Microfluidic device with brain extracellular matrix promotes structural and functional maturation of human brain organoids

Ann-Na Cho1,12, Yoonhee Jin1,12, Yeonjoo An1,12, Jin Kim1, Yi Sun Choi1, Jung Seung Lee1, Junghoon Kim1, Won-Young Choi2, Dong-Jun Koo3, Weonjin Yu4, Gyeong-Eon Chang1, Dong-Yoon Kim3, Sung-Hyun Jo5, Jihun Kim6, Sung-Yong Kim3,7, Yun-Gon Kim5, Ju Young Kim8, Nakwon Choi9, Eunji Cheong1, Young-Joon Kim2, Hyunsoo Shawn Je4, Hoon-Chul Kang6 & Seung-Woo Cho1,10,11✉

Brain organoids derived from human pluripotent stem cells provide a highly valuable in vitro model to recapitulate human brain development and neurological diseases. However, the current systems for brain organoid culture require further improvement for the reliable production of high-quality organoids. Here, we demonstrate two engineering elements to improve human brain organoid culture, (1) a human brain extracellular matrix to provide brain-specific cues and (2) a microfluidic device with periodic flow to improve the survival and reduce the variability of organoids. A three-dimensional culture modified with brain extracellular matrix significantly enhanced neurogenesis in developing brain organoids from human induced pluripotent stem cells. Cortical layer development, volumetric augmentation, and electrophysiological function of human brain organoids were further improved in a reproducible manner by dynamic culture in microfluidic chamber devices. Our engineering concept of reconstituting brain-mimetic microenvironments facilitates the development of a reliable culture platform for brain organoids, enabling effective modeling and drug development for human brain diseases.

1Department of Biotechnology, Yonsei University, Seoul, Republic of Korea. 2Department of Biochemistry, Yonsei University, Seoul, Republic of Korea. 3Institute of Molecular Biology and Genetics, Seoul National University, Seoul, Republic of Korea. 4Signature Program in Neuroscience and Behavioral Disorders, Duke-NUS Medical School, Singapore, Singapore. 5Department of Chemical Engineering, Soongsil University, Seoul, Republic of Korea. 6Division of Pediatric Neurology, Department of Pediatrics, Severance Children’s Hospital, Yonsei University College of Medicine, Seoul, Republic of Korea. 7Department of Chemistry, Seoul National University, Seoul, Republic of Korea. 8Department of Advanced Materials Engineering, Kangwon National University, Samcheok, Republic of Korea. 9Brain Science Institute, Korea Institute of Science and Technology (KIST), Seoul, Republic of Korea. 10Center for Nanomedicine, Institute for Basic science (IBS), Seoul, Republic of Korea. 11Graduate Program of Nano Biomedical Engineering (NanoBME), Advanced Science Institute, Yonsei University, Seoul, Republic of Korea. 12These authors contributed equally: Ann-Na Cho, Yoonhee Jin, Yeonjoo An.✉email: seungwoocho@yonsei.ac.kr
Recent breakthroughs in brain organoid technology have enabled the development of a new in vitro model that promotes significant advancements in the study of nervous system development and diseases. Sasai and co-workers proposed the idea that differentiated pluripotent cells can form multi-layered organized structures that recapitulate embryonic development when grown in three-dimensional (3D) culture. Lancaster et al. developed a 3D culture model termed “cerebral organoids” that recapitulate many key features of the human brain in vivo and develop various distinct and interdependent brain regions. The generation of cerebral organoids depends on the intrinsic ability of pluripotent stem cells (PSCs) to spontaneously self-organize upon precisely timed manipulation of culture conditions even in the absence of external patterning factors. Cerebral organoids representing the whole brain have significant advantages over brain region-specific or extensively patterned organoids due to their ability to generate a diverse range of brain cells and recapitulate the major events in overall brain development. Despite the potential of cerebral organoids in the current technology, there are several challenges. Due to the lack of instructive signals during the generation of human cerebral organoids, they recapitulate only some of the earliest stages of human embryonic brain development and are not able to mimic the later stages of neurogenesis until extended cultivation for 6–9 months. Another critical limitation is the extensive cell death in the developing organoids at later stages due to diffusion limitations in oxygen and nutrient transport.

Several engineering strategies with biomaterials, bioreactors, devices, and genetic modification have been demonstrated to overcome such limitations of current brain organoid culture. For example, synthetic polymer microfilaments enhanced neuroectoderm formation and cortical development by facilitating guided self-organization via neuroepithelium elongation. In other study, miniaturized spinning bioreactors were tested for improving the dynamic culture of brain organoids, which generated more robust disease models with Zika virus infection. To increase the oxygen supply, air–liquid interface culture was adapted for cerebral organoids, resulting in improved survival and morphology with extensive axonal outgrowths. Organ-on-a-chip systems were also employed to improve the oxygen supply to the brain organoids. The vascularization of brain organoids by grafting human brain organoids into the mouse brain or gene editing of vascular transcription factor resulted in progressive neurogenesis with improved neuronal survival. Despite these recent technical improvements, certain progenitor cells still showed low abundance, and the cytoarchitecture of the basal zones and cortical layers was not complete. Moreover, the current protocols based on spontaneous self-organization have exhibited a significant batch-to-batch variation, which in turn results in poor reproducibility. Therefore, cerebral organoids still need to be improved further for neuronal development, structural maturation, and better electrophysiological functionality, as well as for ensuring consistent organoid quality.

Here, we propose a strategy to engineer human PSC-derived cerebral organoids by reconstituting a 3D brain-mimetic microenvironment with a decellularized human brain tissue-derived brain extracellular matrix (ECM) cues together with improved nutrient and oxygen exchange will support cell expansion as well as neuronal differentiation and functional maturation, thereby recapitulating prominent features of human embryonic cortical development in a much precise and reproducible manner.

**Results**

**Characterization of a human brain-mimicking 3D hydrogel matrix.** A bioengineering platform to improve human brain organoid culture was set up with human brain-mimicking 3D hydrogel and a microfluidic system. Human cerebral organoids were generated from human induced pluripotent stem cells (iPSCs) as described in Lancaster’s protocol (Supplementary Fig. 1). When embryoid bodies (EBs) were induced to develop into a neuroepithelial lineage at day 11, they were embedded in a 3D matrix supplemented with human BEM (0.4 mg/ml) (Fig. 1a). Because Matrigel, a common and essential component of the organoid culture, is refractory to the tissue-specific ECM cues that are required by different tissue types, modification of Matrigel-based organoid culture by supplying human BEM would provide enhanced cell growth and more favorable interactions at an early stage of neurogenesis. After four days of culture in BEM-incorporated gel, the organoids were transferred into the microfluidic device under dynamic conditions (Fig. 1a, b). Our microfluidic platform can allow for independent control of the cerebral organoids in much smaller medium volume and precisely controlled medium flow with low fluid shear stress (Supplementary Fig. 2), compared to typical bulk scale bioreactors (e.g. spinner flasks, orbital shakers) which require larger volumes and evoke cell damage due to the high shear stress. With the precisely controlled fluid flow, the effective exchange of oxygen, nutrients, and bioactive molecules in the medium leads to the robust expansion and reduced cell apoptosis at an early stage of organoid development. Consequently, more complex structures with elongated cortical layers would be evident in cerebral organoids.

BEM enriched with brain ECM components was prepared by the decellularization of human brain tissue and subsequent tissue processing. Human brain samples were pooled before decellularization (Supplementary Table 1). Cellular components in brain tissue were removed by treatment with a non-ionic detergent (Supplementary Fig. 3a, b). The decellularization process did not have a significant effect on the glycosaminoglycan (GAG) content (Supplementary Fig. 3c). Histological analysis of Hematoxylin and Eosin (H&E) and Masson’s Trichrome (MT) stained samples suggested that most of the cells were removed, while collagen, one of the major ECM components, remained intact after decellularization (Supplementary Fig. 3d). Proteomic analysis of BEM with mass spectrometry revealed that BEM contains the enrichment of brain-specific ECM components, including various collagen subtypes, proteoglycans (e.g. heparan sulfate, neurocan, versican), and glycoproteins (e.g. laminin, tenasin) (Supplementary Table 2). Collagens, proteoglycans (e.g. neurocan, versican), and glycoproteins (e.g. laminin, tenasin) detected in BEM—can affect various processes involved in neurogenesis, such as neuronal polarization and migration, neurite outgrowth, axon guidance, and synapse development. Thus, BEM can provide a variety of biological components that influence the development and function of brain organoids. Rheological analysis of BEM and Matrigel (Mat) hydrogels identified a higher level of storage (elastic) modulus than loss (viscous) modulus in all tested frequency ranges (0.1–10 Hz), indicating the formation of a stable internal network in both hydrogel systems (Supplementary Fig. 4a). The average storage modulus of Mat and BEM hydrogels at 1 Hz was 115.56 ± 5.55 Pa and 126.17 ± 13.52 Pa, respectively.
(Supplementary Fig. 4b), indicating that there is no significant difference in terms of mechanical properties between these two hydrogels. These data suggest that the positive effects of BEM hydrogel on the development of brain organoids are more likely to be due to the biochemical signals rather than due to mechanical cues.

The ECM profiles in BEM were compared with those in Mat and human brain tissue. All identified proteins were categorized as either ECM or its associated proteins, commonly referred as Matrisome. We checked ‘Human Protein Atlas’ to examine whether BEM has comparable ECM profiles to the normal human brain (Fig. 1c–f). It appears that BEM and human brain tissue contain a similar percentage of matrisome proteins out of total proteins (2–5%), whereas majority of proteins in Mat are matrisomes (~90%) (Fig. 1c). Significant percentages of collagens, glycoproteins, and proteoglycans are contained in BEM and human brain tissue, but Mat primarily consists of glycoproteins (Fig. 1d). We identified 90 ECM proteins in BEM, whereas 747 are found in human brain tissue in Human Protein Atlas (Supplementary Table 3). We found that 85 out of 90 ECM proteins (94%) in BEM are expressed in the native brain tissue (Fig. 1e). The lists of identified proteins in Mat and BEM were also compared with the list of proteins that are known to show elevated expression in the human brain tissue at least four-fold.
higher compared to other tissue types. Only 9 brain tissue-enriched proteins were found in Mat, whereas 352 proteins were identified in BEM (Fig. 1f). A gene ontology biological process (GOBP) analysis indicates that the brain tissue-enriched proteins only identified in BEM are involved in nervous system development and neurogenesis (Fig. 1g). Overall, our proteomics data revealed that the portion and types of matrisome proteins in BEM are much closer to those of human brain tissue than Mat.

The variability of BEM batches arising from the isolation of BEM from different sources should be addressed to standardize the BEM for brain organoid culture. The proteomic analysis of different batches of BEM showed that portion and compositions of matrisomal contents are quite similar in three batches of BEM (batch 1, 2, 3) (Fig. 1h, i). Additionally, the top 10 proteins with the highest intensity-based absolute quantification (iBAQ) values completely overlapped in all three batches (Fig. 1i). Although each batch of BEM was pooled from 2–3 patient samples with different age and gender, batch-to-batch variability of BEM was not significant in our study. It was previously reported that brain ECM composition is altered in cortical dysplasia and temporal lobe epilepsy. For example, upregulation of neurocan and tenascin-C was observed in a murine model of temporal lobe epilepsy, while major ECMs, such as glycoproteins, laminin, and fibronectin that are implicated in tissue remodeling, showed no significant change. Although the expression of some of the ECM proteins might be altered in BEM derived from epilepsy patients, apparent proteins that elicit pathological signals (e.g. pro-inflammatory cytokines, matrix metalloproteinase-9) were not identified in our BEM samples. The GOBP analysis of the total proteins as well as non-matrisome proteins also suggested that BEM does not contain proteins that are involved in inflammation (Supplementary Table 4).

