

Vascularized brain organoids enable reconstruction of the human blood-brain barrier and neurovascular disease modelling

Loic LE GUENNEC

`loic.le-guenneec@inserm.fr`

Leukemia and Niche Dynamics laboratory, Institut Cochin, Université Paris Cité UMR-S1016, INSERM U1016, CNRS UMR8104. <https://orcid.org/0000-0001-8146-6256>

Rana Znaidi

Leukemia and Niche Dynamics laboratory, Institut Cochin, Université Paris Cité UMR-S1016, INSERM U1016, CNRS UMR8104.

Kamal Bouhali

Université Paris Cité, Institut Jacques Monod, CNRS, Epigenetics and Cell Fate, F-75013 Paris, France

Housseem Zitoun

Leukemia and Niche Dynamics laboratory, Institut Cochin, Université Paris Cité UMR-S1016, INSERM U1016, CNRS UMR8104.

Aléria Duperay-Susini

Institut Cochin

Sandrine Adiba

Université Paris Cité, Institut Jacques Monod, CNRS, Epigenetics and Cell Fate, F-75013 Paris, France

Valentina Crippa

Leukemia and Niche Dynamics laboratory, Institut Cochin, Université Paris Cité UMR-S1016, INSERM U1016, CNRS UMR8104.

Mikaelle Bocel

Université Paris Cité, Institut Jacques Monod, CNRS, Epigenetics and Cell Fate, F-75013 Paris, France

Jason Ziveri

Vascular Cell Biology in Infection Inflammation and Cancer - Cochin Institute- INSERM U1016, Paris, France

Youssef Diallo

Vascular Cell Biology in Infection Inflammation and Cancer - Cochin Institute- INSERM U1016, Paris, France

Isabel Dos Santos Souza

Vascular Cell Biology in Infection Inflammation and Cancer - Cochin Institute- INSERM U1016, Paris, France

Bertrand Mathon

Sorbonne Université

Pierre-Olivier COURAUD

Tiziana Bachetti

University of Genova

Sandrine BOURDOULOUS

Institut Cochin <https://orcid.org/0000-0003-2852-4765>

Diana Passaro

Institut Cochin <https://orcid.org/0000-0002-2488-3755>

Resource

Keywords: Blood-brain barrier, Brain organoid, Bioengineering, vascularization, Endothelial cell, Neurovascular unit, Xenograft, Spatial transcriptomics, Neisseria meningitidis, Infection, Genetic disease, Alexander Disease, Permeability

Posted Date: October 23rd, 2026

DOI: <https://doi.org/10.21203/rs.3.rs-11073943/v1>

License:  This work is licensed under a Creative Commons Attribution 4.0 International License.

[Read Full License](#)

Additional Declarations: There is **NO** Competing Interest.

Abstract

The human blood-brain barrier (BBB) is a dynamic neurovascular interface essential for brain development and homeostasis, yet human-specific mechanisms governing its formation and dysfunction remain difficult to study. Here we establish a scalable platform of vascularized human brain organoids that recapitulates key features of the neurovascular unit and BBB organisation. Early integration of endothelial and mural cells generates stable vascular networks with junctional architecture and BBB-associated transporter expression while preserving cortical development. Spatial transcriptomic and functional analyses reveal coordinated neurovascular signalling, reduced hypoxic stress and improved tissue integrity. Following xenotransplantation into immunodeficient mice, human vessels connect to the host circulation, become perfused and undergo further maturation *in vivo*. This platform also reconstructs human-specific vascular colonization and thrombosis during *Neisseria meningitidis* infection and reveals neurovascular alterations associated with Alexander disease. Vascularized brain organoids therefore provide a versatile human model for investigating BBB biology, development and disease across physiological and pathological contexts.

INTRODUCTION

The human blood-brain barrier (BBB) is a highly specialised vascular interface that maintains central nervous system (CNS) homeostasis by regulating the exchange of molecules and cells between the circulation and the brain parenchyma¹. This function relies on the coordinated architecture of the neurovascular unit (NVU), in which endothelial cells (EC) connected by tight and adherens junctions interact with pericytes (PC) embedded in a shared basement membrane and with astrocytic endfeet that ensheath the vascular wall^{2,3}. Beyond acting as a physical and metabolic barrier, the BBB is increasingly recognised as an active regulator of brain development and neural function, modulating angiogenesis, neurogenesis and neurovascular signalling⁴. Disruption of these neurovascular interactions contributes to a wide range of neurological disorders, including neurodegenerative diseases, inflammatory syndromes, cerebrovascular injury, metabolic disorders and pathological consequences of CNS infection^{5–10}. Despite its central physiological and pathological roles, deciphering human-specific mechanisms governing BBB formation, integrity and dysfunction remains challenging due to the limited availability of physiologically relevant experimental models.

Conventional *in vitro* BBB systems, including endothelial monolayers, of non-human or human origin, in transwell inserts and microfluidic BBB-on-chip devices, have provided valuable insight into endothelial transport and permeability mechanisms^{11,12}. However, they lack integrated perivascular components or physiological morphology, resulting in incomplete barrier polarization and reduced transendothelial electrical resistance compared with native tissue¹³. Human brain organoids derived from induced pluripotent stem cells (iPSC) have emerged as powerful models of human neurodevelopment, recapitulating aspects of cortical organisation, neuronal diversification and regional patterning^{14–16}.

However, those organoids remain largely avascular and rely on passive diffusion for oxygen and nutrient supply, leading to hypoxic stress, central necrosis and delayed maturation ¹⁷.

Several approaches have sought to introduce vascular elements into brain organoids. Ectopic ETV2 overexpression can induce endothelial differentiation and generate vascular-like networks within brain organoids, but these endothelial populations often display immature or hybrid identities and lack perfusion ¹⁸. More recently, vascularized brain assembloids have been developed by integrating brain organoids with exogenous endothelial and vascular components, including iPSC-derived vascular organoids or endothelial and mural cell populations ^{19,20}.

Although these systems recapitulate several aspects of human microvascular architecture, they do not fully establish physiological neurovascular interactions and remain largely non-perfused ^{19,20}. These limitations may arise from the absence of coordinated spatiotemporal development between the vascular and neural compartments.

Here, we describe the generation of a robust and accessible platform for generating vascularized human brain organoids in which primary endothelial and mural cells are incorporated into iPSC-derived neural aggregates during early development. This simple and scalable strategy does not require complex genetic engineering or specialised differentiation protocols; in addition, it consistently drives the formation of stable vascular networks displaying key structural and functional features of the human BBB. We used this system to examine how vascularization influences neural development and tissue homeostasis, to assess the maturation of human vessels following xenotransplantation *in vivo*, and to model different neurovascular pathologies ranging from the immunological meningococcal infection to the neurodegenerative Alexander disease. Together, these findings establish vascularized brain organoids as a valuable human platform to investigate BBB biology and neurovascular disease mechanisms.

RESULTS

Development of a human vascularized brain organoid platform

We set out to build a robust, reproducible platform for vascularized brain organoids (vBO). Brain organoids were generated using a standard cerebral organoid protocol ¹⁵. To render these organoids vascularized, we modified the standard workflow: at d7 of differentiation, as human iPSC-derived embryoid bodies (EB) begin their neuroepithelial transition, they were embedded in Matrigel loaded with human vascular cells. The complete workflow, comprising iPSC maintenance, EB formation, neural induction, vascular embedding and organoid maturation, yielded organoids ready for collection between d40 and d60 (Fig. 1a).

To establish a reliable vascularization strategy combining physiological relevance, robustness and scalability, five different human EC sources were tested: (i) primary human umbilical vein EC (HUVEC), (ii) HUVEC transduced with a cassette encoding the E4-ORF1 gene from human adenovirus (E4ORF1 + EC)²¹, referred here as HUVEC^{E4}, (iii) iPSC-derived vascular cells (iEC), (iv) primary co-culture of human brain microvascular EC (BMEC) + pericytes (PC) isolated from cortical samples, and (v) immortalised human brain microvascular EC (hCMEC/D3, referred here to as D3)²².

We first profiled all five sources in 2D for canonical vascular markers (VE-Cadherin, vWF, Claudin-5, PDGFR- β ; Extended Data Fig. 1a). VE-Cadherin and vWF marked every source, confirming an endothelial identity. The discriminating marker was Claudin-5, a tight-junction protein critical for BBB integrity and strongly enriched in central nervous system endothelium^{4,23}, which was restricted to the two CNS-derived sources, primary BMEC+PC²⁴ and D3²². PDGFR- β , a pericyte/mesenchymal marker, was detected in iEC, suggesting a mixed endothelial–mesenchymal phenotype¹⁸, and labelled a subset of cells in the BMEC as expected (Extended Data Fig. 1b). For the iPSC-derived EC, we additionally confirmed loss of the pluripotency marker SOX2, supporting loss of the undifferentiated state (Extended Data Fig. 1c).

We then optimized the culture conditions for vascular development, using EB + HUVEC^{E4} condition. Three conditions were compared: BO maturation medium alone; BO maturation medium supplemented with human VEGF (10 ng ml⁻¹) from d7 to d21; and a 1:1 ratio of BO medium and endothelial medium throughout the maturation phase. The 1:1 ratio mix was deleterious, as premature organoid regression was observed from d40, and vascular structures likewise failed to persist beyond d40 (Extended Data Fig. 1d). Vascularization success rate differed across the three media conditions (14/14 with VEGF, 5/8 with BO medium alone and 0/3 with the 1:1 mixture; Fisher exact test, $P = 0.0005$; Extended Data Table 1). We therefore adopted BO maturation medium supplemented with VEGF for all subsequent experiments.

When comparing vBO generation with the aforementioned EC sources, we observed that all except D3 were able to generate 3D vascular structures within BO (Extended Data Fig. 1f). Vascularization success differed across endothelial sources (Fisher exact test, $P = 0.0006$; Extended Data Table 2), with HUVEC^{E4} reaching 14/14 success rate, compared with 4/7 for BMEC + PC, 2/4 for iEC, 2/4 for HUVEC and 0/3 for D3.

The vBO generated with HUVEC^{E4} showed visible endothelial networks as early as d20 and retained neuroepithelial progenitors marked with SOX2 + at d40 and d60 (Fig. 1b; Extended Data Fig. 1g for non-vascularized controls). This confirmed the ability of the system to support concomitant neural and vascular development. High-resolution confocal microscopy at d40 and d60 revealed branching tubular endothelial networks labelled with VE-Cadherin, including structures suggestive of ongoing lumen formation (Fig. 1c). Organoids beyond d60 (Extended Data Fig. 1h) showed continued growth of the neural compartment, as expected, but a noticeable reduction in vascular network density, suggesting a plateau in endothelial expansion.

To improve vascular stability and physiological relevance, we co-embedded EC with primary human PC isolated from cortical tissue. These PC expressed PDGFR- β but not VE-Cadherin, Claudin-5, or vWF (Extended Data Fig. 1a), reflecting their mural identity. The presence of PC significantly increased the vascular network surface compared to HUVEC^{E4} alone at d40, and was also higher than BMEC + PC (Fig. 1d- e). Moreover, in the BMEC + PC condition, vascular structures appeared to form a vascular-like compartment that remained largely segregated from the neural tissue, with limited penetration into the organoid (Fig. 1d). We therefore conclude that the inclusion of PC in vBO generated with HUVEC^{E4} (HUVEC^{E4}+PC vBO) promotes the formation of a bigger and better integrated vascular network. Immunofluorescence analysis of HUVEC^{E4}+PC vBO confirmed the presence of mural cells (PDGFR- β^+ , α -SMA⁺) surrounding or in close proximity to vessels (Fig. 1f). As expected, PC were also visualised in the BMEC + PC condition, but not in HUVEC^{E4}-only organoids (Fig. 1f).

In human embryonic development, astrocytic endfoot coverage of cerebral vasculature start to emerge before birth and continues to mature during the postnatal period^{2,25}. Thus, we analysed the astrocytic compartment and its association with the vessels. In non-vascularized BO, astrocyte-like cells (GFAP+) appeared in discrete clusters, variably distributed throughout the organoid (Extended Data Fig. 1i). In vBO, isolated astrocyte clusters were still detectable in discrete regions (Fig. 1g), alongside areas where astrocytic processes established physical interactions with blood vessels (Fig. 1h), consistent with the emergence of perivascular astrocyte-vessel interactions.

We next evaluated whether these vessels displayed features of the human BBB. Immunostaining at d40 showed strong expression of Claudin-5 at endothelial junctions in vBO generated with HUVEC^{E4}+PC (Fig. 1i), despite its absence in 2D cultures (Extended Data Fig. 1a). This supports the notion that brain microenvironmental cues promote BBB-like endothelial specification²⁴. We also assessed the expression of the tight junction proteins Occludin and Zonula occludens-1 (ZO-1) (Fig. 1i) and of BBB hallmark transporters: glucose transporter 1 (GLUT1) and the efflux transporters P-glycoprotein (P-gp) and breast cancer resistance protein (BCRP) (Fig. 1j). Occludin, ZO-1, P-gp and BCRP were detected within vessels by immunofluorescence, indicating that the vascular network developed not only structural but also transporter-associated molecular features of the human BBB. In contrast, GLUT1 was not detected within vessels *in vitro*, suggesting incomplete metabolic maturation of EC at this stage (Fig. 1j).

Multimodal spatial and functional analyses reveal preserved cortical development and activity in vascularized brain organoids

To assess whether vascularization influences the morphological and cellular development of BO, we first quantified organoid surface area across the five culture conditions from d20 to d60 and observed no significant differences in organoid size between conditions at any analysed time point (Extended Data Fig. 2a- b).

To achieve high-throughput definition of cellular identities and organisation, we performed spatial transcriptomics at d60 in non-vascularized BO, HUVEC^{E4}+PC and BMEC + PC vBO (Fig. 2a; Extended Data Fig. 2c- d). Multiple tissue sections were analysed from each organoid as spatial subsamples to expand the analysed surface area. UMAP embedding combined with UCell signature scoring and marker-gene analysis resolved eight broad transcriptional domains comprising two progenitor states, together with neuronal, astrocytic, endothelial, pericytic, fibroblastic and hypoxia-associated states (Fig. 2b- c). Notably, in agreement with previous reports, non-vascularized BO also contained a small subset of spots exhibiting an endothelial-like transcriptional signature^{26,27}. Top genes associated with each cluster confirmed their cellular identity (Fig. 2d; Extended Data Fig. 2e), and pseudotime inference revealed a continuous progression from progenitors to neurons and astrocytes, reflecting organized cortical-like maturation (Fig. 2e- f; Extended Data Fig. 3a). Low pseudotime values were predominantly associated with progenitor populations, whereas higher values corresponded to differentiated neuronal and astrocytic states, supporting a developmental ordering from progenitor-like to differentiated neural states within the analysed organoids. Neurodevelopmental progression was further validated at the protein level via expression of PAX6 (apical progenitors) and MAP2 (post-mitotic neurons). PAX6 expression decreased over time while MAP2 increased between d40 and d60, consistent with the transition from progenitor-rich to neuron-enriched tissue (Extended Data Fig. 3b-d).

