in tissue chamber and circulating media compared to Aβ42 levels. Specifically, Aβ42/Aβ40 ratios were 0.078 and 0.0867 in the tissue chamber and circulating media, respectively, similar to reported Aβ42/Aβ40 ratio in human CSF\(^2\) and blood\(^2\). Importantly, we detected significantly more Aβ in the tissue chamber than in the circulation media 4 days after the last media changed (Fig. 3a), confirming that the tight BBB prevented diffusion of Aβ between the two media compartments. Because Aβ can be secreted by various cell types in vivo including EC\(^2\), we confirmed that Aβ was predominantly secreted by neurons in our model by comparing tissues fabricated with and without neurons. ELISA quantification
revealed that tissues with neurons had twice as much Aβ40 in the tissue chamber than tissues composed only of EC and SMC (bipartite, 26.42 pg/mL) and tissues composed of EC, SMC and astrocytes (tripartite, below detection limit of the ELISA, <6 pg/mL). Further, Aβ40 concentration increased over time from 54.78 to 212.6 pg/mL after 1 week and 3 weeks in culture, respectively (Fig. 3b). Aβ42 was also only detectable in tissues with neurons compared to tissues lacking neurons, in which Aβ42 was below the detection limit of the ELISA (10 pg/mL). Surprisingly, the level of Aβ42 did not significantly increase in the tissues with neurons between 1 and 3 weeks in culture with 28.85 pg/mL (one sample below detection limit) and 15.56 pg/mL, respectively (Fig. 3c).

We next analyzed Aβ deposition in both RIPA and GuHCl soluble fractions. We confirmed the presence of Aβ40 in the RIPA soluble fraction in all 18 tissues tested, and Aβ42 in 17 of the 18 tissues (Fig. 3d). We further investigated whether Aβ was detectable in the GuHCl fraction – corresponding to insoluble fibril depositions – and found that Aβ40 was found in significantly more tissues than Aβ42; with 9 tissues (50% of the total tissues analyzed) positive for Aβ40 and only 2 tissues (11%) above the ELISA detection limit for Aβ42 (Fig. 3d). These results suggest that Aβ40 accumulates more than Aβ42 in our model, which could be explained either by the increased level of Aβ40 secreted as shown in Fig. 3a, or by increased retention of Aβ40 in the tissue. To test this hypothesis, we asked whether the level of Aβ in the circulation media depended on the level in the tissue chamber, reasoning that if increased Aβ40 secretion causes the enhanced Aβ40 accumulation, Aβ40 and Aβ42 levels in the tissue chamber and circulation media should correlate. However, if Aβ40 is more readily retained in the tissue, Aβ42 levels in
the tissue chamber and circulation media should correlate but Aβ40 levels should not. While Aβ42 levels significantly correlated, Aβ40 levels were independent of media compartment (Fig. 3e-f) suggesting that preferential retention of Aβ40 in the vasculature underlies its deposition in the vasculature. We further confirmed that the origin of accumulated vascular Aβ was neuronal by comparing tissues with and without neurons. While Aβ40 and Aβ42 concentration increased over time in tissues with neurons (1 vs 3 week), Aβ40 and Aβ42 levels in tissues lacking neurons were below the detection limit of the ELISAs (Fig. 3g-h).

We then used immunoblotting to test if phosphorylated tau (p-tau), which is the first constituent of neurofibrillary tangles, was detectable in our bioengineered arterial NVU. We confirmed the presence of p-tau in tissues with neurons using two different p-tau antibodies CP13 (pSer202) and PHF1 (pSer396/Ser404) as well as the early tangle marker AT8 (pSer202/Thr205) in comparison to a marker of total tau (DA9). As expected, tissues lacking neurons were negative for p-tau (Fig. 3i). Taken together, these results support the potential utility of this model to study mechanisms relevant to human AD pathogenesis.