**BEM increases the neuronal population and enhances neurogenesis in the brain organoid.** The potential of BEM for promoting brain organoid formation and development of the neuronal population was investigated. Brain organoids grown in BEM hydrogel (BEM-incorporated Matrigel) were compared with those in Mat alone at 30 days of culture. BEM organoids were significantly larger (p < 0.05) than Mat organoids (1.56 ± 0.44 and 1.84 ± 0.35 mm in diameter for Mat and BEM organoids, respectively) (Fig. 2a, b). Some samples in the BEM group grew up to 4–5 mm by 30 days of culture (Fig. 2a, bright-field image). Immunostaining analysis revealed the presence of laminin-rich basement membranes at the outer border of the brain organoids in both the BEM and Mat groups (Fig. 2c, d), which is an important phenomenon in neocortical development in vivo. The formation of a laminin layer in BEM organoids was consistently relatively thicker than in Mat organoids. Although both BEM and Mat organoids initially formed a laminin-rich basement membrane in early neuroepithelium at day 30 (Fig. 2c), only BEM organoids maintained a thick laminin basement membrane at day 75 (Fig. 2c). Mat organoids showed only sparse and punctate signals in laminin staining (Fig. 2c), which indicates that they failed to maintain the basement membrane upon the generation and basal migration of neurons. This observation suggests that BEM contributed to the maintenance of a laminin basement layer that could induce the organization of radially aligned neurons in the cortical layers of the organoids. In the proteomic analysis, BEM was found to contain more abundant subtypes of laminin (α2, α4, α5, and β2) than Mat except for α1, β1, and γ1, but Mat contains approximately 80 times higher relative intensity-based absolute quantification (riBAQ) values of total laminin subtypes (0.594 for Mat and 0.007 for BEM) (Fig. 2e). Given that Mat is much more enriched with laminins than BEM, the thicker laminin layer formation in the BEM organoids (Fig. 2c, d) is more likely to be derived intrinsically from the developing organoids rather than ectopically supplied from BEM.

To examine the generation of the neural population and neuronal differentiation, the expression of neural progenitor (NP) markers [Nestin, sex-determining region Y-box 2 (SOX2)] and neuronal markers [class III beta-tubulin (Tuji)], microtubule-associated protein 2 (MAP2) was analyzed (Fig. 2f–g and Supplementary Fig. 5a, b). The areas positive for Tuji and MAP2 were more apparent in the BEM organoids, indicating that BEM increased the neuronal populations and enhanced neuronal differentiation in the organoids (Fig. 2f, g). In the BEM organoids, Tuji-expressing neuronal cells were present throughout the whole constructs, whereas a layer of neurons expressing MAP2—a marker of mature neurons—was mainly located at the basal side of the ventricle-like structures in the peripheral regions of the organoids (Supplementary Fig. 5b, c). The apicobasal axis formation was more evidently observed in brain organoids grown in BEM hydrogel than in organoids produced in Mat (Fig. 2f and Supplementary Fig. 5d), suggesting that BEM organoids better mimicked a distribution of neuronal population showing apicobasal migration in vivo. In Mat organoids, smaller ventricle-like structures were developed, and apicobasal axis polarity was less frequently observed (Fig. 2f). These collective observations demonstrated that the exposure of developing organoids to BEM resulted in the formation of highly dense ventricle-like structures with few vacant spaces, their integration into well-organized structures, and enhanced neuronal differentiation with radial polarity.

Compared to organoids cultured in Mat and neurospheres of human fetal brain-derived neural stem cells (NSCs), human brain organoids produced in the BEM hydrogel exhibited gene expression profiles that are indicative of enhanced neurogenesis (Fig. 2h). As analyzed by quantitative real-time polymerase chain reaction (qPCR) at 30 days of culture, BEM organoids displayed approximately two-fold upregulation of an NP marker, SOX2, compared to Mat organoids and NSCs. The highest expression of the Enhancer of zeste homolog 2 (EZH2) was found in NSCs, and the lowest expression was found in Mat organoids. EZH2 involved in stem cell renewal and maintenance is highly expressed in NSCs or progenitor cells of the cortex, but its expression decreases in differentiated neurons. The expression of CDH1—a co-factor that is required for the regulation of cortical neurogenesis and survival—was noticeably higher in the BEM group than in other groups. BEM organoids displayed the highest TUBB3 (Tuji)1 expression, and Mat organoids and NSCs showed similar levels of TUBB3 expression. The expression of tyrosine hydroxylase (TH), a dopaminergic neuron marker, was also the highest in the BEM group. Immunostaining for a forebrain marker FOXG1 showed that BEM organoids at 75 days of culture had larger FOXG1+ brain lobules than those in Mat organoids, suggesting enhanced forebrain identity (Supplementary Fig. 5e). BEM organoids are likely to have more NPs than Mat organoids, while undergoing enhanced neurogenesis at the same time, as indicated by upregulated expression of both progenitor marker (SOX2) and neuronal marker (TUBB3) (Fig. 2h). This might be because proliferation and differentiation of NPs simultaneously occur at early phase of brain organoid development (~day 30). The rates of proliferation and neurogenesis continuously change during brain development, and their balance is thought to determine the brain size. Thus, enhanced proliferation of NPs in BEM organoids may contribute to the increased size of brain organoids (Fig. 2b).

Surprisingly, an increase in BEM dose did not improve organoid development (Supplementary Fig. 6). We determined 0.4 mg/ml BEM concentration as an optimal dose for human brain organoid
culture. Microscopic observation and immunohistochemical staining for Tuj1 and SOX2 indicated that higher BEM concentrations (1 and 2 mg/ml) than 0.4 mg/ml BEM did not enhance neuronal marker expression and neuroepithelial outgrowth in brain organoids (Supplementary Fig. 6a). qPCR analysis at 30 days of culture revealed that the expression of neuronal differentiation markers (Nestin, PAX6, TUBB3, MAP2) was generally higher in brain organoids grown in 0.4 mg/ml BEM hydrogel than in hydrogel with other BEM concentrations (Supplementary Fig. 6b). These data suggest that an optimal concentration of BEM is required for the development of brain organoids with sophisticated neurogenesis and neuroectoderm-like morphology.
**Fig. 2** BEM improves neurogenesis and organization of cortical layers in cerebral organoids. **a** Bright-field images and hematoxylin and eosin (H&E) staining of Mat- and BEM-embedded brain organoids at day 30 (scale bars = 500 μm for bright-field and 100 μm for H&E staining images, independent replicates = 3). **b** Quantification of the longest diameter of Mat and BEM organoids based on the bright-field images of whole organoids (n = 20, Mat versus BEM p = 0.0349, independent replicates = 3). **c** Laminin staining of Mat and BEM organoids at 30 and 75 days (scale bars = 100 μm), and **d** quantification of the thickness of laminin-rich basement membrane covering the outer surface of the organoids at day 30 (n = 15 for Mat and n = 12 for BEM, Mat versus BEM p = 0.0066, independent replicates = 5). **e** Comparison of relative intensity-based absolute quantification (riBAQ) values of laminin subtypes identified in Mat and BEM. **f** Immunohistochemical staining of neural progenitor marker (Nestin) and neuronal markers (Tuj1 and MAP2) at 30 days of culture (scale bars = 500 μm, independent replicates = 1-4). **g** Image-based quantification of the Tuj1- and MAP2-positive area in the Mat and BEM organoids at day 30 (n = 10 for Tuj1 and n = 15 for MAP2, Mat versus BEM p = 0.0001 for Tuj1; Mat versus BEM p = 0.0012 for MAP2, independent replicates = 2-7). **h** The quantitative real-time polymerase chain reaction (qPCR) analysis to compare gene expression between Mat, neural stem cells (NSCs), and BEM organoids at day 30 (n = 3 for SOX2, EZH2, and TH and n = 4 for CDH1 and TUBB3, 10-15 brain organoids collected as one sample batch, Mat versus BEM p = 0.0061, NSC versus BEM p = 0.0027 for SOX2, Mat versus NSC p < 0.0001, NSC versus BEM p = 0.0371 for EZH2; Mat versus BEM p = 0.0004, Mat versus NSC p = 0.0039, NSC versus BEM p = 0.0001 for CDH1; Mat versus BEM p < 0.0001, NSC versus BEM p < 0.0001 for TUBB3; NSC versus BEM p = 0.0346 for TH, independent replicates = 3). **i** Immunohistochemical staining for mitotic radial glia marker phosphorylated vimentin (p-Vim), SOX2, Nestin, and PAX6, and neuron marker MAP2 in Mat and BEM organoids at day 30 (scale bars = 100 μm, independent replicates = 6). Note that some p-Vim+/SOX2+ cells are located above the apical domain (white arrowheads). **j** Immunohistochemical staining for Tuj1 and NeuN at day 30, CTIP2 and PAX6, and N-cadherin (N-Cad) at day 45 in Mat and BEM organoids (scale bars = 100 μm, independent replicates = 2-5). **k** Immunohistochemical staining images for deep-layer neuron markers MAP2 and CTIP2 at 45 and 75 days (scale bars = 50 μm, independent replicates = 1). **l** Immunostaining images for SOX2 at day 75 (scale bars = 500 μm, independent replicates = 1). **m** Nucleus-stained (Cyto16) light-sheet microscopic images of Mat and BEM organoids at day 75 (scale bars = 500 μm, independent replicates = 5). Quantification of **o** the thickness of ventricle-like structures (n = 30 for Mat and n = 45 for BEM, Mat versus BEM p = 0.0007) and **p** the total organoid volume (n = 6 for Mat and n = 9 for BEM, Mat versus BEM p = 0.0035) based on nucleus-stained (Cyto16) light-sheet microscopic images of Mat and BEM organoids at day 75 (independent replicates = 5). **q** 3D reconstructed images of 30-day BEM organoids obtained by tissue clearing and subsequent immunostaining for Tuj1 and MAP2 (scale bars = 1 mm, independent replicate = 1). **r** 3D images of Mat and BEM organoids stained for Tuj1 and MAP2 after tissue clearing at day 75 (scale bars = 200 μm, independent replicates = 3-5). Mat and BEM organoids were cultured in a dish on an orbital shaker. All data are expressed as mean ± standard deviation (SD). Statistical differences between the groups were determined with a two-sided t-test (*p < 0.05, **p < 0.01, ***p < 0.001). Source data are provided as a Source Data file.

**BEM increases the radial glial cell population and promotes cortical layer development in brain organoids.** Brain organoids grown in BEM hydrogel contain a larger radial glial cell (RGC) population along the ventricular zone (VZ) (Fig. 2i). RGCs represent a major progenitor pool during early development that can give rise to neurons and glia, and act as an axis to guide neuronal migration44,45. RGCs also play a key role in gyriﬁcation46. Immunostaining for radial glial markers phosphorylated vimentin (p-Vim), SOX2 and PAX6 at day 30 showed more densely packed cells in BEM organoids than in Mat organoids (Fig. 2i). The majority of p-Vim+ mitotic radial glial-like cells were located at the apical surface of the VZ in both Mat and BEM organoids. In BEM organoids, radial glial-like cells that extend basal cellular processes toward the outer surface were frequently observed, and some were located above the apical domain, suggesting basal (or outer) radial glia (Fig. 2i). Mat organoids had sparse populations of p-Vim+ RG-like cells (Fig. 2i). Furthermore, a larger number of PAX6+ radial glial progenitor populations were observed in BEM organoids than in Mat organoids (Fig. 2i). At day 45, BEM organoids displayed formation of more densely populated CTIP2+ deep-layer above PAX6+ VZ, compared to Mat organoids (Fig. 2j). This may be due to the presence of an organized layer of laminin-rich basement membrane in the BEM organoids (Fig. 2c), which supports cortical layer formation. Immunostaining of brain organoids cultured for 45 days showed that N-cadherin, which is involved in the radial migration of multipolar cells45 and maintaining the normal architecture of the neuroepithelial or RGCC46, was more highly expressed in BEM organoids than in Mat organoids (Fig. 2i).