Cell-cell communication analysis of the HUVEC^{E4}+PC spatial dataset revealed an asymmetric pattern of inferred signalling between vascular, perivascular and neural compartments (Fig. 2g; Extended Data Fig. 3e-f). Network reconstruction identified the vascular compartment as a prominent source of outgoing signalling towards progenitor, glial, neuronal and stromal domains. Pathway-level analysis highlighted canonical neurovascular signalling routes, including NOTCH (DLL4-NOTCH1), TGFβ (TGFB1-TGFB2) and WNT (WNT4-FZD6) (Fig. 2g), together with junction-, extracellular-matrix- and vascular-maturation-associated pathways, including JAM, SPP1, ANGPTL, MMP and THBS (Extended Data Fig. 3e-f). Spatial projection of representative ligand-receptor pairs showed complementary or adjacent expression patterns across vascular and neural territories, whereas chord diagrams summarized the directionality and relative strength of the inferred interactions (Fig. 2g). Together, these findings infer coordinated, pathway-specific neurovascular communication programs within the analysed HUVEC^{E4}+PC organoids.

Finally, we ensured vascularization did not alter spontaneous neuronal activity by measuring spiking networks with HD MEA. Although cortical-like electrophysiological activity is generally limited in human BO before d100²⁸, HD-MEA recordings at d80 detected spontaneous neuronal activity across all conditions. The proportion of active organoids, firing rates, and number of active electrodes were comparable between groups, confirming that early vascularization did not impair electrophysiological activity (Extended Data Fig. 3g-j).

Vascularization improves tissue integrity and mitigates hypoxic stress in brain organoids

To determine whether vascularization influenced BO tissue integrity and viability, we first examined the distribution of annotated spatial states across the three profiled conditions (Fig. 3a). The relative abundance of spots assigned to each state differed between the datasets, with a prominent central hypoxia-associated domain in the non-vascularized organoid. (Fig. 2a; Fig. 3a). This state was less represented in the HUVEC^{E4}+PC vBO, whereas the BMEC + PC organoid retained extensive hypoxia-associated domains, consistent with the limited vascular penetration observed by immunofluorescence (Fig. 1d).

To independently assess whether vascularization was associated with improved tissue integrity, we quantified nuclear pyknosis in an independent set of organoids. We observed an increased proportion of pyknotic nuclei over time in all conditions, but at a significantly lower rate in vBO at both time points (Fig. 3b- c), indicating that the presence of a vascular compartment helps preserve cell survival in 3D during long-term culture.

Differential gene expression and pathway enrichment analyses show that vBO up-regulated programs related to blood vessel development, angiogenesis, extracellular matrix organisation, and VEGF-associated signalling (Fig. 3d). This was supported at the gene level by increased expression of vascular and matrix-associated components, including *COL1A1*, *COL1A2*, and *CALD1*, as well as signalling mediators involved in cell-cell adhesion and guidance (*EFNB3*) and WNT/ β -catenin activity (*CTNNB1*; Extended Data Fig. 4a). These transcriptional changes are consistent with the emergence of vascular and perivascular compartments: *COL1A1*/*COL1A2* and *CALD1* are associated with perivascular fibroblast and mural-cell programs, whereas endothelial WNT/ β -catenin signalling is a major regulator of CNS angiogenesis and BBB specification ²⁹.

In contrast, non-vascularized BO displayed higher expression of glycolytic enzymes (*PFKL*, *GPI*, *PFKFB3*) and of the extracellular-matrix/proteoglycan-associated genes *CHPF* and *SDC3*, compatible with enrichment of glycolysis, HIF-1 signalling, and cellular stress-related pathways (Extended Data Fig. 4a). Thus, rather than representing a BBB-specific transcriptional signature per se, these changes indicate a shift from a hypoxia-associated metabolic state in non-vascularized BO toward vascular/perivascular and BBB-relevant developmental programs in vBO.

Together, these transcriptional differences indicate that vascularization induces coordinated metabolic, structural, and signalling shifts reflecting enhanced tissue maturation and neurovascular remodelling.

To validate these observations at the protein level we assessed the expression of the neuroprogenitor marker SOX2 and the neuronal differentiation marker HuC/D in comparative staining of BO and vBO (HUVEC^{E4}+PC) at d40 and d60 (Fig. 3e). In vBO, the progressive decrease in SOX2-positive progenitors and concurrent increase in HuC/D-positive neuronal layers indicated enhanced and sustained neuronal differentiation relative to non-vascularized controls (Fig. 3f).

To further characterize the spatial organisation of neural populations relative to the vascular network, we quantified the minimum distance between VE-Cadherin⁺ vessels and either neural progenitors (SOX2⁺) or

post-mitotic neurons (TUJ1⁺) (Extended Data Fig. 4b). In HUVEC^{E4} organoids, vessels were located in closer proximity to SOX2⁺ progenitor domains than to TUJ1⁺ neurons. The addition of PC shifted this spatial relationship, with vessels becoming more closely associated with TUJ1⁺ neuronal populations while exhibiting increased distances from SOX2⁺ progenitor regions. In contrast, BMEC + PC organoids displayed larger vessel-to-cell distances for both SOX2⁺ and TUJ1⁺ populations, consistent with the reduced vascular penetration observed in these organoids (Fig. 1d). These observations suggest that neurovascular spatial organisation may vary with both pericyte addition and endothelial cell source.

Together, these findings indicate that stable vascularization limits the emergence of central hypoxia and necrosis, providing a more physiologically stable microenvironment that supports sustained neural development.

In vivo transplantation allows perfusion and further maturation of vascularized brain organoids

Among the conditions tested, HUVEC^{E4}+PC combined robust vascular penetration with stable pericyte-supported vascularization and close association with neuronal populations and was therefore selected for subsequent functional and *in vivo* studies. To establish vascular perfusion, vBO collected between d40 and d60 were xenografted subcutaneously into immunodeficient NOD-SCID-IL2R^γnull (NSG) mice (Fig. 4a). Organoids were maintained *in vivo* for 3 weeks before explant and analysis (Fig. 4b).

Following xenografting, human VE-Cadherin-positive vessels established direct anastomosis with the host murine vascular network, as shown by the continuity between human and murine endothelial structures (Fig. 4c-d), consistently with inoculated HUVEC^{E4} in implanted Matrigel plugs²¹. Human vessels displayed well-defined lumens containing murine red blood cells (Fig. 4e-f), consistent with access to the host circulation. Functional perfusion was further demonstrated by intravital imaging following intravenous administration of UEA-I lectin, which labelled vascular structures within the graft (Fig. 4g). Together, these observations demonstrate functional integration of the human vascular network with the host circulation. These observations are in line with previous findings emphasizing the role of blood flow and mechanical pulsatility in promoting lumenogenesis³⁰.

We next examined the organisation of neuronal, glial, and vascular mural elements forming the human neurovascular unit *in vivo*. The human vascular network remained closely associated with PDGFR-β-positive perivascular mural cells after xenografting (Fig. 4h), indicating persistence of the mural-cell compartment *in vivo*.

GFAP staining identified persistent astrocytic clusters *in vivo* distributed across the vBO (Fig. 4i). Higher-magnification imaging revealed GFAP-positive astrocytic processes closely apposed to human vessels and MAP2-positive neurons (Fig. 4j), indicating persistence of organised neurovascular interactions after xenografting. SOX2 + neuroepithelial progenitors remained detectable in discrete peripheral zones, while TUJ1 + neurons were interspersed throughout the parenchyma (Fig. 4k). Thus, both progenitor and differentiated neuronal compartments persisted after three weeks *in vivo*.

We next examined whether the BBB-associated molecular features observed *in vitro* were maintained after xenografting. Human vessels retained expression of the tight-junction proteins Claudin-5, ZO-1 and Occludin (Fig. 4l) and of the BBB-associated transporters BCRP and P-gp (Fig. 4m). Notably, GLUT1, which was barely detectable in the vascular compartment *in vitro*, became readily detectable in human vessels after xenografting (Fig. 4m; Extended Data Fig. 5c). Comparison with adult human cortical vessels further showed higher normalized vascular colocalization values for several BBB-associated proteins *in vivo* than *in vitro* (Extended Data Fig. 5a-c). These measurements are consistent with further endothelial maturation following exposure to the host circulation and physiological blood flow^{30,31}.

Reconstructing human-specific BBB invasion by *Neisseria meningitidis*

Neisseria meningitidis is a human pathogen that specifically targets human EC via type IV pili and other adhesins, engaging species-specific receptors^{32,33} which enable BBB crossing and consequent meningitis. To investigate the temporal dynamics of meningococcal infection within a human neurovascular environment, NSG mice xenografted with human vBO were systemically infected with *N. meningitidis* and analysed at early post-infection time points (Fig. 5a). This approach allowed the *in vivo* assessment of bacterial localization, vascular responses, and tissue alterations within human endothelial structures embedded in a perfused brain microenvironment.

Immunofluorescence analyses revealed bacterial colonization of human-derived vessels, whereas bacterial signal was not detected on adjacent murine capillaries in the examined fields (Fig. 5b-c), consistent with the human tropism of *N. meningitidis*. Quantitative analysis demonstrated a significant increase in vascular bacterial burden between 1 h and 2 h post-infection (Fig. 5d), consistent with progressive vascular colonization. High-resolution imaging revealed a characteristic honeycomb-like reorganisation of the human endothelial membrane around bacterial adhesion sites (Fig. 5e), consistent with membrane remodelling previously described in endothelial infection models *in vitro*³².

In parallel, infected vessels displayed prominent intravascular CD41⁺ platelet aggregates colocalised with bacterial foci (Fig. 5f), and quantitative analysis showed a significant increase in CD41-positive intravascular platelet burden between 1 h and 2 h post-infection (Fig. 5g). Von Willebrand factor (vWF) staining further revealed focal vWF-rich intravascular accumulations associated with bacterial aggregates, including visibly occluded vessel segments (Fig. 5h), consistent with infection-associated endothelial activation and thrombotic remodelling³⁴.

At 2 h post-infection, bacteria were detected beyond the human collagen IV-positive vascular boundary, with orthogonal imaging confirming bacterial aggregates within the surrounding organoid parenchyma (Fig. 5i), consistent with local disruption of vascular barrier integrity in heavily colonized regions.

Together, these observations reconstruct successive features of meningococcal vascular pathogenesis within a perfused human neurovascular microenvironment, including human-selective vascular colonization, endothelial membrane remodelling, platelet accumulation and local disruption of vascular barrier integrity.

Modelling Alexander disease in vascularized human brain organoids

Alexander disease (AxD) is a rare astrocytopathy caused by dominant mutations in the astrocytic intermediate filament protein GFAP³⁵, and it is considered a primary astrocytic disorder³⁶. These mutations lead to astrocyte dysfunction and the accumulation of Rosenthal fibres (RF), corkscrew-shaped astrocytic inclusions composed primarily of GFAP, ultimately disrupting brain homeostasis and white matter integrity³⁷. Because astrocytes play a central role in the development and maintenance of the blood-brain barrier (BBB)³⁸, we hypothesised that *GFAP* mutations may disrupt neurovascular organisation and BBB function, a possibility that remains largely unexplored³⁹.

To address this question, we generated vBO with two independent non-isogenic patient-derived iPSC lines carrying two distinct pathogenic *GFAP* mutations associated with AxD, M73K and R79C⁴⁰, alongside a healthy donor-derived iPSC line (IMR90)⁴¹ serving as experimental control (Fig. 6a-b).

We first assessed whether AxD-vBO recapitulated a characteristic astrocytic pathological feature of the disease. Prominent Rosenthal fibre-like GFAP aggregates were detected in both M73K and R79C vBO but were not observed in IMR90 controls (Fig. 6c-d), consistent with the GFAP aggregation phenotype previously described in these patient-derived lines⁴⁰.

We next assessed overall organoid development across the three lines. Bright-field imaging revealed progressive organoid growth across conditions throughout differentiation (Extended Data Fig. 6a). Vascularized BO carrying the R79C mutation displayed a significant increase in size compared with control BO under both non-vascularized and vascularized conditions. In contrast, no significant increase in organoid size was observed for the M73K line. Notably, vascularization did not significantly affect BO growth within any genotype (Extended Data Fig. 6b), in agreement with our previous observations (Extended Data Fig. 2a). By d40, R79C BO had already reached a size comparable to that of control BO at d60. All subsequent *in vitro* analyses were therefore performed at d40 to minimize differences in organoid size between the three lines and limit potential size-related confounding.

We next investigated whether the AxD patient-derived lines displayed alterations in vascularization or astroglial organization. Immunofluorescence analysis showed comparable VE-Cadherin-positive vascular coverage and overall GFAP-positive astroglial area across IMR90 control, M73K and R79C vBO (Fig. 6b,e-f), indicating no gross difference in vascular coverage or GFAP-positive tissue area between the three lines. We therefore asked whether *GFAP* mutations instead affected the spatial organisation of astrocytes relative to the vascular compartment. Quantification of nearest-neighbour distances showed greater astrocyte dispersion in the R79C line than in IMR90 and M73K vBO (Fig. 6g). In addition, the shortest distance from individual GFAP-positive astrocytes to the nearest VE-Cadherin-positive vessel was increased in both AxD patient-derived lines compared with IMR90 controls (Fig. 6h), consistent with reduced astrocyte-vessel spatial association. These findings indicate altered astrocyte-vessel spatial organization in the AxD patient-derived lines despite preserved overall vascular coverage.

We next investigated whether these structural neurovascular alterations could translate into functional BBB defects. Day 40 AxD-vBO were xenografted into NSG mice and allowed to establish functional vascular anastomoses with the host circulation (Extended Data Fig. 6c). BBB permeability was then assessed by injecting intravenously EZ-Link Sulfo-NHS-LC-Biotin 20 minutes before vBO collection and staining (Fig. 6i). Quantification of tracer extravasation demonstrated significantly increased vascular leakage in R79C vBO compared with IMR90 controls, whereas the M73K mutation produced only a modest, non-significant increase (Fig. 6j). These results indicate that disruption of astrocyte-vascular organisation induced by the R79C mutation is associated with impaired BBB integrity *in vivo*.

Together, these findings establish vBO as a valuable human platform to investigate how disease-causing GFAP mutations alter neurovascular organisation and suggest that astrocytic pathology is associated with impaired blood–brain barrier function.