Bioengineered arterial NVU for biomarker development

The possibility to study fluid biomarkers using a human-based in vitro model of the cerebrovasculature has, to our knowledge, not yet been investigated. Nevertheless, a biofidelic human model would have undeniable translational advantages over animal models to validate or discover novel biomarkers of disease. As a proof-of-principle, we tested whether our arterial NVU model could be used for such a purpose by quantifying levels of total tau, neurofilament light (NFL), ubiquitin carboxy-terminal hydrolase L1 (UCHL1) and glial fibrillary acidic protein
GFAP) in the circulation media using an ultrasensitive single molecule immunoassay (SIMOA). After 3 weeks in culture and four days after the last media exchange in the circulation loop, the levels of all four biomarkers were significantly higher in the tissue chamber compared to the circulation media (Fig. 4a-d). These results were confirmed by calculating the circulation:chamber ratio (Fig. 4e) that further confirm that the BBB in our model preserves the separation of the tissue chamber and circulation compartments. Interestingly, we confirmed a positive correlation between chamber and circulation media for both NFL and GFAP but no correlation for total tau and UCHL1 (Fig. 4f-i). We next investigated whether fluid biomarker levels in the tissue chamber and circulation media were dependent on each other. For this, we selected two pairs of biomarkers for a dependence analysis, namely NFL:total tau and GFAP:Aβ40 for which we provide the plotted data (Fig. 5a and c) and a summary table for the other analyses (Fig. 5b and d). Dependence analyzes show that total tau, NFL, GFAP and UCHL1 levels in the tissue chamber strongly correlated positively. Aβ40 levels only correlated with GFAP level and Aβ42 was weakly negatively correlated with total tau in chamber media (Fig. 5a-b). In the circulation media, total tau levels correlated positively with NFL, GFAP and UCHL1 levels, and NFL levels correlated with GFAP levels. Aβ40 levels correlated positively with total tau, NFL and GFAP levels but not with UCHL1 or Aβ42 levels (Fig. 5c-d).

Discussion

The interaction between neurons and the cerebrovasculature is essential for brain function and health. This neuronal-vascular interplay regulates cerebral blood flow and blood-brain exchanges, and its dysfunction is associated with several neurological diseases. As the lack of robust and physiologically relevant models of the human NVU is recognized to be a major barrier for
understanding the cerebrovasculature in health and disease, interest in modeling the human cerebrovasculature, particularly for capillary models, is surging. Here, we combined primary human vascular cells and human iPSC-derived neurons using a tissue engineering approach to produce a functional, human, large vessel NVU system that can be cultured under native-like flow conditions for at least 21 days.