To determine whether developing organoids in the BEM hydrogel could recapitulate the cortical spatial organization, the organoids were stained for cortical layer markers on days 45 and 75 in the culture. At day 45, the deep layer marked by TBR1+ (cortical layer V1) and CTIP2+ (cortical layer V) neurons started to express (Fig. 2k). A thicker layer of TBR1+ and CTIP2+ neurons was found in the BEM organoids than in the Mat organoids at both 45 and 75 days (Fig. 2k). Radial glia gave rise to intermediate progenitor cells (IPCs) that express TBR2 and move to the subventricular zone (SVZ)44,45,49. A well-organized layer containing a mixed population of TBR2+ IPCs and CTIP2+ neurons was reproducibly observed above VZ in the BEM organoids at day 75 (Fig. 2l). Similarly, a thicker layer containing CTIP2+ neurons and SATB2+ late-born neurons was observed in the BEM organoids at day 75 (Fig. 2m). At this stage, the BEM organoids contained different neuronal subtypes expressing SVZ, upper- and deep-layer markers that did not show distinguishably separate layers but given a distribution pattern of dorsal cortical subtype-specific neuronal populations that resembles in vivo regional sub-specification, BEM organoids could model the organization of the neocortex in vivo more closely than the Mat organoids. Bright-field images revealed more elongated neuroepithelial structures in the BEM organoids, which was rarely observed in the Mat organoids (Supplementary Fig. 7a). The BEM organoids displayed a more complex 3D architecture with larger cortical layers than the Mat organoids (Fig. 2n and Supplementary Video 1). The brain organoid culture in BEM hydrogel induced the significant enlargement of individual ventricle-like structure thickness as well as the overall volume of the organoid (Fig. 2o, p). The organization of cortical regions in the BEM organoids was further confirmed by tissue clearing using the CUBIC protocol30 and subsequent staining for neuronal markers (Tuj1, MAP2) (Fig. 2q, r, Supplementary Fig. 7b, c, and Supplementary Video 2). The elongated neuroepithelial structures were observed, while maintaining a high density of cells, and neurons were strongly polarized along their apicobasal axis (Fig. 2q and Supplementary Fig. 7b, c). BEM organoids contained much larger lobes than the Mat organoids (Fig. 2r).
Fig. 3 The genome-wide transcriptome analysis of 75-day brain organoids. (a) Heatmap showing the expression of differentially expressed genes (DEG) between Mat and BEM organoids (n = 3 per group, independent replicate = 1). (b, c) Heatmaps of Pearson’s correlation analysis of RNA-sequencing datasets of the Mat and BEM organoids for comparison with published transcriptome datasets of (b) three different human frontal regions across different stages and (c) five different regions of the brain at fetal and postnatal stages. Values in the heatmaps of Mat and BEM groups dictate Pearson’s correlation coefficients (PCC). Values in the heatmaps of BEM-Mat indicate the differences in PCC between BEM and Mat groups. (d) The 35 enriched gene ontology (GO) terms of upregulated genes in BEM organoids versus Mat organoids (shown in terms of p-values). Numbers on the right-hand side of the bar indicate the number of DEGs within the GO terms. (e) The gene set enrichment analysis (GSEA) of the astrocyte and oligodendrocyte probe sets in the BEM group versus the Mat group. Mat and BEM organoids were cultured in a dish on an orbital shaker. Source data are provided as a Source Data file.

brain organoid development and to compare the developmental level of BEM organoids with the human brain tissue, an RNA-sequencing analysis of global transcriptomes was conducted with three batches of BEM and Mat organoids at 75 days of culture (Fig. 3). Compared to Mat organoids, 1323 genes were downregulated, and 845 genes were upregulated in BEM organoids (Fig. 3a). We compared organoid transcriptional profiles with datasets of brain-specific regions of fetal and adult human brains; three different parts of the human frontal brain and five regions of the brain, including the cerebellar cortex, hippocampus, cerebellum, striatum, and mediodorsal nucleus of the thalamus. A Pearson correlation analysis of three regions of the frontal cortex revealed that both the brain organoid groups generally correlated more closely with the fetal brains (Fig. 3b). In all three regions, BEM organoids showed stronger correlations with human brains than the Mat organoids. Similarly, gene expression patterns related to five other brain regions in the organoids were more strongly correlated with those in the fetal brains, where BEM organoids also exhibited higher correlation coefficient values with human brains than the Mat organoids (Fig. 3c). These findings suggest that the organoids generally resembled identities of various human fetal brain regions in the gestational weeks, and BEM organoids showed a higher correlation with the human brain than the Mat organoids. Gene ontology (GO) performed to assess the differentially expressed genes between BEM and Mat organoids supported the effectiveness of BEM for brain organoid maturation. The GO terms involved in nervous system development, neurotransmitter secretion, axon, and synapse were significantly upregulated in BEM organoids (Fig. 3d). These results confirm our earlier observations by immunostaining and qPCR that BEM promoted brain development and differentiation of neuronal populations. Gene set enrichment analysis (GSEA) revealed the significantly increased expression of gene sets for astrocyte and oligodendrocyte, both of which start to appear at a later stage during brain organogenesis, in BEM organoids compared to that in Mat organoids (Fig. 3e). Interestingly, differentially expressed genes in developing BEM organoids significantly overlapped with known risk genes related to schizophrenia, autism, epilepsy, and stroke (Supplementary Fig. 8). Therefore, brain organoids generated with BEM might be useful for disease modeling and disease mechanism studies during human brain development.

Importantly, BEM organoids exhibited improved electrophysiological functionality and neurotransmitter response over Mat organoids. The level of calcium channel activation was examined in response to glutamate-induced depolarization using a calcium influx indicator Fluo-4 AM at day 45 (Supplementary Fig. 9a, b). Upon exposure to glutamate, greater calcium changes were detected in BEM organoids than those in Mat organoids (Supplementary Fig. 9a, b). The quantification of the influx intensity indicated more noticeable changes in the cytoplasmic calcium influx in individual cell populations of BEM organoids (Supplementary Fig. 9c). The percentage of glutamate-responsive cells was also much higher in BEM organoids (Supplementary Fig. 9c), indicating the existence of electrically more functional neural cell population in the cerebral organoids grown in the BEM hydrogel. Immunohistochemical analysis for vesicular glutamate transporter 1 (VGLUT1), an excitatory neuronal marker, (Supplementary Fig. 9d) indicated that BEM organoids
at 45 days contained VGLUT1+ mature excitatory neurons that are known to emerge during the second trimester of the neonatal brain51. In contrast, the Mat organoids contained only a few VGLUT1+ cells at this stage. To assess the electrophysiological properties of cells in brain organoids, a whole-cell patch analysis was performed (Supplementary Fig. 9e, f). A voltage-dependent tetrodotoxin (TTX)-sensitive sodium current was detected and action potential (AP) was also evoked with current injections. A physiologically functional recording was barely observed in the Mat organoids at the same time point. Specific cell populations in BEM organoids responded to γ-aminobutyric acid (GABA) treatment (Supplementary Fig. 9g)53. Glutamate decarboxylase 1 (GAD1)+ GABAergic neurons that produce GABA for inhibitory neuronal activity were detected in BEM organoids (Supplementary Fig. 9h). Therefore, our findings demonstrate that BEM organoids mimic some features of the later developmental stages of the human brain with more functional neuronal properties than the control organoids grown in Matrigel. Overall, the exposure of organoids with neuroectoderm identity to the complex networks of BEM at an early developmental stage triggered NP expansion, cortical layer organization, and enhanced neurogenesis, leading to the structural maturation and functional improvement of organoids.

The tissue-specific effects of BEM on brain organoid generation. BEM can provide complex networks of brain-specific ECM optimal for brain organoid development. The effects of ECM on lineage specification and organoid development from stem cells could be tissue-specific. Our previous studies have reported that the tissue-specific microenvironments provided by a decellularized tissue matrix improved the survival, differentiation, and function of various types of stem cells, reprogrammed cells, and primary cells52–56. The biochemical compositions of ECM hydrogel vary depending on the tissue source from which the ECM is isolated. Therefore, the tissue-specific effects of BEM for supporting the development of brain organoids were investigated by comparing the brain organoid supporting the ability of BEM-supplemented hydrogel with that of Matrigel incorporated with ECM derived from other types of tissues, including intestine (IEM), liver (LEM), and heart (HEM) (Fig. 4). At day 30, the size of organoids generated in Mat and other tissue-derived ECM hydrogels was similar (Mat; 1385 ± 255.7 μm, IEM; 1241 ± 319.5 μm, LEM; 1130 ± 327.4 μm, HEM; 1355 ± 431.7 μm), whereas the brain organoids cultured in the BEM hydrogel were larger in diameter (BEM; 1723 ± 239.4 μm) than the control groups (Fig. 4a, c). In addition, BEM facilitated the formation of larger neuroepithelial structures and increased Tuj1+ neuronal populations (Fig. 4a, b). Next, the gene expression profiles of the brain organoids cultured in various matrices at day 30 were compared (Fig. 4d). The expression of repressor element 1 silencing transcription factor (REST) and DNMT3B—which are known to suppress neuronal differentiation—was the lowest in BEM organoids among the tested groups. When compared with the organoids in Mat and other ECM control groups, the expression of neuronal markers TUBB3 and MAP2 was significantly upregulated in the BEM group, demonstrating the neuronal differentiation accelerated by the BEM signals. Overall, these results prove the critical role and necessity for tissue-specific ECM in organoid development.

Microfluidic BEM conditions improve the survival of brain organoids. A low level of fluid flow existing in the cerebrospinal and interstitial spaces of the brain has been found to play an essential role in supporting the development of ventricle-like structures and the formation of the neuronal layers in the cerebral cortex57. Cerebrospinal fluid circulation induces pulsatile and bi-directional flow exchange at the blood barrier and the borders between cerebrospinal fluid and interstitial fluid spaces58,59. Therefore, we hypothesized that the presence of fluid flow that mimics the bi-directional cerebrospinal fluid would be able to amplify the effects of BEM for brain organoid development by facilitating molecular diffusion in and out of the organoids, leading to further improvement of neuronal differentiation and ventricle-like structure formation in developing organoids. Based on these considerations, a multi-layered chamber microfluidic device was developed that can generate precisely controlled fluid flow (Fig. 5a and Supplementary Fig. 2a, b). The device consists of three layers with five chambers that are fluidically connected by microchannels, enabling continuous fluid perfusion between the chambers without the need for external tubing and pumping. Two chambers are designed for brain organoid cultures and three chambers for medium reservoirs. Fluid flow through the microchannels connecting the chambers could be generated by hydrostatic pressure created by different medium levels in the chambers (Supplementary Fig. 2c), which was simply achieved by placing the device on a bi-directional laboratory rocker. Simulations preceding the design of the device (Supplementary Table 5) confirmed that our device could mediate the effective transfer of nutrients, such as glucose, into large organoids embedded in hydrogels (Supplementary Fig. 10). Four different designs of the device were simulated: (1) z1-channel model, (2) z3-channel model, (3) y5-channel model, and (4) y5-z3-channel model comprising a combination of z3- and y5-channel designs. Based on the simulation results of glucose transfer under gravity-driven medium flow, the y5-z3-channel model was adopted as a final device design for brain organoid culture, which shows more uniform glucose gradient profiles in the vertical axis (Supplementary Fig. 10a) and greater glucose transfer to the organoids (Supplementary Fig. 10b). As our approach does not require complicated settings with pumps and tubing, a large number of devices could be operated in parallel using a single rocker system. Thus, large-scale organoid cultures and high-throughput assays and screening are possible with our device under microfluidic conditions.

To check whether our device platform can improve molecular diffusion into organoids, after stabilization of brain organoids in BEM hydrogel for four days, the organoids embedded in BEM hydrogel were transferred into a 24-well plate (BEM-plate) or microfluidic device (BEM-device) on the laboratory rocker (Fig. 1a). The computational simulation of glucose diffusion through the brain organoids in the device predicted that at a given fluid flow, the glucose present in the medium is actively transferred to the brain organoids, which equilibrated with the glucose concentration in the medium within 60 min of dynamic culture (Fig. 5b). However, a low level of glucose was diffused into the organoids in the well plate under static conditions without medium flow (Supplementary Fig. 11a). Next, intra-organoid oxygen levels were visualized on day 30 by incorporating oxygen-sensing phosphor nanoparticles composed of Pt(II) meso-tetra(pentafluorophenyl)porphine (PtTFPP)-poly(urethane acrylate nonionomer) (PUAN)60. Higher oxygen levels lead to lower phosphorescence of the nanoparticles, which is attributed to collisional quenching between oxygen molecules and PtTFPP (Supplementary Fig. 11b). When the oxygen-sensing nanoparticles were incorporated in the BEM-plate and BEM-device organoids on day 30, the normalized mean phosphorescence intensity of the BEM-plate organoids was more than two-fold higher than that of the BEM-device organoids (Fig. 5c, d). This significant difference was more pronounced in the core region of the organoids. Therefore, our results indicate that the BEM-device conditions allowed for significantly higher and more
uniform intra-organoid oxygen levels. This observation together with glucose diffusion simulation demonstrates that the precise control of the medium flow at a microscale level may be more effective for oxygen/nutrient supply and molecular exchange in organoids than irregular dynamic flow under bulk scale conditions.