DISCUSSION

Here we establish a vascularized human brain organoid model that reconstructs key features of the NVU. Early integration of endothelial and mural cells enables the formation of stable vascular networks that acquire BBB-associated properties and further mature with perfusion. A key step in establishing this system was the systematic evaluation of multiple endothelial sources. Although all displayed canonical vascular markers in two-dimensional culture, their behaviour differed markedly within developing organoids: iPSC-derived and primary brain microvascular endothelial cells produced only partial vascular structures, immortalised lines failed to generate stable 3D networks, and HUVEC^{E4} reproducibly generated interconnected networks with high efficiency. Endothelial identity in 2D therefore does not predict the ability of these cells to self-organize within a three-dimensional neural environment; robust vascularization instead appears to depend on angiogenic competence, adaptability during tissue integration and potentially the vascular-bed origin of the endothelial cells.

Within vascularized organoids, endothelial cells acquired BBB-associated features, including Claudin-5, Occludin and major efflux transporters, some of which were not detectable in the same endothelial populations in 2D culture. This supports the ability of neural and perivascular cues to promote BBB-like endothelial specialization^{25,42,43} and is consistent with the well-recognised plasticity of endothelial cells^{44,45}. Our findings extend this concept to organoid systems, indicating that the neural tissue context can initiate aspects of BBB differentiation even in endothelial cells that do not originate from the brain.

However, the extent to which HUVEC-derived endothelium fully recapitulates bona fide brain endothelial identity remains uncertain. HUVEC^{E4} cells may be particularly permissive to microenvironmental instruction owing to their sustained angiogenic capacity²¹, whereas additional cues, including controlled hemodynamic forces, additional NVU elements or longer-term maturation, may be required for more complete BBB differentiation.

Spatial transcriptomics identified vascular compartments as prominent signalling hubs interacting with neural progenitors, astrocytes and neurons through pathways including WNT, NOTCH and TGF β . These networks are consistent with developmental mechanisms regulating neurovascular coupling and BBB formation^{23,46,47} and with recent human brain vascular atlases⁴⁸. Their spatial organisation further supports the emergence of structured neurovascular niches within vBO.

Recent clonal lineage tracing showed that cortical organoids can retain a unitary transcriptional trajectory while losing the stereotyped temporal lineage progression observed *in vivo*⁴⁹. The vascular compartment in vBO provides non-cell-autonomous signals to progenitors, astrocytes and neurons, but whether these cues restore faithful temporal lineage progression remains to be tested.

Vascularization was also associated with reduced hypoxia-related gene expression and tissue degeneration, particularly in HUVEC^{E4}+PC vBO, whereas BMEC+PC vBO with poorly integrated vessels retained hypoxia-associated domains. This is consistent with diffusion-limited oxygen supply as a major constraint in conventional organoids¹⁷ and suggests that integrated vascular networks improve metabolic stability and support neural maturation.

A major limitation of current organoid systems is the absence of physiological vascular flow. Following xenotransplantation, human vBO vessels anastomosed with the host circulation, became perfused and showed increased vascular localization of several BBB-associated markers, most notably GLUT1, which was barely detectable *in vitro*. As flow and shear stress promote endothelial specialization³⁰, these findings support a contribution of hemodynamic forces to further vBO maturation and call for future integration with controlled microfluidic perfusion.

Perfused vBO also enabled modelling of systemic neurovascular disease. Whereas NVU-on-chip systems capture bacterial adhesion and endothelial remodelling under controlled flow⁵⁰, they lack a living host circulation and the multicellular neurovascular tissue context provided by xenografted vBO. Following *N. meningitidis* infection, bacteria selectively colonized human endothelial vessels and were associated with platelet accumulation, thrombosis and barrier disruption, reproducing key features of meningococcal vascular pathology⁵¹. This human-selective phenotype highlights the value of vBO for studying species-specific host-pathogen interactions that are difficult to reproduce in conventional animal models.

Genetic neurological disorders provide a complementary application of this platform. Alexander disease is particularly relevant because astrocytic GFAP pathology may perturb neurovascular homeostasis. Mouse models show that mutant GFAP causes reactive gliosis and early abnormalities of perivascular astrocytic processes⁵², while patient-derived astrocytes carrying the M73K and R79C variants used here accumulate GFAP aggregates and dense filament bundles resembling early Rosenthal fibres⁴⁰.

In vBO, AxD-associated astrocytic pathology was accompanied by altered astrocyte-vessel spatial organisation in both patient-derived lines and by impaired barrier integrity *in vivo* in the R79C line,

extending these observations to a multicellular human neurovascular system. These findings support vascularized brain organoids as a platform for investigating neurovascular alterations associated with Alexander disease and other astrocytopathies.

Beyond the diseases examined here, vascularized brain organoids could provide a human platform to investigate other pathologies involving the neurovascular unit, including primary brain tumours and brain metastases. Incorporating tumour or metastatic niches into vBO may enable studies of vascular remodelling, invasion and therapeutic response within a controlled human neurovascular environment.

Declarations

Artificial intelligence use statement

OpenAI ChatGPT (GPT-5.6 Sol; OpenAI, accessed September 2026) was used to assist with the graphical refinement of selected schematic workflow illustrations originally designed by the authors in Microsoft PowerPoint (version 2608, build 20326.20132; Microsoft). AI assistance was limited to graphical presentation and did not generate, modify or interpret experimental data, microscopy images or quantitative results.

Ethics statement

Animal experiments

Animal experiments were approved by the local ethics committee and performed in accredited SPF facilities (ISO 9001), in compliance with European and French regulations on animal care and experimentation (APAFIS#27215-2020041513522374 v6)

Primary human umbilical vein endothelial cells

Primary human umbilical vein endothelial cells (HUVEC) were isolated at AP-HP, Hôpital Saint-Louis, Paris, France. Their transfer and use for research purposes were covered by authorization AC-2024-6640, issued by the French Ministry of Higher Education and Research.

Human cortical tissue

Human cortical tissue was obtained from cortical resections performed during epilepsy surgery at the Department of Neurosurgery, Pitié-Salpêtrière Hospital (AP-HP, Paris, France), under a research collaboration agreement between AP-HP and INSERM (VAL 2021-498/01) and covered by the INSERM CODECOH DC-2021-4604. Tissue samples, which would otherwise have been discarded after routine clinical care, were used for the isolation of primary brain microvascular endothelial cells (BMEC), pericytes, and immunohistochemical analyses. Written informed consent was obtained from all patients in accordance with French legislation and institutional requirements

WTC11 cell line

The WTC11 iPSC line (GM25256) was obtained from the Coriell Institute for Medical Research under a Material Transfer Agreement with Université de Paris, covered by the INSERM CODECOH DC-2021-4604 and used exclusively for research purposes in accordance with the approved Statement of Research Intent.

Alexander disease and IMR90 cell lines

Human iPSC lines used in this study included the healthy control line IMR90 and two Alexander disease patient-derived lines carrying GFAP mutations (AxD997, R79C; and AxD999, M73K). These cell lines were obtained from the laboratory of Dr. Yanhong Shi (Beckman Research Institute, City of Hope, Duarte, CA, USA) under a Material Transfer Agreement between the Beckman Research Institute and INSERM. Their use complied with French regulations governing research involving human biological material and was covered by the INSERM CODECOH declaration DC-2023-6121.

Data availability

The spatial transcriptomic data generated in this study, including raw sequencing data and processed spot-level gene-expression matrices, have been deposited in the NCBI Gene Expression Omnibus (GEO). Previously published single-cell and single-nucleus transcriptomic datasets used to guide cell-type annotation and marker-gene selection are described and cited in the Methods and were not computationally integrated with the datasets generated in this study. Source data underlying the quantitative analyses presented in the main and Extended Data figures will be provided with the paper. All other data supporting the findings of this study are available within the Article and its Supplementary Information.

Code availability

Custom image-analysis pipelines used for nuclear segmentation, fluorescence quantification and neurovascular spatial analysis are publicly available through Zenodo and are cited in the Methods. Additional custom scripts used for the analyses reported in this study will be deposited in a public repository and archived with a persistent DOI before publication. Standard bioinformatic analyses were performed using publicly available software and packages as described in the Methods.

Funding

The enSCORE platform was supported by the Labex Who Am I? (ANR-11-LBX-0071; Idex ANR-11-IDEX-0005-02). The ImagoSeine and IMAG'IC core facilities are members of the France BioImaging infrastructure, supported by the French National Research Agency (ANR-24-INBS-0005 FBI BIOGEN) and GIS-IBiSA. The work in "Leukemia and niche dynamics" is supported by CNRS, INSERM and Université Paris Cité, and this project was supported by grants from Laurette Fugain (R23197KK), Canceropôle IDF (RPH23177KKA), Fondation de France (RAF23152KKA) and "Proof of Concept" grant from IC (2023).

Acknowledgements

We thank the MOUSE'TIC, IMAG'IC, GENOM'IC and HISTIM core facilities of Institut Cochin for their technical support. We thank the ePHYSE platform, particularly Carine Dalle and Charlotte Deleuze, for assistance with electrophysiological recordings. We thank the enSCORE platform for access to instruments and technical advice. We acknowledge the ImagoSeine core facility of the Institut Jacques Monod, a member of the France BioImaging infrastructure (<https://ror.org/01y7vt929>), for imaging support. We thank AP-HP, Hôpital Saint-Louis, Paris, France, for the isolation and provision of primary HUVECs, transferred for research use under authorization AC-2024-6640 issued by the French Ministry of Higher Education and Research. We thank the Normal and Pathological Hematopoiesis team of Institut Cochin for the productive scientific discussions and insightful advice. We thank Vanessa Ribes from Institut Jacques Monod for productive scientific discussion and reading the manuscript. We also thank the Department of Neurosurgery of Pitié-Salpêtrière Hospital, AP-HP, particularly Pr Bertrand Mathon, and the patients who contributed human cortical tissue used in this study.

We thank Dr. Yanhong Shi for providing the IMR90 and Alexander disease iPSC lines, and Drs Rafii and Itkin for providing HUVEC^{E4} cells.

References

1. Wu, D. *et al.* The blood–brain barrier: Structure, regulation and drug delivery. *Sig Transduct Target Ther* **8**, 217 (2023).
2. Abbott, N. J., Rönnebeck, L. & Hansson, E. Astrocyte-endothelial interactions at the blood-brain barrier. *Nat Rev Neurosci* **7**, 41–53 (2006).
3. Armulik, A. *et al.* Pericytes regulate the blood-brain barrier. *Nature* **468**, 557–561 (2010).
4. Obermeier, B., Daneman, R. & Ransohoff, R. M. Development, maintenance and disruption of the blood-brain barrier. *Nat Med* **19**, 1584–1596 (2013).
5. Le Guennec, L., Marois, C., Demeret, S., Wijdicks, E. F. M. & Weiss, N. Toxic-metabolic encephalopathy in adults: Critical discussion and pragmatical diagnostic approach. *Rev Neurol (Paris)* **178**, 93–104 (2022).
6. Le Guennec, L., Balcerac, A. & Weiss, N. A Letter Concerning a Role for Blood-Brain Barrier Dysfunction in Delirium following Noncardiac Surgery in Older Adults. *Ann Neurol* **95**, 410–411 (2024).
7. Le Guennec, L., Mouri, S., Thabut, D. & Weiss, N. Blood-brain barrier dysfunction in hepatic encephalopathy: pathophysiology, diagnostic assessment and therapeutic perspectives. *Metab Brain Dis* **40**, 223 (2025).
8. Le Guennec, L. & Weiss, N. Blood-brain barrier dysfunction in intensive care unit. *J Intensive Med* **3**, 303–312 (2023).
9. Sweeney, M. D., Sagare, A. P. & Zlokovic, B. V. Blood-brain barrier breakdown in Alzheimer disease and other neurodegenerative disorders. *Nat Rev Neurol* **14**, 133–150 (2018).

10. Lopes Pinheiro, M. A. *et al.* Immune cell trafficking across the barriers of the central nervous system in multiple sclerosis and stroke. *Biochim Biophys Acta* **1862**, 461–471 (2016).
11. Ribecco-Lutkiewicz, M. *et al.* A novel human induced pluripotent stem cell blood-brain barrier model: Applicability to study antibody-triggered receptor-mediated transcytosis. *Sci Rep* **8**, 1873 (2018).
12. Ahn, S. I. *et al.* Microengineered human blood–brain barrier platform for understanding nanoparticle transport mechanisms. *Nat Commun* **11**, 175 (2020).
13. Hajal, C. *et al.* Engineered human blood–brain barrier microfluidic model for vascular permeability analyses. *Nat Protoc* **17**, 95–128 (2022).
14. Quadrato, G. *et al.* Cell diversity and network dynamics in photosensitive human brain organoids. *Nature* **545**, 48–53 (2017).
15. Lancaster, M. A. *et al.* Cerebral organoids model human brain development and microcephaly. *Nature* **501**, 373–379 (2013).
16. Qian, X. *et al.* Brain-Region-Specific Organoids Using Mini-bioreactors for Modeling ZIKV Exposure. *Cell* **165**, 1238–1254 (2016).
17. Lancaster, M. A. & Knoblich, J. A. Generation of Cerebral Organoids from Human Pluripotent Stem Cells. *Nat Protoc* **9**, 2329–2340 (2014).
18. Cakir, B. *et al.* Engineering of human brain organoids with a functional vascular-like system. *Nat Methods* **16**, 1169–1175 (2019).
19. Dao, L. *et al.* Modeling blood-brain barrier formation and cerebral cavernous malformations in human PSC-derived organoids. *Cell Stem Cell* **31**, 818-833.e11 (2024).
20. Sun, X. *et al.* Vascularized Brain Assembloids With Enhanced Cellular Complexity Provide Insights Into the Cellular Deficits of Tauopathy. *Stem Cells* **42**, 107–115 (2024).
21. Seandel, M. *et al.* Generation of a functional and durable vascular niche by the adenoviral E4ORF1 gene. *Proc Natl Acad Sci U S A* **105**, 19288–19293 (2008).
22. Weksler, B. B. *et al.* Blood-brain barrier-specific properties of a human adult brain endothelial cell line. *FASEB J* **19**, 1872–1874 (2005).
23. Langen, U. H., Ayloo, S. & Gu, C. Development and Cell Biology of the Blood-Brain Barrier. *Annu Rev Cell Dev Biol* **35**, 591–613 (2019).
24. Stewart, P. A. & Wiley, M. J. Developing nervous tissue induces formation of blood-brain barrier characteristics in invading endothelial cells: a study using quail–chick transplantation chimeras. *Dev Biol* **84**, 183–192 (1981).
25. Daneman, R., Zhou, L., Kebede, A. A. & Barres, B. A. Pericytes are required for blood-brain barrier integrity during embryogenesis. *Nature* **468**, 562–566 (2010).
26. Stankovic, I. *et al.* Schizophrenia endothelial cells exhibit higher permeability and altered angiogenesis patterns in patient-derived organoids. *Transl Psychiatry* **14**, 53 (2024).
27. Fumadó Navarro, J. *et al.* Cerebral Organoids with Integrated Endothelial Networks Emulate the Neurovascular Unit and Mitigate Core Necrosis. *Adv Sci (Weinh)* **12**, e07256 (2025).