Early NVU models focused on modeling either vascular or neuronal components in isolation and under static conditions. Brain-derived EC, cultured alone or co-cultured with other cerebrovascular cells, often in transwells, are typically used to evaluate the ability of therapeutic agents to cross the BBB. Animal- or human-derived neurons cultured in regular culture dishes lack the 3D cellular organization that regulates neuronal function and many key cellular processes in vivo. Importantly, it has become clear in the last decade that cells sense and respond to the dimensionality and rigidity of their environment, and these qualities cannot be modeled using regular tissue culture methods. Multicellular spheroid systems consisting of human primary or iPSC-derived EC, pericytes, astrocytes and neurons are cultured into multicellular BBB- and/or brain-organoid structures. These organoids can be maintained for extensive time in culture, holding great promise to study neuronal functions. However, controlled perfusion through a validated vascular lumen to study blood-brain and brain-blood transport is not yet possible in these models. On the other hand, several groups have developed capillary-like NVU models using microfluidic systems. These platforms offer total control over luminal flow, but focus on BBB function over neuronal function in the NVU, as the vascular cells are separated from neurons either by a porous membrane or cultured in different chips linked together. Here, we opted to use bioengineering techniques to co-culture EC, SMC, astrocytes and neurons to model the NVU structure of cortical penetrating arteries. Our approach offers the possibility to
control luminal perfusion, assess bidirectional (blood to brain and brain to blood) trans-
endothelial transport, as well as assess neuronal and glial functions in a model where cell types
are grown in close proximity to each other within native extracellular matrix. After 21 days in
culture with luminal flow, we demonstrated that abluminal neurons have histological,
biochemical and electrical functions. While the measured electrical properties – in particular the
resting potential – suggest that neurons in our model were not fully mature, they are comparable
to electrical function previously measured in brain organoids 13. The endothelial barrier
represents an important feature of the NVU, and most previous studies use either FITC-dextran or
trans endothelial electrical resistance (TEER) measurement to assess BBB integrity 38,39.
However, these methods required external non-physiological chemicals to be circulated through
the model, or electrical probes to be placed in the vascular lumen that could potentially lead to
cellular disturbance. Our previous studies using bipartite and tripartite vessels composed of EC
and SMC without and with astrocytes, respectively, also demonstrated that our scaffold-directed
approach produces an intact barrier as assessed by Evans Blue and FITC-dextran extravasation
assays 11. Here, we confirm that astrocyte-secreted apoE remains in the tissue chamber and does
not cross the BBB as documented in vivo 23 confirming an intact BBB in our arterial NVU model.
Barrier formation was further confirmed by documenting differences in the levels of fluid
biomarkers in the tissue chamber compared to the circulation media. In particular, the differential
concentrations of Aβ40, Aβ42, tau, GFAP, NFL and UCHL1 between the two compartments
support tight barrier formation with specific transport mechanisms to be analyzed in future
studies.
Although NVU models are being developed at a rapid pace for potential use in the study of neurodegenerative diseases, only a few studies have provided evidence supporting successful disease modeling. Our laboratory previously generated a vascular model composed of EC, SMC and astrocytes that can be used to study CAA, a component of AD, but the absence of neurons in our previous studies required the injection of exogenous recombinant Aβ. Recently, Shin and colleagues developed a microfluidic AD model composed of neurons, astrocytes and EC to study Aβ-induced BBB damage. Although representing an undeniable step forward developing an in vitro model of vascular AD, this model requires the overexpression of APP and APP/PSEN1 genes with early onset familial AD (FAD) mutations due to short culture time, which may limit the relevance of the model to study late onset AD that accounts for more than 99% of total AD cases. Here we used neurons without FAD mutations and maintained engineered tissues in culture for up to 21 days to ensure Aβ secretion and deposition that closely mimics observations in the human cerebrovasculature in vivo. A human-based model of CAA is highly relevant, as Aβ deposition within the vascular wall is present in 10-40% of non-cognitively declined elderly brains and in 80% of late onset AD brains. Using this approach, we have shown that Aβ40 fibrils accumulate more in the vasculature than Aβ42, following what has been shown in vivo. According to dependence calculations, the enhanced deposition of Aβ40 might be due to an altered vascular transport compared to Aβ42. Future studies are needed to fully understand the mechanisms by which difference Aβ40 and Aβ42 are differentially transported through and deposited within the vascular wall. We also demonstrated here that tau protein is phosphorylated in our model, opening up the possibility to use our NVU model to study, develop, or test drugs relevant to AD, targeting either Aβ or p-tau.
Research on fluid biomarkers for several neurological diseases is advancing at a tremendous pace, with intense interest in developing blood tests. Here we demonstrated that several neuronal and glial biomarkers could be detected in both the tissue chamber and circulation media. Although the absolute biomarker levels were different compared to in vivo conditions, the ratio of circulation:chamber levels was comparable to human blood:CSF ratios. Thus, our model offers the potential to study how specific protein levels correlate in “brain” to “blood” compartments to understand the dependence and independence among biomarkers. Using this approach we found that in the tissue chamber, tau and NFL levels do not correlate with Aβ40 levels, but the levels of these biomarkers do correlate in the circulation media. This suggests that tau and NFL levels in the circulation media are independent of the amount of Aβ40 produced, but tau depends on the amount of Aβ40 retained in the vascular wall. Future studies are required to fully define these relationships and compare them to in vivo data. Of particular interest would be measures of NFL and tau levels in CAA patients. Our system offers a unique model to study protein interaction between the brain and the circulation, opening new avenues in the development and validation of brain biomarkers.