Consequently, BEM-device organoids exhibited higher cell proliferation and less cell death, and a notably larger size than BEM-plate organoids (Fig. 5e–j, and Supplementary Fig. 12a–c). Proliferative cells mainly undergo anaerobic glycolysis converting glucose into lactate, whereas postmitotic neurons undergo a switch towards mitochondrial metabolism.11. Quantification of metabolite glucose and lactate at day 30 revealed that BEM organoids cultured in the device consumed more glucose and secreted more lactate, compared to those ones cultured in the plates, indicating that hypoxia in the whole organoid at day 30 (Supplementary Fig. 12c). Interestingly, the presence of BEM is likely to prevent the formation of necrotic clusters that were seen in Mat organoids (Supplementary Fig. 12d, e). To confirm that the microfluidic device allows better oxygen supply to the core region of the organoids, immunohistochemical staining for a hypoxia marker hypoxia-inducible factor-1α (HIF-1α) was performed at day 120 (Supplementary Fig. 12f, g). HIF-1α-positive area in the BEM-device organoids was significantly smaller than that of Mat and BEM organoids cultured in the plates, indicating that hypoxia in the organoid core was alleviated by microfluidic culture. These results support again that the microfluidic device facilitates oxygen transfer to the core of large organoids, resulting in the prevention of necrotic core formation via improved cell survival and decreased apoptosis. BEM-device organoids contained a larger number of FOXG1+ lobes than BEM-plate organoids (Supplementary Fig. 12h). The periodic application of bi-directional medium flow in the microfluidic device contributed to improving the overall quality of the brain organoids. When we examined the expression of a choroid plexus (CP) epithelial cell marker transthyretin (TTR) in Mat, BEM, and BEM-device organoids, all groups were found to contain similar TTR+ area (Supplementary Fig. 12i, j), suggesting that BEM did not affect CP development.
Importantly, the use of a microfluidic device reduced the variability in the brain organoids grown in the 3D BEM. The gene expression profiles of organoids in the BEM-plate and BEM-device conditions were analyzed using a single organoid for each group (Fig. 5k). The relative expression of key neuronal markers outside the brain organoids grown in BEM hydrogel. The relative expression of neuronal markers, device conditions were analyzed using a single organoid for each group. Computational simulation of glucose concentration within the brain organoid and its surroundings in a culture chamber under the fluid flow conditions (left). Comparison of glucose concentration within the brain organoid in the presence and absence of fluid flow (right). CO Representative merged images showing organoids (gray) and phosphorescence of oxygen-sensing (PtTFPP-PUAN) nanoparticles at 754 nm (red) for BEM-plate (top) and BEM-device (bottom) organoids on day 30 (scale bars = 200 μm, independent replicates = 4). CO The normalized mean phosphorescence intensity in BEM-plate and BEM-device organoids (n = 3 per group, BEM-plate versus BEM-device p = 0.032, independent replicates = 4). Measurement of glucose level in organoids (e) and lactate level in the medium at days 30 and 45 (n = 4 for D30 and n = 5 for D45, BEM-plate versus BEM-device p < 0.0001 at D30 and p < 0.0001 at D45, independent replicates = 2 for day 30 and 1 for day 45). CO Immuno-staining for the proliferation marker Ki67 and the progenitor marker Nestin in the BEM-plate and BEM-device organoids (scale bars = 50 μm, independent replicates = 3). CO The quantification analyses of Ki67+ and Nestin+ cells in the BEM-plate and BEM-device organoids (n = 10 for BEM-plate group, and n = 4 for Ki67+ and n = 7 for Nestin+ in BEM-device group, BEM-plate versus BEM-device p = 0.0004 for Ki67 and p = 0.005 for Nestin, independent replicates = 3). CO Brain organoids stained with ethidium homodimer-1 (EthD-1) to label dead cells at day 30 and cleaved caspase-3 (cCasp3) at day 45 (scale bars = 500 μm). CO Quantification of EthD-1+ and cCasp3+ area per organoid (n = 4 for BEM-plate group, n = 4 for EthD-1+ and n = 3 for cCasp3+ in BEM-device group, BEM-plate versus BEM-device p = 0.0056 for EthD-1 and p = 0.049 for cCasp3, independent replicates = 3). CO Differential gene expression analyses by qPCR with BEM-plate and BEM-device organoids. One sample was prepared from a single organoid. The coefficient of variations is dictated above the bars for each group. Data are expressed as violin plots. Dark gray dashed lines and black lines indicate 25%–75% quartiles and median, respectively (n = 19 for BEM-plate group and n = 30 for BEM-device group in all markers except for OLIG1, n = 29 for BEM-plate group and n = 35 for BEM-device group in OLIG1, BEM-plate versus BEM-device p = 0.0102 for NES, p < 0.0001 for TUBB3, p = 0.0001 for OLIG1, p < 0.0001 for BCL2, and p < 0.0001 for BAX, independent replicates = 4). All analyses were performed over 30 days in the culture. All data are expressed as mean ± SD, otherwise stated separately. Statistical differences between the groups were determined by unpaired two-tailed t-test (*p < 0.05, **p < 0.01, ***p < 0.001 versus BEM-plate group). Source data are provided as a Source Data file.
Taken together, these results demonstrate that the microfluidic organoid culture platform increased neuroepithelial outgrowth and overall organoid size by promoting cell proliferation and reducing apoptosis and necrotic cluster. In addition, the microfluidic BEM condition might reduce the variation between organoid samples in terms of gene expression profiles, thereby improving the homogeneity of brain organoids that is critically important for precise evaluation and data interpretation during drug testing and disease modeling.

The **microfluidic BEM platform enhances corticogenesis and radial glial cell generation in brain organoids.** Enhancement of organoid growth and cortical structure formation by microfluidic BEM culture was assessed by 3D light-sheet microscopy and subsequent morphological analysis (Fig. 6a–c). Microfluidic culture improved oxygen supply to the interior of the organoids that leads to increased proliferation, expanded NP population, and thicker neuroepithelium. Accordingly, the microfluidic BEM device induced significantly faster growth and development of more complex surface structure in brain organoids than the control culture conditions (Mat-plate, BEM-plate) (Fig. 6a, b). The overall volume of organoids in Mat-plate, BEM-plate, and BEM-device groups was $0.14 \pm 0.02 \text{mm}^3$, $0.31 \pm 0.14 \text{mm}^3$, and $0.56 \pm 0.17 \text{mm}^3$, respectively, indicating a marked difference between the groups (Fig. 6c). The degree of sphericity was $0.57 \pm 0.03$, $0.47 \pm 0.14$, and $0.35 \pm 0.11$ in Mat-plate, BEM-plate, and BEM-device organoids, respectively (Fig. 6d). Mat organoids were found to be morphologically smoother and smaller relative to the BEM organoids (Fig. 6a–d). The sectioned, nuclei-stained images obtained by confocal microscopy also showed larger and more elongated lobes in the BEM organoids compared to that in the Mat organoids (Supplementary Fig. 14a). These results suggest that BEM promoted volume expansion, structural maturation, and the formation of the elongated epithelium in organoids, and microfluidic devices magnified these effects of BEM by providing favorable dynamic microenvironments for the brain organoid cultures. Overall, BEM combined with a microfluidic system facilitated organoid growth and induced a high level of cellular accumulation, leading to the formation of highly complex and more advanced structural developments.$^{62}$

The efficient supply and exchange of nutrients/oxygen by well-controlled bi-directional fluid flow promoted NP proliferation with the expansion of radial glia during the early stage of brain organogenesis. In BEM-device organoids, the increase in proliferation coincided with an expansion of the NP pool, as shown by immunostaining for radial glia markers p-Vim, SOX2, and PAX6 (Fig. 6e and Supplementary Fig. 14b, c). The larger number of p-Vim$^+$ mitotic RGCs was detected in the BEM-device organoids than that in BEM-plate organoids (Fig. 6e and Supplementary Fig. 14d). At 30 days, some of the p-Vim$^+$ and PAX6$^+$ NPs showing a typical radial glial morphology with processes contacting both the apical and basal surfaces of the neuroepithelium were observed in the BEM-device organoids (Supplementary Fig. 14c). Cortical layer formation in vivo depends upon the proper migration of postmitotic neurons from their sites of origin to their final destinations, and neuronal migration is precisely orchestrated by various intrinsic and extrinsic factors.$^{63,64}$ The presence of Cajal-Retzius cells, which are involved in neuronal migration and cortical lamination$^{64,65}$, was examined by immunostaining for Reelin in the BEM-device organoids at day 30 (Fig. 6f). The sectioned organoid images stained for Reelin revealed a wide distribution of Reelin$^+$ signals in the most superficial regions (Fig. 6f, left panel). The 3D imaging analysis indicated more dispersed signals of Reelin as a secreted factor (Fig. 6f, right panel). Therefore, the enrichment of the cell types and their roles supporting cortical layer formation in BEM-device organoids was speculated.

As a result, the microfluidic BEM system significantly promoted corticogenesis in developing brain organoids. The expression of a deep-layer marker TBR1 in organoids at day 30 was examined by confocal microscopy (Fig. 6g–i). Compared to Mat organoids, the BEM organoids contained an increased TBR1$^+$ cell population (Fig. 6g, h). TBR1 expression with a broader spectrum of the z-axis along the cortical layer was detected in the BEM-device organoids (Fig. 6i) and Supplementary Fig. 15a, b). BEM-device organoids formed a thicker deep layer containing CTIP2$^+$ and TBR1$^+$ neurons at day 45 (Fig. 6i). In addition, a thicker layer of TBR2$^+$ cells with a highly packed band of cells in the cortical region of the BEM-device organoids was also observed at day 45 (Fig. 6k), indicating that the formation of the SVZ layer with a high density of cells was promoted in brain organoids cultured under BEM-device conditions. Chondroitin sulfate proteoglycan (CSPG)$^+$ layer indicating preplate splitting was observed in BEM-device organoids (Supplementary Fig. 15c). BEM and BEM-device organoids displayed a mixed population of deep-layer marker CTIP2$^+$ neurons and upper-layer marker SATB2$^+$ neurons 75 days of culture (Fig. 6l). Compared to BEM organoids, a substantially thicker layer of SATB2$^+$ neurons was formed in the BEM-device organoids (Fig. 6l). Both BEM and BEM-device organoids contained SOX2$^+$ and HOPX$^+$ basal radial glia-like populations at day 75. A SOX2$^+$/HOPX$^+$ outer SVZ structure separated from SOX2$^+$/HOPX$^+$ VZ was more frequently observed in the BEM-device organoids (Fig. 6m).

BEM-device organoids innately developed a higher population of ionized calcium-binding adaptor molecule 1 (IBA1)$^+$ and CD68$^+$ microglia (Supplementary Fig. 16a, b). IBA1$^+$ microglia were initially found sparse in small clusters at day 30, but IBA1$^+$ and CD68$^+$ cells were present throughout the BEM-device organoids at day 74. Quantification of microglial population in Mat, BEM, and BEM-device organoids at day 74 indicated the highest numbers of IBA1$^+$ and CD68$^+$ microglia in the BEM-device organoids (Supplementary Fig. 16c, d). By day 60, the BEM-device organoids showed a higher density of Tuj1 and mature neuronal marker VGLUT1 or presynaptic marker Synapsin I (SYNI) co-positive cell populations than BEM-plate organoids (Fig. 6n, o). At this time point, only BEM-device organoids expressed a mature synaptic marker, postsynaptic density protein 95 (PSD95) (Supplementary Fig. 16e). In addition, glial fibrillary acidic protein (GFAP)$^+$ astrocytes, which are detected in the late stages of brain organogenesis, were more abundantly present in BEM-device organoids (Fig. 6p). The combination of BEM with a microfluidic system facilitated the maturation of neural populations in brain organoids even without exogenous neurotrophic factors in a relatively short period. Overall, bioengineering organoid culture with brain ECM- and microfluidic cues increased populations of proliferative progenitors and Reelin-secreting Cajal-Retzius cells, resulting in a robust organoid growth and accelerated cortical layer organization along the radial axis that contains mature neurons and glial cells. This contributes to the formation of elongated and continuous cortical layers and increases the complexity of the structures.