28. Jgamadze, D. *et al.* Structural and functional integration of human forebrain organoids with the injured adult rat visual system. *Cell Stem Cell* **30**, 137-152.e7 (2023).
29. Winkler, E. A. *et al.* Single cell atlas of the normal and malformed human brain vasculature. *Science* **375**, eabi7377 (2022).
30. Gebala, V., Collins, R., Geudens, I., Phng, L.-K. & Gerhardt, H. Blood flow drives lumen formation by inverse membrane blebbing during angiogenesis in vivo. *Nat Cell Biol* **18**, 443–450 (2016).
31. Dessalles, C. A., Leclech, C., Castagnino, A. & Barakat, A. I. Integration of substrate- and flow-derived stresses in endothelial cell mechanobiology. *Commun Biol* **4**, 764 (2021).
32. Bernard, S. C. *et al.* Pathogenic *Neisseria meningitidis* utilizes CD147 for vascular colonization. *Nat Med* **20**, 725–731 (2014).
33. Le Guennec, L. *et al.* Receptor recognition by meningococcal type IV pili relies on a specific complex N-glycan. *Proceedings of the National Academy of Sciences* **117**, 2606–2612 (2020).
34. Mairey, E. *et al.* Cerebral microcirculation shear stress levels determine *Neisseria meningitidis* attachment sites along the blood-brain barrier. *J Exp Med* **203**, 1939–1950 (2006).
35. Grossi, A. *et al.* A systematic review and meta-analysis of GFAP gene variants in Alexander disease. *Sci Rep* **14**, 24341 (2024).
36. Quinlan, R. A., Brenner, M., Goldman, J. E. & Messing, A. GFAP and its role in Alexander disease. *Exp Cell Res* **313**, 2077–2087 (2007).
37. Sosunov, A. A., McKhann, G. M. & Goldman, J. E. The origin of Rosenthal fibers and their contributions to astrocyte pathology in Alexander disease. *Acta Neuropathol Commun* **5**, 27 (2017).
38. Janzer, R. C. & Raff, M. C. Astrocytes induce blood-brain barrier properties in endothelial cells. *Nature* **325**, 253–257 (1987).
39. Zarekiani, P. *et al.* Pathology of the neurovascular unit in leukodystrophies. *Acta Neuropathologica Communications* **9**, 103 (2021).
40. Li, L. *et al.* GFAP Mutations in Astrocytes Impair Oligodendrocyte Progenitor Proliferation and Myelination in an hiPSC Model of Alexander Disease. *Cell Stem Cell* **23**, 239-251.e6 (2018).
41. Nichols, W. W. *et al.* Characterization of a new human diploid cell strain, IMR-90. *Science* **196**, 60–63 (1977).
42. Goldmann, E. E. *Vitalfärbung am Zentralnervensystem: Beitrag zur Physio-Pathologie des Plexus chorioideus und der Hirnhäute.* (Königl. Akademie der Wissenschaften, Berlin, 1913).
43. Ehrlich, P. *Das Sauerstoff-Bedürfniss des Organismus: Eine farbenanalytische Studie.* (August Hirschwald, Berlin, 1885).
44. Dejana, E., Hirschi, K. K. & Simons, M. The molecular basis of endothelial cell plasticity. *Nat Commun* **8**, 14361 (2017).
45. Pasut, A., Becker, L. M., Cuypers, A. & Carmeliet, P. Endothelial cell plasticity at the single-cell level. *Angiogenesis* **24**, 311–326 (2021).

46. Zhou, Y. *et al.* Canonical WNT signaling components in vascular development and barrier formation. *J Clin Invest* **124**, 3825–3846 (2014).
47. Rattner, A., Wang, Y. & Nathans, J. Signaling Pathways in Neurovascular Development. *Annu Rev Neurosci* **45**, 87–108 (2022).
48. Wälchli, T. *et al.* Single-cell atlas of the human brain vasculature across development, adulthood and disease. *Nature* **632**, 603–613 (2024).
49. Stouffer, M. *et al.* Temporal uncoupling of radial glia lineage progression in cortical organoids. *Nature* <https://doi.org/10.1038/s41586-026-10916-7> (2026) doi:10.1038/s41586-026-10916-7.
50. Pinon, L. *et al.* An in vitro human vessel model to study *Neisseria meningitidis* colonization and vascular damages. *eLife* **14**, (2025).
51. Join-Lambert, O. *et al.* Meningococcal interaction to microvasculature triggers the tissular lesions of purpura fulminans. *J Infect Dis* **208**, 1590–1597 (2013).
52. Hagemann, T. L. *et al.* STAT3 Drives GFAP Accumulation and Astrocyte Pathology in a Mouse Model of Alexander Disease. *Cells* **12**, 978 (2023).

ONLINE METHODS

Key reagents, medium, and materials used for the study are shown in Supplementary Table 1, 2 and 3.

Cell lines and culture conditions

WTC11 iPSC: Human induced pluripotent stem cells (WTC11, Coriell Institute) were maintained on Matrigel®-coated plates (Corning) in mTeSR+™ medium (STEMCELL Technologies) at 37 °C and 5% CO₂. Cells were passaged as clumps using Gentle Cell Dissociation Reagent at 60-80% confluency. Y-27632 (10 μM) was added after thawing.

iEC: iPSC-derived vascular cells were generated from WTC11 iPSCs using the STEMdiff™ Endothelial Differentiation Kit (STEMCELL Technologies, catalog no. 08005) according to the manufacturer's instructions. iEC were cultured in complete STEMdiff™ Endothelial Expansion Medium.

HUVEC^{E4}: Primary HUVEC transduced with ^{E4}ORF1 (Angiocrine/Veravec) were cultured in EBM-2 (Lonza).

Primary HUVEC: Primary HUVEC were isolated at AP-HP, Hôpital Saint-Louis, Paris, France, and transferred for research use under authorization AC-2024-6640 issued by the French Ministry of Higher Education and Research. HUVEC were cultured in EBM-2 (Lonza).

hCMEC/D3: Human cerebral microvascular endothelial cells (hCMEC/D3 or D3) were used as an immortalised human brain endothelial cell line. D3 were cultured in EBM-2 (Lonza).

Primary BMEC: Primary human brain microvascular endothelial cells were isolated from cortical tissue obtained from Pitié-Salpêtrière Hospital, Paris, as described below. BMEC were cultured in EBM-2 (Lonza).

Pericytes: Primary human brain pericytes (PC) were isolated from cortical tissue obtained from Pitié-Salpêtrière Hospital, Paris, as described below. PC were cultured in EBM-2 (Lonza).

For Alexander disease modelling:

IMR90 iPSC: Healthy control iPSC line used for Alexander disease modelling.

AxD997 iPSC: Patient-derived iPSC line carrying the pathogenic GFAP R79C mutation.

AxD999 iPSC: Patient-derived iPSC line carrying the pathogenic GFAP M73K mutation.

All three iPSC lines were provided by Dr. Yanhong Shi (Beckman Research Institute, City of Hope, Duarte, CA, USA), maintained under identical culture conditions, and differentiated into brain organoids as described above.

All cells were tested negative for mycoplasma contamination and maintained in humidified incubators at 37 °C, 5% CO₂. Media were supplemented with 1% penicillin/streptomycin.

Isolation of human BMEC and PC

Fresh human cortical tissue was transferred into ice-cold Hank's Balanced Salt Solution (HBSS; Thermo Fisher). The tissue was minced with a scalpel and digested in a mixture containing Dispase (12 mL), DNase I (120 µL), Collagenase II (120 µL), Heparin (120 µL), and FBS (240 µL) in 12.5 mL HBSS at 37 °C. The suspension was triturated every 5 min for 30 min, filtered through a 100 µm cell strainer, and treated with RBC lysis buffer (Sigma) for 5 min. After neutralization with pure FBS and centrifugation (5 min, 1200 rpm), the cell pellet was resuspended in EBM-2 and plated in T-25 flasks. After 10-14 days, the confluent primary mixed BMEC + PC culture was detached with trypsin (Sigma). A fraction of this mixed vascular culture was used directly for EB embedding (BMEC + PC condition), whereas the remaining cells were further expanded to obtain a pericyte-enriched population, typically from the second passage onwards. These expanded PC were subsequently used for co-embedding with HUVEC^{E4} cells. PC identity was confirmed by immunostaining for PDGFR-β, with absence of VE-Cadherin, Claudin-5, and vWF.

Embryoid body formation

iPSC were dissociated into single cells with Gentle Cell Dissociation Reagent (GCDR) and resuspended at 9×10^5 cells ml⁻¹ in EB Seeding Medium (STEMCELL Technologies). 9000 cells per well (in a 100µl volume) were seeded into each well of a U-bottom ultra-low-attachment 96-well plate (Corning). After 5 days in EB Formation Medium, embryoid bodies (EB) with diameters of 100-200 µm were transferred into 48-well ultra-low-attachment plates and cultured in Induction Medium for 2 additional days.

Generation of vascularized brain organoids

At d7 (neuroepithelial transition), each EB was embedded in 15 μ L of liquid Matrigel® mixed with 1×10^5 HUVEC^{E4} and 5×10^3 PC, resuspended in 5 μ L EBM-2. Embedding was performed on STEMCELL Embedding Sheets and polymerised for 30 min at 37 °C. Embedded EB (12 per well) were transferred to 6-well ultra-low-attachment plates and cultured for 3 days in Expansion Medium. For HUVEC, HUVEC^{E4}, iEC and D3 conditions, 1×10^5 endothelial cells were embedded per EB, either alone or together with 5×10^3 expanded primary cortical PC, as indicated. For the BMEC + PC condition, 1×10^5 cells from the primary mixed BMEC + PC culture were embedded per EB without addition of separately expanded PC. At d10, organoids were switched to Brain Organoid Maturation Medium (STEMCELL Technologies) and placed on an orbital shaker at 37 °C. Medium was changed every 3-4 days. Unless otherwise indicated for the medium-optimization experiments, all subsequent BO and vBO cultures, including non-vascularized controls, were supplemented with human recombinant VEGF-165 (10 ng ml⁻¹; Thermo Fisher Scientific) from d7 to d21. We therefore adopted BO maturation medium supplemented with VEGF for all subsequent experiments. The same VEGF supplementation was applied to non-vascularized BO used as controls in all subsequent comparative experiments.

Vascularization success was assessed at d40 as a binary outcome: an organoid batch was scored as vascularized when three-dimensional VE-Cadherin-positive or CD31-positive endothelial structures were detected within the neural tissue by confocal microscopy, irrespective of the extent of the vascular network. The vascularization success rate is the proportion of independent differentiation batches meeting this criterion (Extended Data Tables 1 and 2).

Subcutaneous xenografting in NSG mice

Male or female NOD-scid IL2R γ null (NSG) mice aged 8-12 weeks were used under SPF conditions (MOUSE'TIC facility, Institut Cochin, ISO 9001 certified). Anaesthesia was induced with 4% isoflurane and maintained at 1.5-2%. Buprenorphine (0.05 mg Kg⁻¹, s.c.) was used for analgesia. The dorsal skin was shaved, disinfected, and a 1 cm incision was made. Subcutaneous pockets were created using blunt forceps, and vBO collected between d40-d60 were implanted. Incisions were closed with surgical clips removed at d7-10. Mice were monitored daily and maintained for 3 weeks before vBO explantation.

Humanised meningococcal infection model

Bacterial strains and preparation. For the infection, meningococcal strains were grown overnight at 37 °C on GCB agar plates prepared without iron and supplemented with 15 μ M deferoxamine (Desferal, Novartis). Bacterial colonies were harvested and cultured in RPMI media with 1% BSA medium and 0.06 μ M deferoxamine with gentle agitation to reach the exponential phase of growth. Bacteria were then resuspended in physiological saline solution as described previously^{51,53,54}.

Infection procedure. Three weeks after vBO xenografting, NSG-vBO mice received an intravenous challenge of *Neisseria meningitidis* strain 2C4.3 via the tail vein. The inoculum was adjusted

spectrophotometrically at OD₆₀₀ to yield approximately 5×10^6 CFU per injection, ensuring bacteraemia levels comparable to validated humanised infection models⁵¹. Immediately prior to infection, mice received intraperitoneal administration of 10 mg of human holotransferrin to promote meningococcal iron acquisition and systemic dissemination. Mice were euthanised 1 h or 2 h post-infection, depending on experimental endpoints. vBO grafts were then collected, fixed in 4% paraformaldehyde overnight at 4 °C, and processed for immunofluorescence or histological analyses.

Immunofluorescence staining

Vibratome:

Organoids were rinsed twice in PBS and fixed in 4% paraformaldehyde (PFA) at 4 °C for 24 h. Samples were washed, embedded in 8% agarose, and sectioned at 100 µm using a vibratome (Leica).

Sections were permeabilised and blocked in TBS-azide containing 10% FBS, 5% donkey serum, and 0.5% Tween-20 for 24 h at 4 °C. Primary antibodies were incubated 24 h at 4 °C, followed by fluorescent secondary antibodies for 24 h at 4 °C. Nuclei were counterstained with 4',6-diamidino-2-phénylindole (DAPI).

Microtome: Organoids were rinsed twice in PBS and fixed in 4% paraformaldehyde (PFA) for 3 hours, cryoprotected in 30% sucrose, embedded in OCT, and cryosectioned at 14 µm using a microtome. Sections were mounted on slides and stored at -80 °C. For immunostaining, slides were thawed, washed in PBS, permeabilised in 0.1% Triton X-100 (PBST) for 5 min, and blocked in PBST containing 2% bovine serum albumin (BSA) for 30–60 min. Sections were incubated overnight at 4 °C with primary antibodies diluted in PBST with 2% BSA. After washing in PBST, sections were incubated for 1 h at room temperature with secondary antibodies in PBST containing 1 µg ml⁻¹ DAPI. Slides were washed, mounted with Fluoromount, and stored at 4 °C until imaging. Primary and secondary antibodies used for immunofluorescence, including target, host species, clonality, supplier, catalogue number and working dilution, are listed in Supplementary Table 2.