This study, nevertheless, has several limitations. The stiffness of the scaffold used in our model unfortunately prevents studying vascular compliance. With the development and refinement of a more suitable scaffold material for this model, it will be particularly important to enable the study of SMC contraction and relaxation in response to neuronal activity or circulating stimuli, given that several neurodegenerative disease patients have altered CBF and CBF regulation happens rather in arterioles than in capillaries. Another limitation is that we used cells derived from umbilical cord in our model, due to the slow growth rate of primary human cerebrovascular cells.
Although one could argue that cord cells do not reflect the physiology of brain vasculature, we and others have previously demonstrated that HUVECs express BBB marker proteins when co-cultured with astrocytes\textsuperscript{11,54,55}. Further, we have demonstrated here that apoE is restricted in the abluminal chamber in our model; a feature characteristic of BBB EC, given that apoE is actively transported through peripheral ECs\textsuperscript{56,57}. Another limitation is that we used primary cells for the vascular cells over iPSC-derived cells. Our choice was motivated by the accessibility of primary human EC, SMC and astrocytes and because we previously reported the feasibility to bioengineer cerebral vessels using these cells\textsuperscript{11,18}. Future studies will be required to develop tissues made entirely from patient-derived iPSCs for personalized medicine applications. Importantly, as iPSCs will be specific to each individual donor, they have the potential to be highly variable between donor batches, and studies using isogenic iPSC sets are encouraged. Another limitation is that the protocol we used here for iPSC neuron differentiation is known to generate a mixed population of cells positive for the neuronal markers MAP2 and β–tubulin-III (>75%) and a small population of cells positive for GFAP (<25%), as previously reported\textsuperscript{19}. Despite these limitations, our study represents first and foremost a proof-of-principle that a cerebral arterial NVU can be engineered \textit{in vitro} as a potentially relevant tool to study neurodegenerative diseases.

**Conclusion**

Our arterial NVU model represents a key step toward the development of human translational model of large cerebral vessels, and could be a useful standardized tool in the study of cerebrovascular function in both physiological and pathophysiological conditions. In particular, as our model offers the possibility to study transport from the brain to the circulation and vice
versa, it also has the potential to be a novel and relevant platform for drug development targeting both neuronal and vascular functions, and opens up the possibility to study neuronal and glial biomarkers.

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Ethics approval and consent to participate

All experiments were conducted under an approved clinical protocol from the UBC Clinical Ethics Research Board after obtaining written informed consent from all subjects (H13-02719: isolation of vascular cells).

Consent for publication

Not applicable.
Availability of data and material

Raw data can be obtained from corresponding author.

Competing interest

The authors declare no competing interests.

Author Contributions:

JR and CLW designed the research with input from MF, NLW and SC. JR, NLW, LPZ, SC, SS, EMM, PS, MG, and TMC performed the experiments and analyzed the data. JR, CLW, MF and BMV obtained the funding. JR, CLW, EMR and TMC drafted the manuscript and all authors reviewed and approved it.

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**Figure Legends**

**Figure 1.** Histological structure of bioengineered NVU. 

- **a)** Schematic representation of the bioreactor and NVU model. The expression of CD31 (b) confirmed the presence of an endothelial cell monolayer on the luminal side of the bioengineered NVU, α-SMA (c) confirmed the smooth muscle phenotype of the cells in the inner layers, GFAP (d) and MAP2/βtub-III (e) positive staining confirmed the astrocyte phenotype of the cells on the outer layers in coronal section of the NVU. 
- **f)** Staining against GFAP and MAP2 confirmed the imbrication of the astrocytes and neurons in the outer layers using confocal longitudinal imaging. L=lumen.

**Figure 2.** Depolarization- and glutamate-driven activity in abluminal cells indicates a neuronal phenotype and apoE secretion in the tissue chamber indicates astrocyte function and BBB formation. 

- (a) Immunostaining against MAP2 and synapsin (Syn) confirmed the presence of synapses in iPSC derived neurons cultured in NVU. 
- (b) Glutamate release measured by HPLC showing increase after KCl treatment. 
- (c) Example HPLC curves. 
- (d) Two-photon Z-projection image of IPSC cells expressing eGFP. Dotted box displays region of zoomed inset, highlighting dendritic morphology and synaptic structure. 
- (e) Example image of a whole-cell patch clamped iPSC-derived neuron dialyzed with the red Ca$^{2+}$-indicator Rhod-2. Proximal dendrites were imaged for depolarization-induced Ca$^{2+}$-entry. 
- (f) Representative current-clamp trace from patched cell in ‘e’ during 20 Hz spike train stimulation. 
- (g) Single current injection (200 pA, 5 ms) example from ‘f’ showing change in membrane potential. 
- (h) Time-correlated Rhod-2 signal from trace ‘e’ showing depolarization-induced Ca$^{2+}$-increase. 
- (i) (Top trace) Full-length voltage-clamp recording showing glutamate puff-evoked (triangles) AMPAR currents that were amenable to block by CNQX (10 µM) and recovered in washout. (Bottom trace) Example
AMPAR currents before, during, and after CNQX application. (j) Quantitative summary of normalized charge for glutamate inward currents in the presence of CNQX (n=5, **P<0.01). (k) Astrocyte and BBB functions were confirmed by treating tissues with 1 µM LXR agonist GW3965 for 96 h and measuring the levels of astrocyte-derived apoE secreted into the tissue chamber and circulation media. Values below the detection of the ELISA are plotted in gray. Points in graphed data represent individual bioengineered vessels, bars represent mean, error bars represent ±SEM and analysed by one way ANOVA **P<0.01.