The further maturation of brain organoids at the molecular and functional levels by micro-controlled fluid flow. The microfluidic BEM system facilitated the further maturation of brain organoids in terms of transcriptome profiles and electrophysiological properties. To investigate the effects of microscale dynamic flow on the transcriptome profiles in brain organoids, RNA-sequencing was performed with three batches of BEM-plate
Fig. 6 Bioengineering of the brain organoids by the microfluidic BEM system improves radial glial generation and cortical organization. a Light-sheet microscopic bright-field images of 3D brain organoids encapsulated in Mat and BEM cultured in a plate or microfluidic device at 60 days of culture (scale bar = 500 μm, independent replicates = 3). b Reconstructed light-sheet microscopic images of 60-day Mat-plate, BEM-plate, and BEM-device organoids (scale bar = 500 μm, independent replicates = 3). c Quantification analyses for the 3D organoid volume (n = 3, 9, and 13 for Mat-plate, BEM-plate, and BEM-device groups, respectively, Mat-plate versus BEM-device p = 0.0007, BEM-plate versus BEM-device p = 0.0014, independent replicates = 3), and (d) sphericity (n = 3 per group, independent replicates = 3) of brain organoids using IMARIS software. e Representative immunostaining images for mitotic radial glia marker phosphorylated vimentin (p-Vim) and SOX2 in the BEM-plate and BEM-device organoids at day 30 (scale bar = 50 μm, independent replicates = 5). f Expression of the radial glial marker PAX6 and extracellular glycoprotein marker Reelin in BEM-device organoids (left panel, scale bars = 200 μm, independent replicates = 2) and 3D imaging of Reelin expression in BEM-device organoids at day 30 (right panel, scale bars = 200 μm, technical replicates = 5). g 3D plotting and (h) quantification of deep-layer marker TBR1-expressing cells in Mat-plate, BEM-plate, and BEM-device organoids at day 30 using IMARIS software (n = 3 for Mat-plate and BEM-device groups, and n = 4 for BEM-device group, independent replicates = 3). i 3D plotting analysis of TBR1+ cells in BEM-device organoids on different z-positions in the radiometric color spectrum (scale bar = 200 μm, biological replicates = 3). j Immunohistochemically stained sections for TBR1 and CTIP2 at day 45 (scale bar = 200 μm, independent replicate = 1). k Immunohistochemical staining images for subventricular marker TBR2 and PAX6 at day 45 (scale bar = 50 μm, independent replicates = 2). Pink color in the pie-charts indicates the number of cortical structures with separated layers of TBR2+ and PAX6+ cells, and grey color indicates no layer preferences. l Immunostaining images showing CTIP2 and superficial-layer neuron marker SATB2 (scale bars = 50 μm, independent replicate = 1), and (m) basal radial glia marker SOX2 and HOPX at day 75 (scale bars = 50 μm, independent replicate = 1). n Immunostaining of Tuj1 and glutamatergic neuron marker VGLUT1 (independent replicates = 2). o presynaptic marker SYNI (independent replicates = 2), and (p) astrocyte marker GFAP in the BEM-plate and BEM-device organoids at day 60 (scale bars = 100 μm, independent replicates = 3). All data are presented as mean ± SD. Statistical differences between the groups were determined with unpaired two-tailed t-test (**p < 0.01, ***p < 0.001). Source data are provided as a Source Data file.

and BEM-device organoid samples at day 75 (Fig. 7a–c and Supplementary Fig. 17). A heatmap of the differential expression analysis showed that 214 genes were upregulated and 802 genes were downregulated in BEM-device organoids compared with that in the BEM-plate organoids (Supplementary Fig. 17a). Pearson’s correlation coefficient values between samples in the same group and principal component analysis (PCA) demonstrated that there was less variability between the samples in the
BEM-device group than in those in the BEM-plate group (Fig. 7a). GO analysis identified the enrichment of neuronal differentiation, axonal guidance, and neuronal migration among upregulated genes in the BEM-device group compared to the BEM-plate group (Fig. 7b). Especially, the expression of genes involved in mediating the cellular response to hypoxia and cell proliferation increased (Fig. 7c). GSEA analysis showed that genes involved in apoptosis were enriched in the BEM-plate group (Fig. 7d). A few cells in the BEM-device organoids exhibited spontaneous spikes of Ca2+ influx, even in the absence of any neurotransmitters (Fig. 7e and Supplementary Video 4). An electrophysiological study using whole-cell patch clamping demonstrated that recorded neurons in both organoid groups showed Na+ currents in response to voltage steps (Fig. 7f). The average amplitudes of Na+ currents in response to voltage ramps between −30 mV and 20 mV were much higher for neurons in BEM-device organoids than those in BEM-plate organoids (Fig. 7g). Neurons in both organoid groups were capable of eliciting APs in response to depolarizing current injection, which are similar to those of mature functional neurons (Fig. 7f). Neurons in BEM-device organoids were able to produce APs at a

**Fig. 7** The transcriptome profile analyses and electrophysiological function characterization of brain organoids cultured in the microfluidic BEM system. a Pearson’s correlation matrix for whole-genome profiles in BEM-plate and BEM-device organoids (n = 3, independent replicate = 1). Pearson’s correlation coefficient (PCC) values are indicated in each box. b The top 14 enriched gene ontology (GO) terms of upregulated genes in the BEM-device group versus the BEM-plate group (shown in terms of p values). c Lists of differentially upregulated genes classified in two GO functional categories; ‘Cellular response to hypoxia’ and ‘Cell proliferation’. d Ratio metric images of calcium imaging before and after 100 μM glutamate treatment in Fluo-4 AM-loaded BEM-plate and BEM-device organoids (scale bars = 50 μm, independent replicates = 4). The color scale indicates fluorescence intensity of Fluo-4 AM. e A fluorescence image showing spontaneous calcium (Ca2+) transient of cells in BEM-device organoids (left) (scale bar = 100 μm, independent replicates = 3). Representative time-course peaks showing spontaneous changes in Fluo-4 AM fluorescence intensity measured in cells in the BEM-device organoid (right). Traces were obtained from the region of intensity (ROI) marked on the fluorescence image. f Representative current traces recorded in a neuron with a voltage-clamp mode (left two panels) and representative traces of evoked action potentials (APs) recorded in a neuron with a current-clamp mode (right two panels) in brain organoids grown in either a BEM-plate (top panels) or a BEM-device (bottom panels). g Quantification of sodium (Na+) currents in response to increased voltage steps starting from −30 mV to +20 mV (10 mV step size) in neurons within brain organoids grown in either a BEM-plate or a BEM-device (n = 5). h Quantification of AP incidence (BEM-plate: n = 27; BEM-device: n = 39, independent replicates = 7), spike numbers (single versus multi) in each condition, and threshold potentials to evoke AP (BEM-plate: n = 19; BEM-device: n = 33, unpaired two-tailed t-test (*p < 0.05), BEM-plate versus BEM-device p = 0.0380). Brain organoids cultured for (a-e) 75 days and (f-h) 60 days were analyzed. All data are expressed as mean ± SD. Data source are provided as a Source Data file.
higher rate, and higher percentages of these cells fired multispikes of APs compared to those in BEM-plate organoids (Fig. 7h). The threshold levels of APs were also increased in BEM-device organoids (Fig. 7h). Importantly, a postsynaptic current (PSC) was detected only in the BEM-device organoids, even though this was rare, indicating the formation of a postsynaptic compartment and the possible establishment of synaptic networks (Supplementary Fig. 18). Our results support that the organoid culture in microfluidic BEM condition could improve the electrophysiological functional properties of brain organoids. Overall, a precise perfusion culture of human cerebral organoids in our customized bioreactor based on a microfluidic device platform facilitated the efficient exchange of nutrients, oxygen, and waste, leading to volumetric expansion with complex structures, and further improvement in corticogenesis and electrophysiological functionalities of individual neuronal populations.

**Reproducible improvement in brain organoid development by microfluidic BEM platforms.** We examined whether microfluidic BEM culture supports reproducible generation of brain organoids exhibiting improved neurogenesis and structural maturation. To this end, neurogenesis of BEM-device organoids was directly compared with that of BEM organoids (in petri dish) cultured using the standard conventional orbital shaker method. At day 30, BEM organoids cultured in dish on the orbital shaker and BEM-device organoids had approximately 1.2 times higher number of SOX2+ NPCs than Mat organoids in dish on the orbital shaker (Fig. 8a, b). At day 45, BEM-device organoids contained substantially higher number of NeuN+ neurons compared to organoids in other two groups cultured with an orbital shaker (Fig. 8a, c). BEM-device organoids also displayed significantly larger PAX6+ ventricle-like structures than orbital shaker-cultured organoids (Fig. 8a, d). At day 60, the presence of the thickest layer of CTIP2+ deep-layer neurons and the largest area of VGLUT1+ excitatory neurons were observed in the BEM-device organoids (Fig. 8a, e, f). We reproducibly observed that the use of microfluidic device reduces the variation in the size of brain organoids derived from iPSCs with different passage numbers as indicated by the lowest coefficient of variation in the BEM-device organoids (Supplementary Fig. 19).

In addition, the effects of BEM and microfluidic device were further confirmed by generating organoids using another iPSC line (KYOU-DXR0109B, ATCC) (Supplementary Fig. 20). When BEM-device organoids were directly compared with Mat and BEM organoids cultured on an orbital shaker, similar features of BEM-device organoids, including faster growth, substantially longer diameter, and increased neuronal marker expression, were reproducibly observed with the new iPSC line. Combination of BEM and microfluidics enhanced neurogenesis during the development of brain organoids derived from new cell line, as indicated by more densely packed SOX2+ NPCs at the apical side at day 30 (Supplementary Fig. 20a), and thicker CTIP2+ deep layer and SATB2+ superficial-layer neurons observed at day 60 (Supplementary Fig. 20b). SATB2+ neurons were barely observed in Mat and BEM organoids at this time point (Supplementary Fig. 20b). BEM-device organoids from the new iPSC line at day 60 were also notably larger than Mat and BEM organoids without microfluidic culture, while showing the reduced variation in the organoid size (Supplementary Fig. 20c). The expression of several neuronal markers was significantly elevated in the BEM-device organoids compared to Mat and BEM organoids at day 60 (Supplementary Fig. 20d). These data validate the reproducibility of our culture system based on BEM and microfluidics for reliable production of human brain organoids, irrespective of iPSC lines.

Specialized basal radial glia (bRG) in the SVZ is an important hallmark of human cortical development. To analyze the distribution of cortical multi-layer neuronal subtypes in SVZ layer development, we conducted the immunostaining for several markers (TBR2, SOX2, HOPX) in the Mat, BEM, and BEM-device organoids 100 days of the culture (Fig. 8g, h). We observed outer SVZ-like structures containing HOPX+ bRG-like cells (Fig. 8g). The number of SOX2+ HOPX+ bRG-like cells substantially increased in the BEM and BEM-device organoids in comparison to Mat organoids (Fig. 8g, k). The highest populations of SOX2+ HOPX+ bRG-like cells were found in the BEM-device organoids. In the BEM-device organoids, we also observed SOX2+ HOPX+ region clearly separated from the SOX2+ HOPX+ VZ region (Fig. 8g). A thin SOX2+ HOPX+ layer appeared between these two regions, indicating an inner SVZ-like region (Fig. 8g). At day 100, Mat organoids contained a layer of a mixed population of TBR2+ cells and CTIP2+ neurons showing no layer preferences (Fig. 8h). In contrast, BEM and BEM-device organoids displayed CTIP2+ cortical plate-like layer that was formed above TBR2+ SVZ-like layer (Fig. 8h). The highest number of TBR2+ IPCs was observed in the BEM-device organoids among all groups (Fig. 8h, l). The CTIP2+ layers were significantly thicker in BEM and BEM-device organoids than in Mat organoids (Fig. 8m). These results demonstrate that BEM enhances the number of bRG-like cells in the SVZ layer and the microfluidic device improves the formation of multi-layer progenitor zones.

Co-staining of the brain organoids for upper-layer marker SATB2 and deep-layer marker CTIP2+ on day 100 showed that all groups contained partially separated layers of early born CTIP2+ neurons and late-born SATB2+ neurons (Fig. 8i), suggesting a layer specification of deep and upper layers. Thicker CTIP2+ and SATB2+ layers were found in the BEM and BEM-device organoids compared to Mat organoids (Fig. 8i, m, n). In the BEM-device organoids, the thickness of the superficial layer was more consistent and the distribution of SATB2+ neurons was localized more superficially to the CTIP2+ neurons by day 120 (Fig. 8i). Accordingly, this two-layer separation became more distinct in BEM-device organoid by day 120 (Fig. 8i). These data support that the culture of brain organoids using BEM and microfluidic device facilitates the development of upper-layer neurons as well as deep-layer neurons.

**The feasibility of porcine BEM for culturing human brain organoids.** We explored the feasibility of porcine brain tissue-derived BEM (pBEM) for brain organoid culture. Human sources may be more desirable for human brain organoids but due to a limited availability, application of BEM derived from porcine brain tissues, which can be prepared from the same donor conditions (e.g. brain regions, donor ages, etc.), may be a more preferred option in terms of standardization (Supplementary Fig. 21a–h). Proteomic analysis of cortex-derived pBEM confirmed similar numbers and profiles of matrisomal contents to those of human BEM (Supplementary Table 2). pBEM was found to have higher content of glycoproteins and less proteoglycans compared to human BEM, but the overall profiles of matrisome compositions were similar (Supplementary Fig. 21c–h). pBEM contained 457 brain tissue-enriched proteins, and GO analysis revealed that these proteins are involved in synapse signaling, nervous system development, and neurogenesis (Supplementary Fig. 21b). Brain organoid culture using pBEM and microfluidics showed similar improvement as seen in human BEM-based culture (Supplementary Fig. 22). Combination of pBEM and microfluidics promoted organoid growth, and increased the populations of PAX6+ progenitors, CTIP2+ and NeuN+ neurons at 60 days of culture (Supplementary Fig. 22a–f). Gene expression...
analyses by qPCR indicated higher neuronal gene expression (PAX6, TUBB3, and MAP2) in pBEM and pBEM-device organoids compared to Mat organoids (Supplementary Fig. 22g). These results may suggest that pBEM is comparable to human BEM.