Imaging

Organoid images were acquired using an Olympus IXplore IX83 spinning-disk confocal microscope equipped with a Yokogawa CSU-W1 T1 spinning-disk unit and an ORCA-Flash 4.0 V3 sCMOS camera (Hamamatsu) (IMAG'IC facility, ISO 9001 certified). Excitation was performed using 405, 488, 561, and 640 nm laser lines for DAPI and antibody fluorophores. Tiles were stitched using cellSens Dimension software (Olympus) to reconstruct complete images. To confirm perfusion of human vessels, Ulex europaeus agglutinin I (UEA-I)-rhodamine (Vector Laboratories) was injected intravenously (100 µl at 2 mg ml⁻¹) 30-60 min before imaging. For intravital imaging, mice were maintained under anaesthesia with inhaled isoflurane on a thermostatically controlled heating pad throughout the procedure. Body temperature was continuously monitored using a rectal probe connected to the heating system and maintained at approximately 37 °C. Before incision, buprenorphine was administered at the planned

incision site. A small skin incision was then made over the subcutaneous graft, and the surrounding tissue was gently retracted to expose the vascularized organoid for direct optical access while preserving its vascular connections. The skin was secured to minimize respiratory motion and associated movement artefacts during image acquisition. The exposed graft was kept moist with PBS throughout the procedure. The eyes were regularly lubricated to prevent corneal drying during anaesthesia. Multiphoton imaging was performed using a Leica TCS SP8 multiphoton system (IMAG'IC facility, ISO 9001 certified) controlled with LAS X software (v3.5.7.23225) and equipped with an HCX IRAPO L 25×/0.95 water-immersion objective. Multiphoton excitation was provided by a 1040-nm laser and a tunable multiphoton laser set to 850 nm, with emitted fluorescence collected using external non-descanned PMT/HyD detectors. Images were acquired at 512 × 512 pixels with a 0.75× scan zoom. To assess blood-brain barrier permeability within vBO, EZ-Link™ Sulfo-NHS-LC-Biotin (Thermo Scientific, catalog no. 21217), a membrane-impermeant biotinylation tracer, was injected intravenously (100 µL at 1 mg/mL) 20 min before sacrifice. Xenografted vBO were then collected, fixed, sectioned, and biotin distribution was revealed using fluorescently conjugated streptavidin. Vascular leakage was assessed by quantifying biotin fluorescence outside and within human VE-Cadherin-positive vessels and expressed as the ratio of extravascular to intravascular fluorescence intensity. Higher ratios therefore indicate greater tracer extravasation. Whole organoid images were acquired using a Zeiss Axio Observer Z.1 confocal microscope equipped with a 25×/0.8 Plan-Apochromat immersion objective and an Airyscan detector module (Airyscan Fast 4Y–8Y) (IMAGOSEINE facility, ISO 9001 certified). Excitation was performed using 405, 488, 561, and 639 nm laser lines for DAPI and antibody fluorophores. Tiles were stitched using ZEN software (Zeiss) to reconstruct complete images.

Meningococcal infection readouts

Quantification of bacterial vascular colonization and thrombosis was performed as described previously^{53,54}. Confocal images of infected vBO sections were acquired at 20× magnification under identical acquisition conditions using a Yokogawa CSU-W1 spinning-disk microscope. Each quantified imaging field was represented as an individual data point. In each experiment, one mouse was analysed at 1 h and one mouse at 2 h post-infection, with two xenografted vBO per mouse. Because human vessels were heterogeneously distributed within the grafts, imaging fields containing identifiable human vascular structures were selected for quantitative analysis.

For each image, the human vascular compartment was manually delineated in ImageJ (v1.53) based on human VE-Cadherin immunostaining. The vascular colonization index was calculated as the surface area occupied by *N. meningitidis* signal within the delineated human vascular compartment, normalized to the total human vascular area. The thrombosis index was calculated as the surface area occupied by CD41-positive platelet signal within the same delineated human vascular compartment, normalized to the total human vascular area. All measurements were performed on background-subtracted and thresholded images using the same analysis criteria across experimental conditions. Each quantified imaging field was represented as an individual data point. Comparisons between 1 h and 2 h post-infection were performed using a two-sided Mann-Whitney U-test on imaging-field values.

Spatial transcriptomics and computational analyses

Samples were fixed in 4% PFA for 24 h at 4°C with agitation, followed by dehydration and storage at -80 °C in methanol. Prior to embedding, samples were partially rehydrated in 70% ethanol and processed for paraffin embedding. Several serial sections of 20 µm were generated and precisely arranged on the same Visium Slide V1 using the Visium Spatial Gene Expression Kit for FFPE, Human Transcriptome (10x Genomics).

Spatial transcriptomic profiling included one non-vascularized BO (4 sections), one BMEC+PC vBO (2 sections) and two HUVEC^{E4}+PC vBO (2 sections per organoid), for a total of 10 tissue sections from four organoids. Sections and Visium spots originating from the same organoid were considered spatial subsamples (pseudoreplicates) and not independent biological replicates.

Tissue permeabilization, cDNA synthesis, and library construction were performed using the Visium v1.1 kit, followed by paired-end sequencing on an Illumina NovaSeq 6000 platform (R1: 28 bp, i7: 10 bp, R2: 90 bp). Raw data were processed with Loupe Browser (v5.1.0) and SpaceRanger (v1.3.1) using the GRCh38 reference genome to generate spot-level expression matrices and spatial barcode annotations.

Computational analysis was performed on Seurat v4.3.0. Since one slide can contain more than one section of the tissue, each section was retrieved using spatial coordinates separately with no quality control thresholds applied. Each sample was normalized separately using SCTransform in Seurat v4. Then, to assess the batch effects in the datasets, merging was performed using merge function. (Extended Data Fig. 2c). The observation of batch effects between the samples led to a necessary integration approach based on CCA algorithm (Canonical Correlation Analysis) of Seurat. The number of selected features for integration is set to 3000. Dimensionality reduction was performed using UMAP on 1:50 dims with default number of PCs based on euclidean algorithm. (Extended Data Fig. 2c). Clustering was performed using the Louvain algorithm at multiple resolution from 0.5 to 2.5. The resolution 2.3 was selected based on the number of clusters and the differentially expressed genes retrieved at each resolution and how much these results were in correlation to the composition of the tissue. Louvain clustering identified 15 transcriptional clusters, which were subsequently consolidated into eight broad cell-state annotations based on UCell signature scores, differentially expressed marker genes and spatial distribution. Cell-type annotation and the marker-gene sets used to construct UCell signatures were guided by published single-cell and single-nucleus transcriptomic datasets of human brain tissue and cerebral organoids^{55–59}, together with brain mural-cell transcriptomic datasets for perivascular populations⁶⁰. These reference datasets were used to define representative lineage- and cell-state-associated gene signatures and to support biological interpretation of the clusters; they were not computationally integrated with the present spatial transcriptomic dataset. Neural progenitor, neuronal and astrocytic annotations were primarily informed by developmental human brain and cerebral organoid datasets^{55,58,59}. Endothelial, pericytic and fibroblast/stromal annotations were additionally informed by human brain vascular atlases^{29,48} and mural-cell transcriptomic datasets^{56,60}. The hypoxia-associated state was defined by enrichment of glycolytic, hypoxia-response and cellular-stress genes

and was supported by previously described hypoxia- and unfolded-protein-response signatures in cerebral organoids⁵⁹.

Cluster annotation was based on Ucell signature, differentially expressed genes and string analysis network. Differentially expressed genes of the different samples were identified based on the FindAllMarkers function where thresholds of $p_val_adj < 0.05$ and $|avg_log2FC| > 0.25$ were used to filter the markers. The list of differentially expressed genes was used as input to identify the enriched pathways in each cluster and which cell type they belong to, even multiple clusters can belong to the same cell type (Fig. 3d). As for the UCell signature, lists of populations of interests created in-house were used to calculate the signature of cell types in the different clusters (Fig. 2c). Differential expression analysis on these annotated cell types was conducted and Z-Score expression of the top markers of each cell type was plotted on a heatmap (Fig. 2d). The spots belonging to the different cell types were plotted on the images of the tissues to see at what degree they match the expected spatial localizations on the tissue (Fig. 2a). Proportions were calculated for each sample per cell type and for the variation between the different conditions across all cell types (Fig. 3a).

Monocle3 v1.4.27 R package was used for the pseudotime analysis in those 4 cell types: Astrocytes, Neurons, Progenitors 1 and Progenitors 2. All the calculations of pseudotime values were done on default parameters with Progenitor 1 cell type selected as the root cells (Fig. 2c).

Cellchat v2 R package was used to perform cellular interactions and pathway analysis of the HUVEC^{E4}+PC dataset, comprising four sections from two organoids. CellChat v2 was run using default parameters, except during communication-probability estimation, for which `distance.use = FALSE` was specified because the four tissue sections were physically separate on the Visium slide and did not share a meaningful continuous spatial coordinate system. The number of incoming and outgoing signalling patterns was determined from the cophenetic coefficient plots. Selected signalling pathways were visualized using chord diagrams (Extended Data Fig. 3f) and projected onto tissue coordinates (Fig. 2g; Extended Data Fig. 3e). Normalized expression of genes associated with these pathways was also mapped onto the tissue sections (Fig. 2g).

Differential expression gene analysis for Non vascularized and HUVEC^{E4}+PC was conducted using FindMarkers function in Seurat with `ident.1` as HUVEC^{E4}+PC BO and `ident.2` as Non vascularized BO. Significant markers were selected based on those thresholds $p_val_adj < 0.05$ and $|avg_log2FC| > 0.25$.

Electrophysiological recordings (HD-MEA)

At d80, spontaneous neuronal activity was recorded using high-density multi-electrode arrays (HD-MEA, 3Brain, Arena HD-MEA platform). Organoids were maintained in their culture medium for at least 48-72 h after the last medium change before recording, to allow stabilization of spontaneous activity. All recordings were performed at 37 °C in culture medium characterized by a potassium concentration higher than the physiological level, as previously described for cortical organoid preparations.

Acquisition parameters.

Electrical signals were acquired in “spike” readout mode with a sampling frequency of 20 kHz and a high-pass filter of 100 Hz. Recordings were performed in the native culture medium without perfusion. Each session lasted at least 5 min, and, when required, neuronal excitability was pharmacologically enhanced by acute application of KCl to induce or increase spontaneous spiking activity.

Spike detection and signal processing.

Spikes were detected using the Precise Timing Spike Detection (PTSD) algorithm, with a threshold set at nine times the standard deviation of baseline noise. Automatic filtering by the integrated AI-based module was used to exclude noisy or unstable electrodes. Spike sorting was not applied in this study, and all analyses were performed on unsorted extracellular spikes.

Analysis parameters and readouts.

For each electrode, a firing rate (FR) was computed over a 5-min recording window defined by the experimenter. The following parameters were extracted:

- Fraction of active organoids,
- Number of active electrodes (defined as electrodes exhibiting ≥ 5 spikes/min),
- Mean firing rate (MFR, average FR across active electrodes),

Data analysis.

Quantitative analyses were performed using BrainWav^{E4} (3Brain’s proprietary software) and custom R scripts developed by François-Xavier Lejeune (Data Analysis Core, ICM). Raster plots, activity heatmaps, and distribution curves were generated for each condition (non-vascularized, HUVEC^{E4}, and HUVEC^{E4}+PC vBO). At least three independent organoids per condition were included in the final quantitative analyses.

Data curation.

Datasets from organoids with fewer than 3 active electrodes were excluded from downstream analyses to ensure reliability and reproducibility of spike quantification.

Necrosis quantification

Nuclei segmentation was performed using QuPath (v0.5)^{61–63} combined with the StarDist extension and the pretrained model *dsb2018_heavy_augment.pb*, available at : <https://github.com/qupath/models/tree/main/stardist>.

For each acquisition series, a pre-processing step was applied to the segmented nuclear masks to exclude aberrant detections. Objects were filtered according to the following thresholds:

- $area_nuclei < threshold_min$,
- $area_nuclei > threshold_max$,
- $max_intensity < threshold_min$.

Necrotic areas were quantified from H&E- or DAPI-stained sections using a custom analysis pipeline⁶⁴. Threshold values were empirically determined for each imaging batch using the *Show Measurement Map* function in QuPath to account for variations in staining and acquisition parameters.

Pyknotic nuclei identification (Fig. 3b-c).

Pyknotic nuclei were detected based on combined morphometric (area and circularity) and intensity criteria. Pyknotic nuclei typically displayed reduced nuclear area and increased maximum intensity compared to non-pyknotic nuclei. Because DAPI signal intensity varied across images, a dynamic intensity threshold was computed for each field of view, ensuring robust identification of hypercondensed (pyknotic) nuclei.

The pyknosis index was calculated as the percentage of pyknotic nuclei among all segmented nuclei. Nuclear pyknosis was used as a morphological proxy for tissue degeneration and cell death-associated nuclear condensation.

Quantification of vascular parameters

Quantification of colocalization between human vascular structures and blood-brain barrier-associated markers was performed in Imaris (v10.1, Oxford Instruments) using the Coloc module. For each confocal z-stack, fluorescence channels corresponding to human VE-Cadherin and the BBB-associated marker of interest (Claudin-5, Occludin, ZO-1, GLUT1, P-glycoprotein or BCRP) were analysed directly from the three-dimensional voxel-level fluorescence data, without surface reconstruction or segmentation masks.

Because fluorescence background differed between tissue preparations and imaging fields, intensity thresholds were determined independently for each image rather than applying a fixed absolute threshold across datasets. For each channel, the threshold was adjusted to exclude local background signal while preserving specifically labelled structures. Threshold selection followed the same predefined visual criteria across all experimental conditions and was validated by overlaying the resulting colocalization mask onto the corresponding raw fluorescence image. Thresholds resulting in detectable colocalization outside anatomically defined vascular structures were rejected and readjusted.

Once thresholds were defined, colocalization was calculated using the Imaris voxel-based algorithm. Human VE-Cadherin was systematically defined as channel A and the BBB-associated marker as channel B. The primary quantitative readout was the percentage of channel B signal above threshold that colocalized with channel A (% volume B colocalized), providing a measure of the relative localization of the BBB-associated marker within human vascular structures.

Analyses were performed on three-dimensional confocal z-stacks acquired with a 60× oil-immersion objective, covering the vascular network over a depth of approximately 40-60 µm, with identical voxel-size settings within each acquisition series. Multiple imaging fields from at least three distinct organoids were analysed for each experimental condition.

To provide a physiological reference for BBB marker localization, adult human cortical tissue sections were analysed using the same procedure. For each marker, the median vascular colocalization value obtained from the human cortical reference samples was defined as 100%. Values obtained in vitro and xenografted vBO were normalized to this reference and expressed as a percentage of the corresponding human cortical value.

Quantification of neural populations and neurovascular spatial relationships

Quantification of neural markers (SOX2, HUCD, TUJ1) (Fig. 3e-f, Extended Data Fig. 3b) were performed using a custom analysis pipeline⁶⁵. For each nucleus, intensity statistics (mean and maximum intensity) were extracted for all fluorescence channels. Positive nuclei were identified using image-specific dynamic thresholds computed from per-image intensity distributions:

$$Threshold_{max} = \text{mean}(\text{max_intensity}) + factor_{max} \times \text{std}(\text{max_intensity})$$

$$Threshold_{mean} = \text{mean}(\text{mean_intensity}) + factor_{mean} \times \text{std}(\text{mean_intensity})$$

where mean and std correspond to the mean and standard deviation of the respective nuclear intensity measurements. Channel-specific parameters (factor_max and factor_mean) were defined independently for each fluorescence channel.