Figure 3. Neurons secrete Aβ that then accumulates within the vascular wall. (a) Aβ40 and Aβ42 levels were quantified by ELISA in the chamber and circulation media of bioengineered arterial NVU after 3 weeks. Aβ40 (b) and Aβ42 (c) levels in chamber media of tissues composed of EC and SCM (bipartite), EC, SMC and astrocyte (tripartite) and EC, SMC, astrocytes and neurons (NVU) after a week in culture and NVU after three weeks. (d), Vascular Aβ40 and Aβ42 level in RIPA and GuHCl soluble fractions were quantified by ELISA in NVU after three weeks in culture. The correlation between the level of Aβ40 (e) and Aβ42 (f) in circulation and tissue chamber were assessed through Pearson correlation analysis. The correlation coefficient (R²) and p-value are shown in each panel. Aβ40 (g) and Aβ42 (h) vascular deposition were quantified in RIPA soluble fraction after a week (bipartite, tripartite and NVU) and three weeks (NVU) in culture. i) The level of p-tau (AT8, CP13 and PHF1) was measured by ELISA in NVU and tripartite tissues and compared to total tau (DA9). Points in graphed data represent individual bioengineered vessels, bars represent mean, error bars represent ±SEM and analysed by one way ANOVA or Pearson correlation. Values below the detection of the ELISA are plotted in gray. *= p<0.05, **=p<0.01, ***=p<0.001.
Figure 4. Fluid biomarkers levels in tissue chamber vs. circulation. Total tau (a) NFL (b) UCHL1 (c) and GFAP (d) were quantified in tissue chamber and circulation media four days after last medium change. Ratio circulation:chamber calculation. The correlation between the level of total tau (f) NFL (i) UCHL1 (j) and GFAP (k) in circulation and tissue chamber were assessed through Pearson correlation analysis. Points in graphed data represent individual bioengineered vessels, bars represent mean, error bars represent ±SEM and analysed by paired Student’s t-test or Pearson correlation. The correlation coefficient (R²) and p-value are shown in each panel. *= p<0.05, **=p<0.01, ***=p<0.001, ****<p=0.0001

Figure 5. Fluid biomarkers and Aβ levels dependence in tissue chamber and circulation. The correlation between the level of total tau, NFL, UCHL1, GFAP, Aβ40 and Aβ42 in tissue chamber (a-b) and circulation (c-d) were assessed through Pearson correlation analysis. The correlation coefficient (R²) and p-value are displayed and significant correlations are graphed. *= p<0.05, **=p<0.01, ***=p<0.001, ****<p=0.0001
Figure 1

**a** BIOREACTOR SYSTEM

- gas exchange
- circulation loop
- medium bottle
- pump

**NVU MODEL**

- neuron
- astrocyte
- scaffold
- SMC
- EC

**NEURONS/ASTROCYTES**

- GFAP
- MAP2
- DAPI

**NEURONS**

- β-tub III

**SMC**

- CD31
- DAPI

**b** EC

**c** SMC

**d** ASTROCYTES

**e** NEURONS

**L**

**f** NEURONS/ASTROCYTES

- GFAP
- MAP2
- DAPI

**scaffold**

- 100 μm

**Figure 1**

- 50 μm
Figure 2

(a) MAP2-Syn-DAPI

(b) Glutamate (nM)

(c) Before KCl

(d) eGFP

(e) rhod-2 dendrite

(f) 20 Hz

(g) 200 pA, 5 ms

(h) 40 mV

(i) +CNQX WASH

(j) Normalized Charge (% Baseline)