We also examined (1) batch variation (different pBEM batches derived from the same donor), (2) sample variation (pBEM samples derived from different donors), and (3) region variation (pBEM derived from different brain regions). The content and composition of matrisome proteins in pBEM derived from the
cortex of the same donor were similar between different preparation batches of pBEM (porcine A cortex #1 versus porcine A cortex #2) (Supplementary Fig. 21c, d). In addition, the top 10 matrisome proteins with the highest expression completely overlapped between different batches of pBEM (red highlighted) (Supplementary Fig. 21e). The variability of pBEM derived from porcine brain cortical tissues of different donors was then investigated (porcine A cortex versus porcine B cortex). The content and composition of matrisome proteins were similar, and 9 out of the top 10 proteins with the highest expression were matched (Supplementary Fig. 21f–h). When the ECM profiles of pBEM derived from different brain regions were compared (cortex versus cerebellum), the content and composition of matrisome proteins in pBEM prepared from cerebellum were different from those in pBEM derived from cortex (Supplementary Fig. 21i, j). Cerebellum-derived pBEM contained higher content of matrisome proteins (~18%) than cortex-derived pBEM (~8%). However, both samples shared 8 out of 10 proteins in the top 10 proteins with the highest expression (Supplementary Fig. 21k). A heatmap of Spearman’s rank correlation coefficients and PCA plot of all samples confirm higher degree of similarity in the pBEM derived from the same brain regions, regardless different batches or donor samples. (Supplementary Fig. 21l, m). There was less similarity between pBEM derived from cortex and cerebellum. Volcano plots of matrisome proteins identified in cortex pBEM versus cerebellum pBEM also demonstrated the substantial numbers of differentially expressed proteins (52 out of 108 matrisome proteins differentially expressed by ≥ 2 folds with p < 0.05) in both types of pBEM samples (Supplementary Fig. 21n). When we compared cerebral brain organoids cultured in porcine cortex- and cerebellum-derived pBEM hydrogels, interestingly they showed differential effects on the development of FOXG1+ forebrain regions. Brain organoids grown in cerebellum pBEM displayed smaller FOXG1+ area than organoids cultured in cortex pBEM, which was comparable to Mat organoids (Supplementary Fig. 23a, b), indicating that cerebellum ECM is not effective for forebrain region development. Therefore, BEM derived from different brain regions may affect the compositions of developing brain regions in the cerebral organoids.

Overall, proteomic analysis demonstrates that the ECM profile variation arising from different brain regions exists, but the batch-to-batch variation from BEM preparation and different donor samples is relatively low in the case of BEM derived from the same brain region. Given that porcine tissues with varied ages are available and also not strictly limited to specific brain regions, pBEM derived from porcine brain tissue may be able to provide a reliable source for standardization and practical applications in brain organoid culture.

Combination of microfluidic BEM culture platform with other advanced brain organoid protocols. Finally, we tested a combination of microfluidic BEM platform with other advanced brain organoid protocols. Previously, the original cerebral organoid protocol could be improved by using poly(lactide-co-glycolide) (PLGA) fiber scaffolds and treating with short pulses of CHIR9902120. Thus, we combined our platform with PLGA microfilament-engineered cerebral organoid (enCOR) method by encapsulating PLGA-incorporated EBs into BEM hydrogel and culturing the constructs in our microfluidic device (enCOR-BEM-device) (Supplementary Fig. 24). We observed higher SOX2+ progenitor population at day 30 and higher number of NeuN+ neurons at day 50 in the enCOR-BEM-device organoids than in the enCOR-Mat organoids or enCOR-BEM organoids. Although these preliminary data may indicate a potential of our microfluidic BEM system to be integrated with enCOR method, more in-depth investigation would be required to check epithelium elongation and cortical layer development. For the improvement of generating region-specific organoids, our platform may also be combined with region-selective differentiation culture methods4,9,66,67 by supplementing BEM in the medium instead of Mat and culturing the constructs under the microfluidic device at a later stage.

Discussion

Here, we develop a brain-mimetic 3D organoid culture platform by combining two basic tissue-engineering elements; matrix (human brain tissue-derived ECM) and bioreactor (microfluidic chamber device). This combination aims to solve problems of current organoid culturing, which have hindered the practical application of brain organoids for disease modeling and drug screening, by providing brain-specific ECM cues and a precisely controlled brain fluid-mimetic dynamic environment. The
organoid culture with BEM-integrated microfluidics enables the crucial features of human brain development that have been limitedly demonstrated in the cerebral organoids previously reported. Our bioengineered human cerebral organoids exhibit structural, phenotypic, and functional features being observed during whole human brain development as follows: (1) the structural features of spontaneous brain morphogenesis (e.g. widespread corticogenesis, organization of highly complex structures with elongated cortical layers, apicobasal polarization of radial organization, preplate splitting), (2) mature neuronal identities (e.g. abundant expression of mature neuronal markers (NeuN, VGLUT1, GAD, GABA), extensive neural networks (N-cadherin), and synaptogenesis (SYNI, PSD95) at earlier time points), (3) divergent brain cell population (e.g. radial glial cells, microglia, astrocytes, excitatory/inhibitory neurons, Cajal- Retzius cells), and (4) electrophysiologically active properties (e.g. sodium current, multi-peaks of AP, postsynaptic current).

Although improvements in some of the cellular events and features related to whole brain development have been demonstrated in previously reported mature organoids engineered with several technologies, to the best of our knowledge, there have been few cerebral organoids simultaneously exhibiting all those characteristics. For example, genetic modification of PSCs increased NP proliferation and induced expansion and folding in cerebral organoids, but delayed neuronal differentiation. Synthetic polymer fiber microfilaments generated cerebral organoids with more continuous neuroepithelium. However, the maturity of engineered organoids was not confirmed in terms of mature neuronal marker expression, folding structure, and electrophysiological functionality. Moreover, the treatment of CHIR99021, an exogenous patterning factor, led to more reproducible forebrain formation rather than whole brain regions. As our organoids do not involve any small molecules and solely rely on spontaneous development in the presence of brain-specific ECMs, they resemble more closely to the whole brain development methods involve the use of patterning factors at an early stage to specify progenitor fate. These factors are then removed or minimized, and subsequent differentiation follows intrinsic programs. Likewise, biochemical and biophysical cues of ECM at early point seem to be critical because neuroepithelium expansion occurs and fate of progenitors are specified at an early stage of brain organoid development, which affects subsequent maturation stages. In the future study, it may be preferable to provide brain organoids with ECM microenvironments adjusted to developmental stages by applying fetal BEM and adult BEM at early and later time points of organoid culture, respectively. A recent study to compare porcine fetal and adult brain ECMs demonstrated that all types of ECM proteins were included in both ECMs, but fetal brain-derived ECM contained higher contents of fibrillin and biglycan. Therefore, it would be important to investigate any changes in ECM compositions in-depth during brain development and reflect such ECM changes to different stages of brain organoid culture. A fine-tuned consistent dynamic flow provided by the BEM-incorporated microfluidic device not only significantly reduced the formation of the necrotic region and decreased apoptotic cell death throughout the structures of cerebral organoids, but also affected metabolic states and enhanced cell proliferation. Although several methods have been applied to facilitate oxygen/nutrient supply and waste exchange and reduce necrotic region in organoids, our cerebral organoids engineered with BEM-microfluidic device underwent improved structural, phenotypic, and functional maturation compared to previously reported cerebral organoids. A recent study adapted organotypic slice culture at the air–liquid interface to cerebral organoids (ALI-CO), leading to improved neuronal survival and axon outgrowth. However, because the organoids were cultured in slide sections, the whole structure as well as the cortical layers of brain organoid might not be well established. Another recent study generated vascularized human cortical organoids by ectopically expressing the vascular transcription factor in PSCs. The presence of functional vascular-like networks in the organoids alleviated the apoptotic and hypoxic condition of the interior, and resulted in higher incidences of APs, but there was marginal improvement in cortical layer structure formation. This study examined maturity of vascularized cortical organoids by analyzing SYNI expression and incidence of AP. Our bioengineered organoids showed positive expression of SYNI at earlier time point (Fig. 6o) and higher incidence of APs (Fig. 7h). Miniaturized spinning bioractors were developed to overcome some of the major limitations of cerebral organoids (e.g. large batch-to-batch variability, diffusional limitations of oxygen/nutrient, high cost, etc.), but the study did not examine synaptogenesis and the development of the organoids relied on region-specific differentiation protocols. Cerebral organoids generated by our methodology showed positive expression for mature synaptic markers (SYNI, PSD95) (Fig. 6o and Supplementary Fig. 16e). The presence of IBA1 microglia (Supplementary Fig. 16a, b) indicates the diversity of cell population in our cerebral organoids. Importantly, brain organoids cultured in our microfluidic devices exhibited less variation in terms of size and gene expression levels (Fig. 5k and Supplementary Figs. 12a, 13e, f, 19, 20c, 22g) probably owing to uniform flow of medium to reduce variation in culture condition, demonstrating that microfluidic culture could improve the quality control of organoids. Although BEM enhanced overall growth, neuronal differentiation, cortical layer development, and electrophysiological function of brain organoids, BEM alone did not reduce the variation in the size of organoids in the orbital shaker without the use of microfluidic device (Figs. 2b and 4c). As BEM-
incorporated microfluidics could generate cerebral organoids representing the development of the whole human brain with reduced variability, our cerebral organoids could satisfy both diversity and consistency in recapitulating brain development. Due to the significant advantages of the microfluidic system as an in vitro culture platform, a couple of studies have previously demonstrated the application of chip systems for brain organoid cultures. For example, the commercially available milliPore system called Quasi Vivo increases oxygen transport, resulting in an increased proportion of dopaminergic neurons in midbrain organoids for up to 30 days of culture. In another study, cerebral organoid culture using a 5-channel chip was applied in modeling neurodevelopmental disorders under chemical exposure. 

The cryogenic pump-based perfusion method was employed for organoid culture in the chips, which decreases the throughput for culture and analysis due to a need for additional equipment. In addition, only short-term cultures were demonstrated in both studies where cortical layers and the radial organization of cell populations were not observed in the organoids. In contrast, our microfluidic chamber device was established to generate a perfusion-based microfluidic culture platform. Cerebrospinal fluid-mimetic dynamic environments were achieved with a gravity-driven flow by laboratory rocker. Our system not only takes the beneficial features of microfluidic technologies, such as small-medium volumes, effective fluid exchanges, and precisely controlled fluid properties at the microscale, but also overcomes the limitations of current microfluidics for dynamic 3D cell cultures, including a long throughput in culture and analysis, and a requirement for complex pumps, tubing, and expensive external devices.

Collectively, our organoid culture system based on combining material biology and microfabrication technology enables the generation of high-quality cerebral organoids with increased reproducibility, throughput with reduced cost, and feasibility for long-term 3D culture, providing a useful platform advancing the organoid models for studying human organogenesis and disorders. Our bioengineering strategy based on microfluidic brain matrix would be more effective for generating cerebral organoids that enable in vitro recapitulation of spontaneous whole brain development processes than previously reported technologies, while satisfying all important criteria of cerebral organoids, including diversity, maturity, and consistency. The maturation of cerebral organoids without involving exogenous morphogens and genetic alteration would be regarded as a significant advancement in cerebral organoid technology. As region-specific organoids generated by guided methods with supplementation of exogenous patterning factors have great potential for revealing the human-specific aspects of particular brain regions, our culture platform could be applied to modified cerebral organoid protocols with added exogenous factors or regional organoids to expand its applications for disease modeling and drug development in the future. It would also be broadly applicable to other types of 3D tissue organoid culture beyond the nervous system.