Neural tissue regions were defined by morphological expansion to pixels positive for neural markers, followed by geometric merging (unary union) and erosion to refine tissue boundaries and reduce segmentation artifacts. Resulting neural tissue contours were converted into polygonal objects and quantified by total area.

Vascular structures were detected using the Frangi vesselness filter (Extended Data Fig 4b), which enhances tubular structures based on eigenvalue analysis of the Hessian matrix. Following filtering, binary thresholding generated vessel masks that were converted into polygonal objects for spatial analysis.

Spatial proximity between vessels and neural cells (Extended Data Fig 4b) was estimated by calculating Euclidean distances using a KD-tree nearest-neighbor search (SciPy spatial library). Vessel coordinates were extracted as point matrices and neural cell centroids obtained from segmentation outputs. A KDTree structure was built from neural centroids, and for each vessel coordinate the distance to the nearest neighbours was computed using Euclidean distance ($p = 2$), enabling efficient spatial analysis across large datasets.

Quantification of Alexander disease-associated phenotypes

Quantification of Alexander disease-associated phenotypes was performed in Imaris (v10.1, Oxford Instruments), using the same general image-analysis framework described above for vascular and neural compartment segmentation. GFAP-positive Rosenthal fibre-like aggregates were identified from background-subtracted fluorescence images using image-specific intensity thresholds and quantified as the total aggregate area normalized to the corresponding imaging-field area (Fig. 6d). Overall vascular coverage and astroglial area were quantified from VE-Cadherin-positive and GFAP-positive masks, respectively, and normalized to the total organoid section area (Fig. 6e-f).

Astrocyte spatial organization was assessed from segmented GFAP-positive astrocytic objects. For each astrocyte, the mean Euclidean distance to its three nearest neighbouring astrocytes was calculated to quantify astrocyte dispersion (Fig. 6g). To assess astrocyte-vessel association, VE-Cadherin-positive vascular structures were segmented to generate vascular masks, and the shortest Euclidean distance from each GFAP-positive astrocyte to the nearest VE-Cadherin-positive vascular coordinate was calculated using a KD-tree nearest-neighbour search, as described above (Fig. 6h).

For the permeability analysis, human vascular structures were delineated from VE-Cadherin staining and intravascular and extravascular regions were defined for each analysed imaging field. EZ-Link Sulfo-NHS-LC-Biotin fluorescence intensity was quantified separately within and outside the human vascular compartment after background subtraction. Tracer extravasation was expressed as the ratio of extravascular to intravascular fluorescence intensity, with higher values indicating greater extravasation (Fig. 6j).

Statistical analyses

Statistical analyses were performed with GraphPad Prism v10.2.2 (GraphPad Software), except for the high-density microelectrode array data, which were analysed with SigmaPlot v16 (Grafiti). The test used for each experiment was chosen according to the experimental design and is stated in the corresponding figure legend. Two groups were compared using a two-sided Mann-Whitney U-test. Three or more groups were compared using a Kruskal-Wallis test followed by Dunn's multiple-comparisons test. Experiments with a two-factor design, combining culture time with either vascularization or cell line, were analysed by ordinary two-way ANOVA followed by Šídák's multiple-comparisons test restricted to the comparisons indicated on the graphs. Electrophysiological recordings were compared by one-way ANOVA after verification of normality (Shapiro-Wilk test) and equality of variances; given the group sizes, these analyses were not powered to establish equivalence between conditions. Data are presented as mean $\pm$ s.e.m. unless otherwise stated. The replicate unit differs between panels and is specified in each figure legend as one organoid, one imaging field or one mouse. Experiments included at least three independent organoids per condition, drawn from at least three independent differentiations, unless otherwise specified in the corresponding figure legend. Animal sample sizes varied according to the experimental design and are specified in the corresponding figure legends. No statistical method was used to predetermine sample size, and no data points were excluded from the analyses.

Vascularization success rates were compared globally across media conditions and endothelial cell sources using exact tests on the corresponding contingency tables (Extended Data Tables 1 and 2). Pairwise comparisons reported in Extended Data Tables 1 and 2 were additionally assessed using two-sided Fisher's exact tests. Pairwise P values were not adjusted for multiple comparisons. For the organoid growth analysis shown in Extended Data Fig. 2b, six organoids per condition were sampled at each time point across multiple independent differentiations. Measurements were cross-sectional and individual organoids were not longitudinally tracked. Groups were therefore compared separately at each time point using a Kruskal-Wallis test followed by Dunn's multiple-comparisons test.

A P value below 0.05 was considered significant and is reported as *P < 0.05, **P < 0.01 and ***P < 0.001. No statistical comparison was performed on the spatial distance distributions shown in Extended Data Fig. 4b or on the barrier-marker quantification relative to the human brain reference shown in Extended Data Fig. 5c.

METHODS-ONLY REFERENCES:

53. Ziveri, J. *et al.* Angiopoietin-like 4 protects against endothelial dysfunction during bacterial sepsis. *Nat Microbiol* **9**, 2434–2447 (2024).
54. Denis, K. *et al.* Targeting Type IV pili as an antivirulence strategy against invasive meningococcal disease. *Nat Microbiol* **4**, 972–984 (2019).
55. Darmanis, S. *et al.* A survey of human brain transcriptome diversity at the single cell level. *Proc Natl Acad Sci U S A* **112**, 7285–7290 (2015).
56. Lake, B. B. *et al.* Integrative single-cell analysis of transcriptional and epigenetic states in the human adult brain. *Nat Biotechnol* **36**, 70–80 (2018).
57. Tran, M. N. *et al.* Single-nucleus transcriptome analysis reveals cell-type-specific molecular signatures across reward circuitry in the human brain. *Neuron* **109**, 3088-3103.e5 (2021).
58. Chiaradia, I. *et al.* Tissue morphology influences the temporal program of human brain organoid development. *Cell Stem Cell* **30**, 1351-1367.e10 (2023).
59. Sato, Y., Asahi, T. & Kataoka, K. Integrative single-cell RNA-seq analysis of vascularized cerebral organoids. *BMC Biol* **21**, 245 (2023).
60. He, L. *et al.* Analysis of the brain mural cell transcriptome. *Sci Rep* **6**, 35108 (2016).
61. Schmidt, U., Weigert, M., Broaddus, C. & Myers, G. Cell Detection with Star-convex Polygons. in vol. 11071 265–273 (2018).
62. Bankhead, P. *et al.* QuPath: Open source software for digital pathology image analysis. *Sci Rep* **7**, 16878 (2017).

63. Adiba, S. Automated Nuclei Detection and Spatial Export in QuPath Using a Pre-Trained StarDist Model. <https://doi.org/10.5281/zenodo.18190032> (2026) doi:10.5281/zenodo.18190032.
64. Adiba, S. Automated Quantification of Nuclear Fluorescence Signals Using DAPI Segmentation Masks from QuPath. <https://doi.org/10.5281/zenodo.18216121> (2026) doi:10.5281/zenodo.18216121.
65. Adiba, S. Vessel in neural tissue for vascularized cerebral organoids. <https://doi.org/10.5281/zenodo.19577290> (2026) doi:10.5281/zenodo.19577290.

Figures

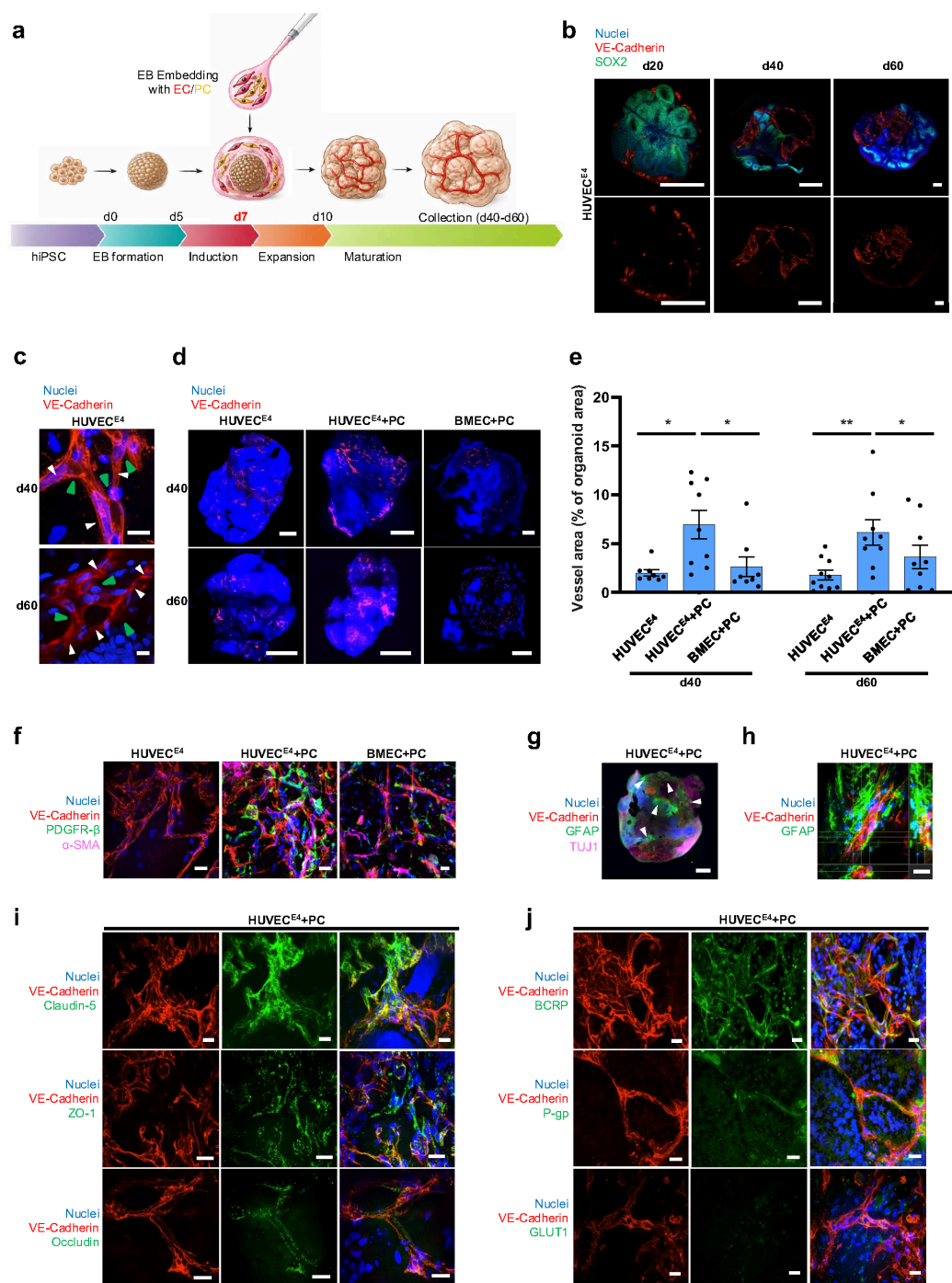


Figure 1

Figure 1

Generation and characterization of human vascularized brain organoids.

a, Workflow for vascularized brain organoids (vBO) differentiation from human iPSC: embryoid body formation, neural induction, embedding at d7 in Matrigel containing human endothelial cells (EC) and pericytes (PC), then expansion and maturation to d60. **b**, Emergence of the vascular and neural

compartments at d20, d40 and d60 in HUVEC^{E4} vBO. VE-Cadherin (red) endothelial networks expand within the neuroepithelial tissue, while SOX2 (green) delineates neural progenitor zones (scale bars, 500 μ m). **c**, Endothelial junctions at d40 and d60 in HUVEC^{E4} vBO. White arrowheads indicate tubular endothelial structures suggestive of ongoing lumen formation, green arrowheads indicate branching points (scale bars, 10 μ m). **d,e**, Vascular area across HUVEC^{E4}, HUVEC^{E4}+PC and brain microvascular endothelial cell (BMEC)+PC conditions: VE-Cadherin (red) networks (**d**; scale bars, 500 μ m) and vessel area fraction per organoid (**e**). Each dot represents one organoid (At d40, n = 8, 9 and 8 organoids for HUVEC^{E4}, HUVEC^{E4}+PC and BMEC+PC, respectively; at d60, n = 10, 9 and 9 organoids, respectively, from at least three independent differentiations), bars show mean $\pm$ s.e.m.; two-sided Kruskal–Wallis test with Dunn's multiple comparisons test, performed separately at each time point ; *P < 0.05, **P < 0.01. **f**, Perivascular mural-cell association. HUVEC^{E4}-only organoids lack detectable mural cells, whereas HUVEC^{E4}+PC and BMEC+PC organoids show PDGFR- β (green) mural cells adjacent to the endothelial network, a subset co-expressing α -smooth muscle actin (magenta) (scale bars, 20 μ m). **g**, Astroglial compartment at d40 stained for GFAP (green), VE-Cadherin (red) and TUJ1 (magenta). White arrowheads indicate emerging GFAP-positive astrocyte clusters (scale bar, 300 μ m). **h**, GFAP-positive astroglial clusters (green) extending towards VE-Cadherin (red) vessels (scale bar, 20 μ m). **i,j**, Tight-junction (**i**) and transporter (**j**) expression at d40: Claudin-5, ZO-1 and Occludin (**i**), BCRP, P-gp and GLUT1 (**j**), all in green, with VE-Cadherin (red) endothelium. GLUT1 was not detected at the vessel surface (scale bars, 20 μ m). Nuclei are counterstained with DAPI (blue) throughout. Images are maximum intensity projections of 50 μ m z-stacks in **b, d** and **g**, and of 30 μ m z-stacks in **c, f, h, i** and **j**.