(k) APOE SECRETION

| apoE (ng/ml) | Cham. veh | Cham. GW | Circul. veh | Circul. GW |
|--------------|-----------|----------|-------------|-----------|
| 0            |          |          |             |          |
| 1            |          |          |             |          |
| 2            |          |          |             |          |
| 4            |          |          |             |          |
| 8            |          |          |             |          |
| 16           |          |          |             |          |
| 32           |          |          |             |          |
| 64           |          |          |             |          | **

** p < 0.01
Figure 3

(a) Aβ (pg/mL) in Tissue chamber and Circulation

(b) Aβ40 and Aβ42 levels in Tissue chamber

(c) Aβ42 levels in Tissue chamber

(d) Aβ (pg/mL) in RIPPA and GuHCl

(e) Aβ40 levels in Tissue (RIPA)

(f) Aβ42 levels in Circulation

(g) Aβ40 levels in Tissue (RIPA)

(h) Aβ42 levels in Tissue (RIPA)

(i) Immunoblots for p-tau and total tau

R² = 0.0021
p = 0.8317

R² = 0.2208
p = 0.0183

ratio p-tau/total tau NVU
**Figure 4**

|       | Tau (ng/mL) | NFL (pg/mL) | UCHL1 (ng/mL) | GFAP (ng/mL) |
|-------|-------------|-------------|---------------|--------------|
| Mean (std) | 0.1367 (0.2847) | 0.01766 (0.009494) | 0.03768 (0.02526) | 0.01388 (0.01497) |
| Median | 0.01718 | 0.01543 | 0.03221 | 0.007492 |

**Statistical Analysis**

- \( R^2 = 0.03461 \) for Tau, \( p = 0.4458 \)
- \( R^2 = 0.3465 \) for NFL, \( p = 0.0080 \)
- \( R^2 = 0.6136 \) for UCHL1, \( p = 0.0006 \)
- \( R^2 = 0.004407 \) for UCHL1, \( p = 0.7871 \)

**Discussion**

- **Tau** indicates a low correlation with \( R^2 = 0.03461 \) and a p-value of 0.4458, suggesting no significant relationship.
- **NFL** shows a stronger correlation with \( R^2 = 0.3465 \) and a p-value of 0.0080, indicating a significant relationship.
- **UCHL1** exhibits a high correlation with \( R^2 = 0.6136 \) and a p-value of 0.0006, suggesting a strong relationship.
- **GFAP** also has a significant correlation with \( R^2 = 0.004407 \) and a p-value of 0.7871, indicating a weak relationship.

**Graphical Representation**

- **a** and **b** show the correlation between tissue chamber and circulation for Tau and NFL, respectively.
- **c** and **d** illustrate similar correlations for UCHL1 and GFAP.

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**Notes**

- The table and graphs provide a comprehensive analysis of the correlation between various proteins in tissue chamber and circulation. The findings suggest differing levels of correlation, with NFL and UCHL1 showing stronger relationships compared to Tau and GFAP.

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**References**

For further details, please refer to the original research paper.
Figure 5

(a) Tissue chamber

(b) Circulation

(c) Correlation

(d) Correlation

| p | R² | Tau | NFL | UCHL1 | GFAP | Aβ40 | Aβ42 |
|---|----|-----|-----|-------|------|------|------|
|   |    |     |     |       |      |      |      |
|   |    | 0.8820 | 0.9667 | 0.2571 | 0.0062 | 0.2022 |
|   |    | 0.0001 | 0.0001 | 0.0097 | 0.5112 | 0.0247 |
|   |    | 0.7404 | 0.8377 | 0.9364 | 0.0004 | 0.1564 |
|   |    | 0.0467 | 0.0771 | 0.0855 | 0.5085 | 0.0558 |
|   |    | 0.3308 | 0.2583 | 0.2371 | 0.2635 | 0.0799 |
|   |    | 0.2022 | 0.1634 | 0.3173 | 0.0036 | 0.1554 |
|   |    | 0.010  | 0.1127 | 0.3634 | 0.0051 | 0.0445 |
|   |    | 0.0263 | 0.1600 | 0.1270 | 0.0051 | 0.0445 |
|   |    | 0.0345 | 0.0397 | 0.1600 | 0.4486 | 0.0467 |
|   |    | 0.0246 | 0.0063 | 0.7721 | 0.0017 | 0.0805 |
|   |    | 0.2409 | 0.3387 | 0.3339 | 0.3222 | 0.1790 |