Methods
Preparation of the BEM. Human brain tissues were collected from the patients by excision surgery (Supplementary Table 1) and stored at −80 °C until use. For the use of human brain tissue for BEM preparation, informed consent was obtained from the participants and the study with human brain tissue was approved by the Institutional Review Board (IRB) (Permit Number: 4-2014-0769) of Yonsei University College of Medicine. Porcine brain tissues were obtained from the commercial market. The decellularization of the brain tissue was conducted following the protocol of our previous report. The frozen brain tissues were slowly thawed, while satisfying all important criteria of cerebral organoids, while satisfying all important criteria of cerebral organoids, while satisfying all important criteria of cerebral organoids, while satisfying all important criteria of cerebral organoids. For organoid culture, a BEM solution of 40 mg/ml (w/v) concentration was washed with distilled water thrice before preceding to the next step. All procedures were performed at 4 °C and agitated at 120 rpm if not stated specifically. The decellularized brain tissues were lyophilized and stored at −80 °C until use. BEM was prepared by directly transferring decellularized brain tissue with RPMI (GIB-07000) in 0.02 M HCl and stirred at 120 rpm at room temperature for two days. For organoid culture, a BEM solution of 40 mg/ml (w/v) concentration was mixed with Matrigel (#354277; Corning Inc., Corning, NY, USA) to adjust the final concentration to 400 μg/ml (w/v) BEM.

Fabrication of microfluidic device. The microfluidic device was fabricated by a standard soft lithography technique. The mixture of poly(dimethylsiloxane) (PDMS) pre-polymer solution (Dow Corning, Inc., Midland, MI, USA) and curing agent (Sylgard® 184, Dow Corning Inc.) was cast at a ratio of 10:1 on the patterned master wafer with a 2.2 mm thickness to mold the microfluidic devices as per the traditional replica molding process. The details on the dimensions of the microfluidic device are provided in Supplementary Fig. 2a. A 8-mm-diameter biopsy punch was used to punch holes into the chambers (Kai Industries Co., Ltd., Gyeonggi-do, Korea) for the samples to be cultured on oxygen plate. To bond the PDMS layers, two layers of the patterned PDMS were stacked on top of each other, and a thin film of PDMS was bonded at the bottom as a seal. For sterilization, the assembled devices were autoclaved and dried under ultraviolet light before use.

Human iPSC maintenance. Human iPSC lines WT3 (kindly provided by the Yonsei University School of Medicine) and KYOU-DXR0109B (#ACS-1023, American Type Culture Collection, Manassas, VA, USA) were cultured in iPSC-feeding medium comprising DMEM/F12 (#11320-082; Thermo Fisher Scientific), 2% (v/v) Knockout Serum Replacement (KSR, #10082-028; Thermo Fisher Scientific), 1% (v/v) penicillin-streptomycin (P/S, #GIB-15140-122, Thermo Fisher Scientific), 1% (v/v) β-mercaptoethanol (#21785-050; Sigma-Aldrich), and 1 μM Rho-associated protein kinase (ROCK) inhibitor. After 5 days, EBs were detached from STO feeder layers by 2 mg/ml collagenase IV (#428160; Gibco) treatment for 45 min at 37 °C. Then, iPSC clumps were dispersed by Accutase (#A1110501; Thermo Fisher Scientific) and 3 × 105 iPSCs were plated onto gelatinized STO (2×) at a density of 1,000 cells/cm². These iPSCs were cultured in ES-medium supplemented with 4 ng/ml basic fibroblast growth factor (bFGF, #4114-TC-01M; R&D System Inc.) and 50 μM Rho-associated protein kinase (ROCK) inhibitor (#Y0503; Sigma-Aldrich). EBs generated from iPSCs were plated onto gelatin-coated Matrigel-coated plates and cultured in a differentiation medium comprising DMEM/ F12 (#11320-082; Thermo Fisher Scientific), 1× N2 supplement (#GIB-17502-048; Thermo Fisher Scientific), and 1× L-glutamine (#GIB-03500061; Thermo Fisher Scientific). EBs were transferred to a low-attachment 96-well plate (Corning, Inc.) after 45 days. After 5–6 days of culture, the formed EBs were transferred into a low-attachment Petri dish (21.5 cm², #10068; SPL Life Sciences, Pocheon, Korea). To induce the neuroepithelium-like structures, the EBs were cultured in suspension in a neural induction medium composed of DMEM/F12 (#11320-082; Thermo Fisher Scientific), 1× N2 supplement (#GIB-17502-048; Thermo Fisher Scientific), 1× Glutamax (#GIB-03500061; Thermo Fisher Scientific), 1× MEM-NEAA (#GIB-1140-050; Thermo Fisher Scientific), and 1 μM Rho-associated protein kinase (ROCK) inhibitor (#Y0503; Sigma-Aldrich). EBs generated from feeder-free conditions were cultured in Essential 8 medium (Thermo Fisher Scientific) and 30 μM ROCK inhibitor. After 5–6 days of culture, the formed EBs were transferred into a low-attachment Petri dish (21.5 cm², #10068; SPL Life Sciences, Pocheon, Korea). For feeder-free conditions, the EBs were cultured in suspension in a neural induction medium comprising DMEM/F12 (#11320-082; Thermo Fisher Scientific) and Neurobasal (#GIB-21103-049; Thermo Fisher Scientific) (1:1) ratio supplemented
characterization of the BEM. Histological analysis of decellularized human brain tissue was performed to confirm the removal of cellular components and the preservation of ECM components. Deseeded human brain tissues were embedded in the OCT compound (HCP-0100-00A; CellPath, Hemel Hempstead, UK), frozen at −80 °C, and sectioned to 6 μm thickness. Hematoxylin and eosin (H&E) and Masson’s Trichrome (MT) staining of the sections were conducted to identify the removal of cells and the presence of collagen, respectively. To further confirm the removal of cellular components, the DNA was quantified in the tissue before and after decellularization. The total DNA was isolated using a DNA extraction kit (K-3032; Bioneer, Daejeon, Korea), according to the manufacturer’s instructions, and the DNA content of each sample was determined by measuring its absorbance at 260 nm on a microplate reader (Infinite M200 Pro, Tecan, Maennergasse, Switzerland). The GAG content was measured in the native brain and decellularized brain tissues using 1,9-dimethyl methylene blue dye solution (341088; Sigma-Aldrich, St. Louis, MO, USA) and chondroitin sulfate A (#C9819; Sigma-Aldrich) as a standard, as previously described53.

Proteomic analysis. Proteomic analysis was performed on total 12 samples of Matrigel (n = 1, biological replicate = 1), human BEM (n = 3, biological replicates = 3), pBEM derived from porcine cortex (n = 4, biological replicates = 4), and pBEM derived from porcine cerebellum (n = 4, biological replicates = 4). Proteomic analysis was performed using A Thermo Scientific Q Exactive Hybrid Quadrupole-Orbitrap equipped with Dionex U 3000 RSLcGn HPLC system (Thermo Fisher Scientific). The purified peptide sample was reconstituted in solvent A (Water/Acetonitrile (98:2 v/v), 0.1% Formic acid), and then injected into LC-MS/MS system. The sample was trapped in an Acclaim Pepmap 100 trap column (100 μm × 2 cm, nanoViper C18, 5 μm, 100 Å, Thermo Fisher Scientific) and washed for 6 min with 98% solvent A at a flow rate of 4 μl/min, and then separated on an Acclaim Pepmap 100 capillary column (75 μm × 15 cm, nanoViper C18, 3 μm, 100 Å, Thermo Fisher Scientific) at a flow rate of 300 nl/min. The LC gradient was run at 2 to 35% solvent B over 30 minutes, then from 35% to 95% over 10 min, followed by 90% solvent B for 5 min, and finally 5% solvent B for 15 min. Xcaliber software version 3.1 was used to collect MS data. The Orbitrap analyzer scanned precursor ions with a mass range of 350–1800 m/z with 70,000 resolution at m/z 200. For collision-induced dissociation, up to the 15 most abundant precursor ions were selected. The normalized collision energy was 32. Mass data were acquired automatically using proteomics discoverer 2.2 (Thermo Fisher Scientific).

Protein identification was performed by Thermo Proteome Discoverer (version 2.4.1.15). Proteins were identified by searching MS and MS/MS data of peptides against the Homo sapiens Uniprot database (2020.10 release) for human BEM, Sus scrofa Uniprot database (2020.12 release) for pBEM, and Mus musculus Uniprot database (2020.12 release) for Matrigel. Trypsin was used as the protease for cleavage and up to two missed cleavages were allowed. Carbamidomethylation of cysteines was set as a static modification. Oxidation of methionine, N-terminal acetylation, and N-terminal methionine excision were set as dynamic modifications. Precursor mass tolerance was set to 10 ppm and fragment mass tolerance was set to 0.02 Da. Proteins and peptides were filtered for false discovery rate (FDR) less than 1%.

We compared the identified proteins in Matrigel and human BEM with an Atlas of Brain Proteome (http://www.brainproteome.org). The contents and composition of matrice proteins in Matrigel, human BEM, pBEM, and human brain tissue were compared based on relative intensity-based absolute quantification (rBAQ) values, which are approximately proportional to the molar amount of proteins present in the matrices. Gene ontology biological process (GOBP) analysis was performed on the proteins reported in the BEM-device organoid. We found that the proteins in Matrigel, which have at least four-fold elevation of expression in human brain tissue compared to other tissues55,78,79. For statistical significance, proteins and peptides were filtered for FDR and p value less than 0.05. Raw p value was determined by Fisher’s exact test.

Histology and immunohistochemistry. At several time points in the organoid culture (30, 45, 60, 75, 100, and 120 days), the brain organoids were fixed in 10% formalin solution (H45501640; Sigma-Aldrich) for one hour at room temperature, washed twice using phosphate-buffered saline (PBS, Bioseasang, Seongnam, Korea), and immersed in 30% (w/v) sucrose dissolved in distilled water overnight at 4 °C for cryoprotection. The organoids were embedded in OCT compound and frozen at −80 °C. The samples were then sectioned in tissue slices of 20 μm thickness using a Leica Inc. (Wetzlar, Germany) cryostat. The histological analysis of the tissue sections were examined with H&E staining to analyze the overall cellular morphology and cavity in the organoids. For immunohistochemical staining, the sectioned tissues were washed with PBS to remove excess OCT and permeabilized with 0.2% (v/v) Triton X-100 (#X1800; Sigma-Aldrich) in PBS for 20 min. Then, the sections were treated with 4% (v/v) bovine serum albumin (#21600088; MP Biomedicals, Santa Ana, CA, USA) and 2% (v/v) horse serum (#16505010; Thermo Fisher Scientific) for one hour to block the non-specific binding of antibodies. The samples were incubated with primary antibodies listed in Supplementary Table 6. The stained samples were then washed with PBS three times and incubated with Alexa Fluor 488 or 594-conjugated secondary antibody (Thermo Fisher Scientific) for signal visualization. The nuclei were counterstained with 4',6-diamidino-2-phenylindole (DAPI, #A2412; TCI America, Portland, OR, USA) for 30 min and washed with PBS. The samples were mounted using a fluorescent mounting medium (H1400; Vector laboratories, Burlingame, CA, USA) and observed under a confocal microscope (LSM 800, Carl Zeiss, Oberkochen, Germany).

Image-based quantification. Fluorescence intensities of Tuji and MAP2 in the whole section of organoids were measured by selecting the marker positive area and using ‘Integrated density’ function in ‘set measurements’ setting in ImageJ. The corrected total organoid fluorescence was calculated by the following equation; Integrated density = (Area of selected cell × Mean fluorescence of background readings). The area and perimeter of PAX6+ ventricle-like structures from 4 representative organoids were measured using ImageJ. The average number of cells positive for SOX2, NeuN, TBR2, HOPX, IBA1, and CD68 per field was manually counted using ImageJ. The average percentage ratio of cells positive for SOX2 per field was quantified by manually counting the positively stained cells and dividing them by the number of DAPI-stained nuclei. The positive areas for EctD1, Ki67, FOXG1, and NeuN were determined by measuring the area stained positively for markers and normalized to DAPI+ area using Image J. For Nestin+, CASP3+, HIF-1α, VGLUT1+ area measurements, the area positively stained for each of the markers was normalized to the total area of the image. The cortical plate, defined as the densely packed CTIP2+ regions, and the superficial layer defined as the SATB2+ regions were measured using ImageJ. Three measurements were taken at 45-degree angles to obtain the mean value.

qPCR analysis. The gene expression of the brain organoids at 30 and 75 days of culturing was investigated using qPCR analysis, as previously described45,57. Total RNA was isolated from approximately 150 organoids per sample using an RNA extraction kit (97676 A; Takara Bio Inc., Kusatsu, Shiga, Japan) following the manufacturer’s instructions. The RNA concentration was calculated by measuring the absorbance of the samples at 260 nm using a spectrophotometer (Infinite M200 Pro, Tecan). The total RNA was reverse-transcribed into cDNA using a TaKaRa PrimeScript II First-Strand cDNA synthesis kit (#6110 A; TaKaRa Bio Inc.). The synthesized cDNA was used for the qPCR reaction with the TaqMan Fast Universal PCR MasterMix (#4366073; Thermo Fisher Scientific). The gene expression was quantified with the Step One Plus Real-Time PCR system (Applied Biosystems, Foster City, CA, USA) for the targets listed in Supplementary Table 7. TaqMan Assay primers were purchased from Thermo Fisher Scientific. The relative gene expression, which sample was calculated by the comparative method normalized and normalized using an endogenous reference glyceraldehyde-3-phosphate dehydrogenase (GAPDH). The gene expressions of the human brain organoids were compared with human neural stem cells (NSCs, provided by Prof. Kook In Park at Yonsei University) and mouse neural cells (provided by Dr. Masayuki Uchida at Osaka University) cultured in a 13-week fetus with full written parental consent and approval of the research ethics committee of Yonsei University College of Medicine (protocol #4-2003-0078).