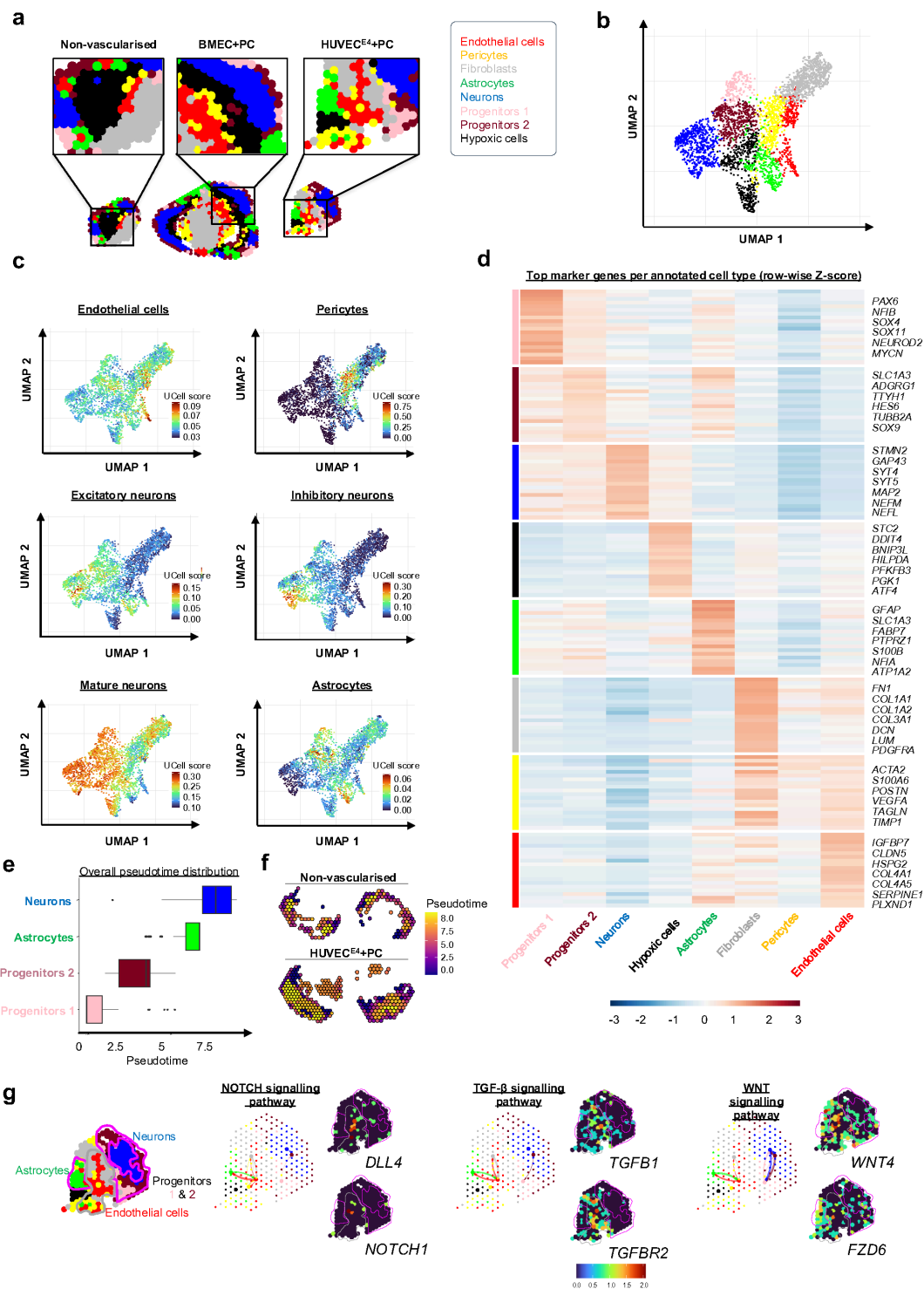


Figure 2

Figure 2

Spatial transcriptomics maps neurodevelopmental trajectories and neurovascular signalling in vascularized brain organoids.

a, Spatial distribution of annotated cell-type clusters in non-vascularized, BMEC+PC and HUVEC^{E4}+PC organoids. Each dot represents one Visium spot, coloured by its cell-type identity, as depicted; insets

show the boxed regions at higher magnification. **b**, UMAP embedding of all spatial spots of 10 organoid slices across 3 experimental conditions integrated with CCA, coloured by annotated cell-type clusters. **c**, Depicted UCell signature scores projected onto the UMAP. Colour scales are independently scaled for each signature. **d**, Top 20 marker genes per cluster (row-wise Z-score), with representative markers depicted for each cell-type. **e**, Pseudotime distribution across the progenitor 1, progenitor 2, astrocyte and neuronal clusters. Boxes span the interquartile range with the centre line at the median, whiskers extend to 1.5 times the interquartile range and dots are values beyond that range. **f**, Hex-binned spatial maps coloured by pseudotime in non-vascularized and HUVEC^{E4}+PC organoids. **g**, Expression-based CellChat analysis of the HUVEC^{E4}+PC spatial dataset. Left, spatial projection of the major annotated cell-type clusters. Right, spatial expression of representative ligand-receptor pairs associated with the corresponding CellChat-inferred interaction spatial networks.

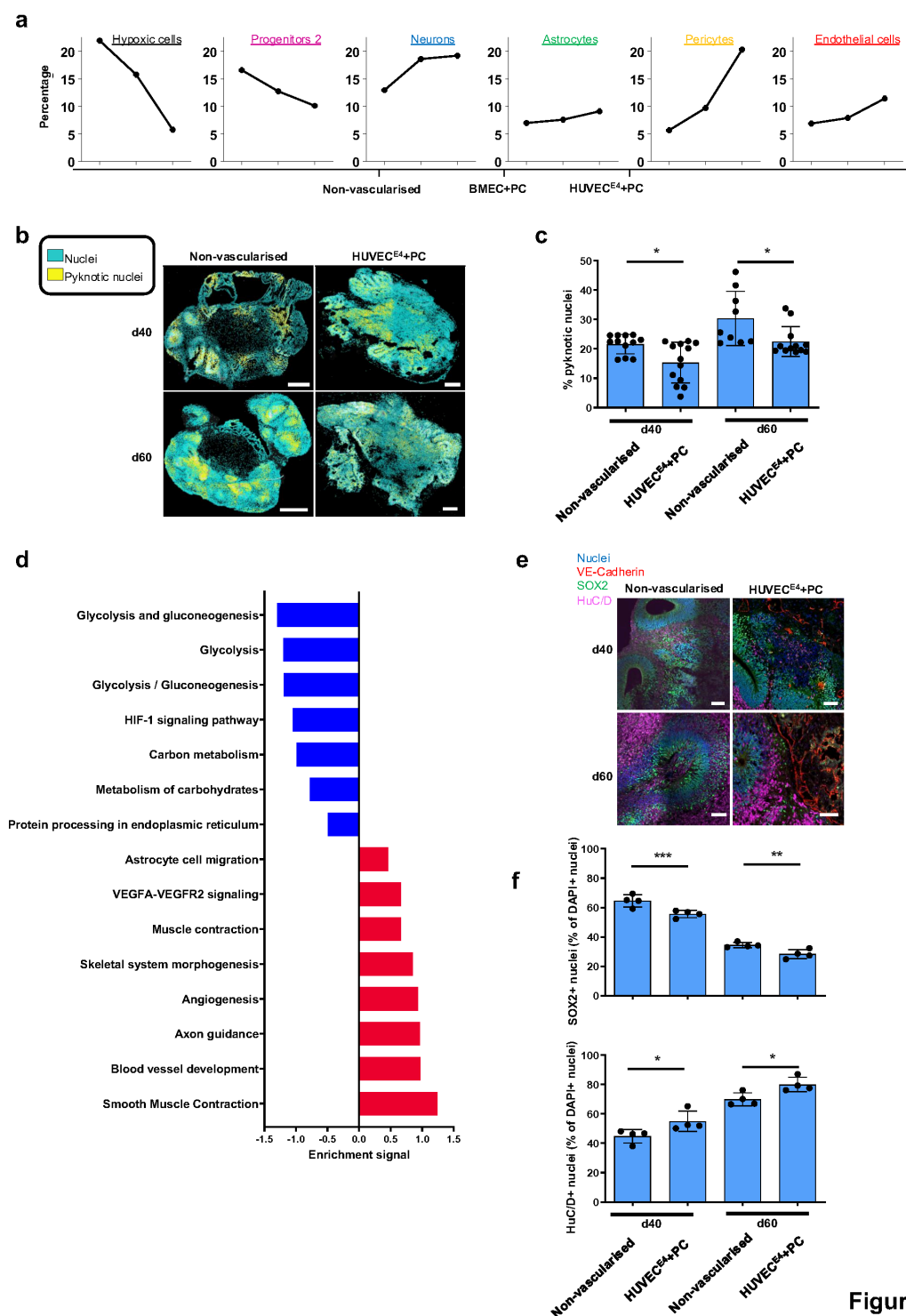


Figure 3

Figure 3

Vascularization improves tissue integrity, reduces hypoxia-associated stress and supports neuronal differentiation in brain organoids.

a, Distribution of selected annotated spatial states in the profiled non-vascularized BO, BMEC+PC vBO and HUVEC^{E4}+PC vBO, expressed as the percentage of Visium spots assigned to each state. One

organoid was profiled per non-vascularized BO (4 slices) and BMEC+PC vBO (2 slices) conditions, and two organoids per HUVEC^{E4}+PC vBO condition (2 slices each). **b**, Whole-organoid nuclear maps of non-vascularized BO and HUVEC^{E4}+PC vBO at d40 and d60. Nuclei segmented and classified by area, circularity and signal intensity are shown in cyan and pyknotic nuclei in yellow (scale bars, 200 μ m). **c**, Percentage of pyknotic nuclei. Each dot represents one organoid (At d40, n = 12 non-vascularized BO and n = 13 HUVEC^{E4}+PC vBO; at d60, n = 9 non-vascularized BO and n = 12 HUVEC^{E4}+PC vBO, from at least three independent differentiations). **d**, Gene Ontology and pathway enrichment among genes upregulated or downregulated in HUVEC^{E4}+PC vBO compared to non-vascularized BO. **e**, Immunofluorescence for the neuroprogenitor marker SOX2 (green), the neuronal marker HuC/D (magenta) and the endothelial marker VE-Cadherin (red) in non-vascularized BO and HUVEC^{E4}+PC vBO at d40 and d60 (scale bars, 80 μ m). **f**, Percentage of SOX2-positive (**top**) and HuC/D-positive (**bottom**) nuclei per organoid in the same conditions. Each dot represents one organoid (n = 4 organoids per condition from at least three independent differentiations). Bars show mean $\pm$ s.e.m.; ordinary two-way ANOVA with time and vascularization as factors, followed by Šídák's multiple comparisons test; *P < 0.05, **P < 0.01. Nuclei are counterstained with DAPI (blue) in **e**. Images in **b** and **e** are maximum intensity projections of 10 μ m z-stacks.

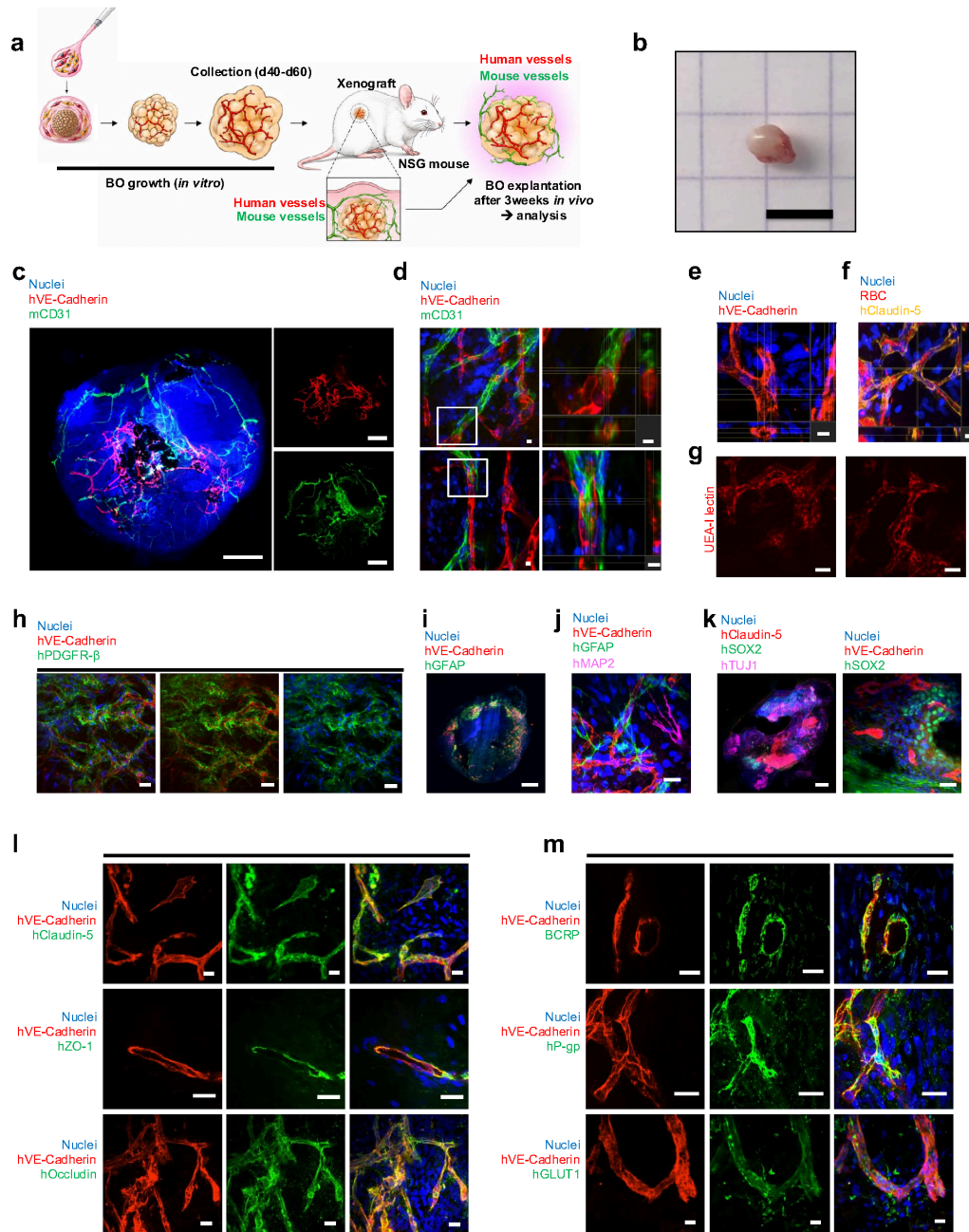


Figure 4

Figure 4

***In vivo* transplantation confirms persistence, perfusion, and maturation of vascularized brain organoids.**

a, Experimental design of the xenograft assay. HUVEC^{E4} + pericyte (PC) vBO collected between d40 and d60 were grafted subcutaneously into NSG mice, maintained for three weeks *in vivo*, then explanted and

analysed. **b**, Explanted graft on millimetre paper (scale bar, 500 μ m). **c**, Human and murine vascular networks within the graft, labelled for human VE-Cadherin (hVE-Cadherin, red) and murine CD31 (mCD31, green). Right, the two channels shown separately (scale bars, 500 μ m). **d**, Orthogonal views of the boxed regions showing continuity between human and murine endothelial structures at anastomosis points (scale bars, 5 μ m). **e**, Orthogonal views of lumenised human vessels (scale bar, 10 μ m). **f**, Perfused human capillary containing autofluorescent red blood cells (RBC, red), with human Claudin-5 (yellow) delineating endothelial junctions (scale bar, 10 μ m). **g**, Two-photon imaging of a xenografted vBO after intravital labelling of fluorescent *Ulex europaeus* agglutinin I (UEA-I) lectin, labelling the endothelium of perfused human vessels. Single optical sections (scale bars, 70 μ m). **h**, Perivascular mural-cell association *in vivo*, showing PDGFR- β -positive cells (green) adjacent to hVE-Cadherin-positive human vessels (red) (scale bars, 20 μ m). **i**, Tile-scan confocal overview labelled for hVE-Cadherin (red) and human GFAP (green) (scale bar, 300 μ m). **j**, Perivascular astrocyte processes adjacent to neuronal process, labelled for hVE-Cadherin (red), human GFAP (green) and human MAP2 (magenta) (scale bar, 20 μ m). **k**, Persistence of neurogenic and neuronal domains. Left, human SOX2-positive progenitor zones (green) and TUJ1-positive neuronal layers (magenta). Right, a rosette-like structure of SOX2-positive progenitors (green) adjacent to hVE-Cadherin-positive vessels (red) (scale bars, 200 μ m and 30 μ m). **l,m**, Blood–brain barrier marker expression in human vessels, labelled for hVE-Cadherin (red) with Claudin-5, ZO-1 and Occludin (**l**) or BCRP, P-gp and GLUT1 (**m**), all in green (scale bars, 20 μ m). Nuclei are counterstained with DAPI (blue) throughout. Images are maximum intensity projections of 50 μ m z-stacks in **c**, **i** and **k** (left), and of 30 μ m z-stacks in **d**, **e**, **f**, **h**, **j**, **k** (right), **l** and **m**.

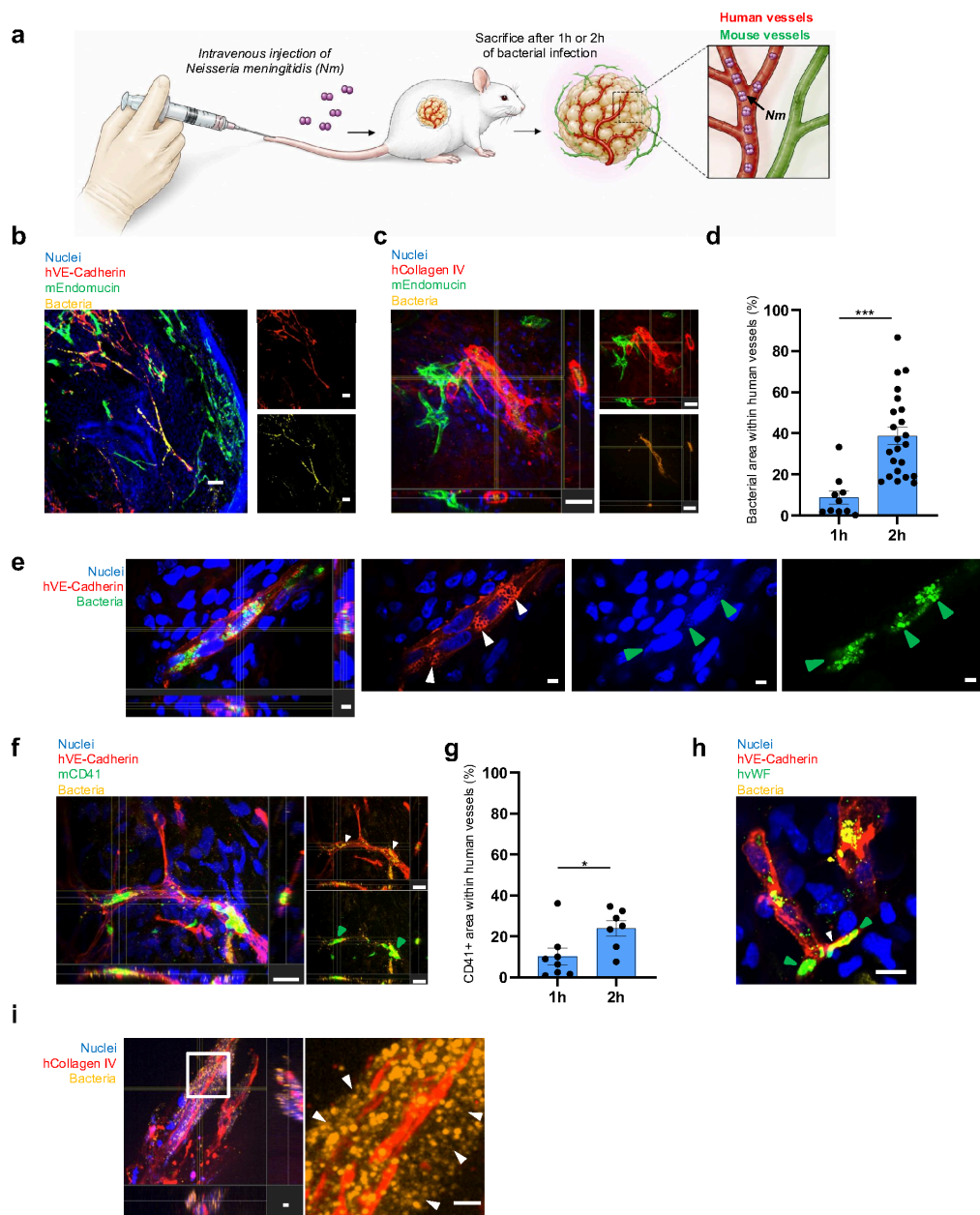


Figure 5

Figure 5

Reconstructing human-specific BBB invasion by *Neisseria meningitidis* using vascularized brain organoids.

a, Experimental workflow. HUVEC^{E4} + pericyte (PC) vascularized brain organoids (vBO) were xenografted subcutaneously into NSG mice for three weeks to allow vascular integration and perfusion, then infected

intravenously with *Neisseria meningitidis* (Nm). Grafts were collected 1 h or 2 h after infection and analysed by immunofluorescence. **b,c**, Bacteria (yellow) associate selectively with human vessels and are not detected on adjacent murine vessels. Human vessels are labelled for human VE-Cadherin (hVE-Cadherin, red) in **b** and for human collagen IV (red) in **c**; murine vessels are labelled for mouse endomucin (green) in both panels. Right, the separated channels in **b** and orthogonal views in **c** (scale bars, 70 μ m in **b**; 30 μ m in **c**). **d**, Percentage of vascular bacterial colonization at 1 h and 2 h after infection. **e**, Honeycomb-like remodelling of the human endothelial membrane at infection sites. White arrowheads indicate the endothelial honeycomb structures and green arrowheads the bacterial foci. Human endothelium is labelled for hVE-Cadherin (red) and bacteria in green (scale bars, 5 μ m). **f**, Platelet aggregates adjacent to intraluminal bacterial foci, labelled for hVE-Cadherin (red), mouse CD41 (green) and bacteria (yellow). White arrowheads indicate bacterial clusters and green arrowheads platelet thrombi. Right, orthogonal views of the same field (scale bars, 15 μ m). **g**, Percentage of vascular thrombosis at 1 h and 2 h after infection. **h**, Human capillary occluded by von Willebrand factor (hvWF, green) within a bacterial focus (yellow), with hVE-Cadherin-labelled endothelium (red). White arrowheads indicate bacterial clusters and green arrowheads indicate vWF-rich intravascular accumulations (scale bar, 10 μ m). **i**, Bacterial extravasation at 2 h. Orthogonal views with human collagen IV (red) delineating the vascular basement membrane and bacteria in yellow. Right, magnification of the boxed region; white arrowheads indicate bacterial aggregates beyond the vascular boundary (scale bars, 2 μ m). Nuclei are counterstained with DAPI (blue) throughout. Images are maximum intensity projections of 30 μ m z-stacks. In **d** and **g**, data were obtained from three independent experiments, each comprising one mouse analysed at 1 h and one mouse analysed at 2 h post-infection, with two xenografted vBO per mouse. 20 $\times$ imaging fields containing identifiable human vascular structures were selected and quantified individually. Each dot represents one imaging field; fields were sampled from two vBO per mouse across three independent experiments. Bars show mean $\pm$ s.e.m.; two-sided Mann–Whitney U-test; * $P < 0.05$, *** $P < 0.001$.

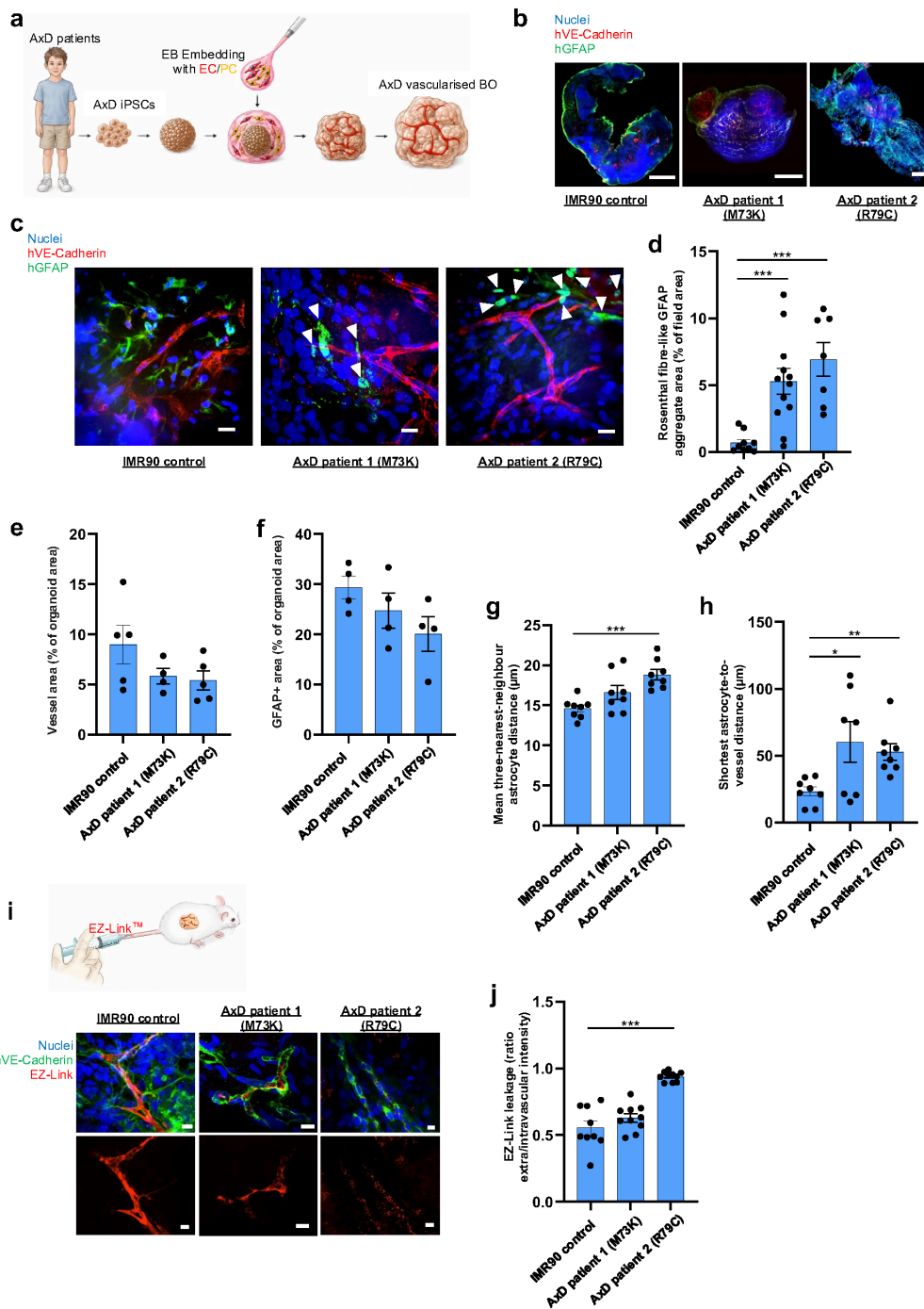


Figure 6

Figure 6

Vascularized brain organoids derived from GFAP-mutant hiPSC model the astrocytic and vascular phenotypes of Alexander disease.

a, Experimental workflow. hiPSC lines from a healthy donor (IMR90) and from two patients carrying pathogenic GFAP mutations (M73K and R79C) were differentiated into brain organoids and vascularized

by embedding with HUVEC^{E4} endothelial cells and primary pericytes at d7. **b**, Whole-organoid views of d40 vBO of the three genotypes, labelled for human VE-Cadherin (hVE-Cadherin, red) and human GFAP (hGFAP, green) (scale bars, 500 μ m). **c**, High-magnification views of the astrocyte–vessel interface. White arrowheads indicate Rosenthal fibre-like GFAP aggregates, which are present in both AxD lines and absent from the control (scale bars, 20 μ m). **d**, Rosenthal fibre-like GFAP aggregate area normalized to imaging-field area. **e,f**, VE-Cadherin-positive vascular area (**e**) and GFAP-positive astrocytic area (**f**) per organoid. Each dot represents one organoid. In **e**, $n = 5, 4$ and 5 organoids for IMR90, M73K and R79C, respectively; in **f**, $n = 4$ organoids per condition. Organoids were obtained from at least three independent differentiations. Bars show mean $\pm$ s.e.m. Statistical comparisons were performed using a two-sided Kruskal-Wallis test followed by Dunn's multiple-comparisons test; no comparison reached statistical significance.

g, Average distance from each astrocyte to its three nearest astrocytes. **h**, Shortest distance from each GFAP-positive astrocyte to the nearest VE-Cadherin-positive vessel. **i,j**, Blood–brain barrier permeability after subcutaneous xenografting of d40 vBO into NSG mice. Mice received an intravenous injection of EZ-Link Sulfo-NHS-LC-Biotin 20 min before collection. Representative images show the tracer (red) with hVE-Cadherin (green), upper row merged with nuclei and lower row showing the tracer channel alone (**i**; scale bars, 10 μ m), and the extravascular-to-intravascular tracer fluorescence ratio is quantified in **j** as an index of tracer extravasation. Nuclei are counterstained with DAPI (blue) throughout. Images are maximum intensity projections of 50 μ m z-stacks in **b** and of 30 μ m z-stacks in **c** and **i**. In **d, g** and **h**, data were obtained from three independent differentiation experiments, and each dot represents one imaging field; in **e** and **f**, each dot represents one organoid. In **j**, each dot represents one imaging field sampled from three xenografted vBO implanted in one mouse per cell line. Bars show mean $\pm$ s.e.m.; two-sided Kruskal-Wallis test with Dunn's multiple-comparisons test; * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$.

Supplementary Files

This is a list of supplementary files associated with this preprint. Click to download.

- [Supplementaryfile.pdf](#)