Tissue-specific ECM experiment. To investigate the tissue-specific effect of ECM on brain organoid development, decellularized ECM derived from porcine organs, including the brain (BEM), intestines (IEM), liver (LEM), and heart (HEM), was tested for brain organoid culture. Decellularization of the porcine liver and heart was performed as previously reported46. The porcine small intestine was decel-

nlarized as follows: the intestine was thoroughly washed and cut laterally along the intestinal wall so that it was in a flat, rectangular form. Then, it was cut into...
segments of 20 cm in length and agitated in the following solutions for the time indicated: distilled water (24 h), decellularization solution containing 1% (v/v) Triton X-100 in 1X PBS (pH 7.4) for 300 min, which explained the low level of residual DNA. Such an aqueous solution was distilled water (24 h), 1% (v/v) penicillin/streptomycin (#G1510-142; Thermo Fisher Scientific) (1 h), and distilled water (1 h). All processes were performed at 4 °C and agitated at 120 rpm. The decellularized intestinal tissues were lyophilized and stored at 4 °C until use. The porcine brain was decellularized using the same protocol for the human brain. At day 11, the EBs with neuroepithelium identity were encapsulated in 30 μl Matrigel (#354277; Corning Inc., Corning, NY) or decellularized ECM gel (0.4 mg/ml ECM; Sigma-Aldrich) before analysis. The ZEN software was used to measure the intensity of phosphorescence signals. The mean phosphorescence intensity of the BEM-device group was normalized to that of the BEM-plat group using Zen software.

### Calcium imaging

To examine the response of brain organoids to neurotransmitters, the organoids were incubated with the intracellular calcium indicator 2 μM Fluo-4 AM (#F14210; Thermo Fisher Scientific) for 40 min at 37 °C and additionally for 30 min at 25 °C. Time-lapse changes of intracellular Ca2+ levels in brain organoids were imaged after treatment with 100 μM glutamate (Sigma-Aldrich), the stock solution of the UAN precursors via a radical reaction among their vinyl groups, 10 μg of UAN was dissolved in dimethyl sulfoxide (DMSO) to obtain a homogeneous UAN-DMSO solution. After adding 0.2 g of azobisobutyronitrile (AIBN), a radical initiator, the solution was heated to 60 °C and maintained for 3 h with gentle stirring. Polymerized UAN-DMSO solution (25.0 g; 0.11 M) was then poured into 1 ml of DMSO containing 10% (w/w) NNNN-tetrakis (2-HP) ethylenediamine, 10% (w/w) urea, 1.3 M MgCl2 saturated with 95% O2 and 5% CO2 at 30–32 °C. Recording glass pipettes (2–4 MΩ) were filled with an intracellular solution containing (in mM): 135 potassium methanesulfonate, 10 KCl, 10 HEPES, 1 EGTA, and 2 Na2ATP (pH 7.2 to 7.4 adjusted with KOH). The whole-cell recordings were performed using an upright microscope (BX51WI, Olympus, Tokyo, Japan) with IR-DIC optics. Signals were recorded using a MultiClamp 700B amplifier, filtered at 3 kHz using a Bessel filter, and digitized at 10 Hz with a Digitaper 1520B analog-to-digital board (Molecular Devices, Sunnyvale, CA, USA). To measure Na+ currents, cells were subjected to a series of voltage steps (1000 msec duration) from −30 to +20 mV (in 10 mV increments) at a holding voltage of −60 mV for 20 s to improve transparency. We measured the resting membrane potential, and then a series of current pulses (500 msec) with increasing amplitude between −5 pA and +25 pA (in 5 pA steps) were applied to evoke APs. The AP threshold was defined as the somatic voltage at which dV/dt exceeds 20 V/s. Synaptic currents were acquired from a neuron at a resting membrane potential (~60 mV).

### Electrophysiology

The whole-cell patch-clamp recording was performed as previously described. During recording, brain organoids were submerged and continuously perfused with an artificial cerebrospinal fluid (aCSF) consisting of (in mM): 119 NaCl, 2.5 KCl, 11 glucose, 26 NaHCO3, 1.25 NaH2PO4, 2.5 CaCl2, and 1.3 MgCl2 saturated with 95% O2 and 5% CO2 at 30–32 °C. Recording glass pipettes (2–4 MΩ) were filled with an intracellular solution containing (in mM): 135 potassium methanesulfonate, 10 KCl, 10 HEPES, 1 EGTA, and 2 Na2ATP (pH 7.2 to 7.4 adjusted with KOH). The whole-cell recordings were performed using an upright microscope (BX51WI, Olympus, Tokyo, Japan) with IR-DIC optics. Signals were recorded using a MultiClamp 700B amplifier, filtered at 3 kHz using a Bessel filter, and digitized at 10 Hz with a Digitaper 1520B analog-to-digital board (Molecular Devices, Sunnyvale, CA, USA). To measure Na+ currents, cells were subjected to a series of voltage steps (1000 msec duration) from −30 to +20 mV (in 10 mV increments) at a holding voltage of −60 mV for 20 s to improve transparency. We measured the resting membrane potential, and then a series of current pulses (500 msec) with increasing amplitude between −5 pA and +25 pA (in 5 pA steps) were applied to evoke APs. The AP threshold was defined as the somatic voltage at which dV/dt exceeds 20 V/s. Synaptic currents were acquired from a neuron at a resting membrane potential (~60 mV).

### RNA-sequencing analysis

Brain organoids in each condition (Mat-plate, BEM-plate, BEM-device) were collected at the indicated time points (n = 3). For sample preparation, 5–10 organoids were collected from the 3D matrix per one batch of samples for each experimental condition. RNA was isolated using an RNA extraction kit (Takara Bio Inc.) according to the manufacturer’s instructions. RNA concentrations were measured using a NanoDrop spectrophotometer (#ND-8000; Thermo Fisher Scientific). The total RNA integrity was checked using an Agilent Technologies 2100 Bioanalyzer (#G29398; Agilent, Santa Clara, CA, USA). A high-quality sample is required for successful library preparation. Bacterial DNA was removed using a Zymplify kit (Agilent Technologies, Santa Clara, CA, USA). RNA was enriched for mRNA sequencing. RNA sequencing was performed on a NextSeq 500 (Illumina, San Diego, CA, USA). RNA was enriched for mRNA sequencing using an Illumina Truseq stranded mRNA library prep kit (#20020595; Illumina, San Diego, CA, USA). NextSeq 500 sequenced the sample library in single-end 100 bp mode. The Illumina Truseq stranded mRNA library prep kit was used to generate a paired-end dataset with the same sequencing length as the single-end data. Sequencing data was analyzed for quality control and trimmed read lengths. After trimming, the reads were mapped to the hg19 human genome using a bowtie2 alignment algorithm. The trimmed reads were then passed through a number of processing steps, with the first step being the removal of low-quality reads. The remaining reads were then aligned to the genome using the bowtie2 algorithm. The resulting alignments were then used to calculate gene expression levels. Finally, the raw counts were normalized to gene lengths and expressed as FPKM (fragments per kilobase of gene model per million sequence reads). The resulting gene expression values were then used for further analysis.

### Measurement of oxygen levels in brain organoids

To monitor the oxygen levels within organoids, the oxygen-sensing nanoparticles were diffused into the brain organoids by incubating the organoids in the medium containing the PtTFPP-PUAN nanoparticles at 37 °C overnight. The stock PtTFPP-PUAN solution in DMSO was pre-mixed with the culture medium at a ratio of 1:1 (v/v). Then, the oxygen-sensitive phosphor remained partitioned within the hydrophobic core of PUAN nanoparticles in the course of formation of micelle-like PUAN nanoparticles dispersed in the aqueous PBS solution. The PtTFPP-PUAN nanoparticles were formed via the nano-precipitation method80. During recording, brain organoids were submerged and continuously perfused with an artificial cerebrospinal fluid (aCSF) consisting of (in mM): 119 NaCl, 2.5 KCl, 11 glucose, 26 NaHCO3, 1.25 NaH2PO4, 2.5 CaCl2, and 1.3 MgCl2 saturated with 95% O2 and 5% CO2 at 30–32 °C. Recording glass pipettes (2–4 MΩ) were filled with an intracellular solution containing (in mM): 135 potassium methanesulfonate, 10 KCl, 10 HEPES, 1 EGTA, and 2 Na2ATP (pH 7.2 to 7.4 adjusted with KOH). The whole-cell recordings were performed using an upright microscope (BX51WI, Olympus, Tokyo, Japan) with IR-DIC optics. Signals were recorded using a MultiClamp 700B amplifier, filtered at 3 kHz using a Bessel filter, and digitized at 10 Hz with a Digitaper 1520B analog-to-digital board (Molecular Devices, Sunnyvale, CA, USA). To measure Na+ currents, cells were subjected to a series of voltage steps (1000 msec duration) from −30 to +20 mV (in 10 mV increments) at a holding voltage of −60 mV for 20 s to improve transparency. We measured the resting membrane potential, and then a series of current pulses (500 msec) with increasing amplitude between −5 pA and +25 pA (in 5 pA steps) were applied to evoke APs. The AP threshold was defined as the somatic voltage at which dV/dt exceeds 20 V/s. Synaptic currents were acquired from a neuron at a resting membrane potential (~60 mV).
applied for sequencing by synthesis on a HISEQ 2500 sequencing system (Illumina) with 100 bp length. The reads were aligned with Tophat 13 (v2.0.13; http://ccb.jhu.edu/software/tophat/) to map the reads and to calculate the expression levels between samples and a reference genome (human genome: hg19). To observe the differentially expressed genes (DEGs), aligned reads were analyzed by Cuffdiff (v2.2.0; http://cole-trapnell-lab.github.io/cufflinks/cuffdiff/). Then, to visualize RNA-seq data, Cuffdiff data were loaded to CummeRbund (v2.8.2; http://comprha.bitbucket.io/manual_2.8.2.html). Aligned reads of the control and cases were subjected to fragments per kilobase of transcript per million (FPKM) estimation with Cufflinks (v1.3.0).

For the comparison of organoids in several brain regions (dorsolateral prefrontal cortex, ventral prefrontal cortex, orbital frontal cortex, cerebellar cortex, hippocampus, cerebellum, striatum, and mediodorsal nucleus of the thalamus), the FPKM expression values of Brainspan were downloaded from the Allen BrainSpan human transcriptome dataset (http://www.brainspan.org/static/download.html). FPKM values were filtered for differential expression and joined with the Brainspan via the gene symbols. The similarity of expression was compared by the rank of the expressed gene via Pearson correlation coefficient (p value). The fold change of gene expression was calculated by searching MS and MS/MS data of peptides against the homop sapiens UniProt database (2020.10 release) for human BEM. Sus scrofa UniProt database (2020.12 release) for pBEM, and Mus musculus UniProt database (2020.12 release) for Mat. Proteins identified in mat and human BEM were compared with the datasets in the Human Protein Atlas portal [www.proteinatlas.org]. The contents and composition of matrisome proteins in human brain tissues were analyzed from the data in the Human Proteome Map [www.humanprotememap.org]. All datasets generated during the current study are available from the corresponding author on reasonable request. Source data are provided with this paper.

Received: 1 July 2020; Accepted: 6 July 2021; Published online: 05 August 2021

References
1. Di Lullo, E. & Kriegstein, A. R. The use of brain organoids to investigate neural development and disease. Nat. Rev. Neurosci. 18, 573 (2017).
2. Eiraku, M. et al. Self-organized formation of polarized cortical tissues from ESs and its active manipulation by extrinsic signals. Cell Stem Cell 3, 519–532 (2008).
3. Lancaster, M. A. et al. Cerebral organoids model human brain development and microcephaly. Nature 501, 373–379 (2013).
4. Lancaster, M. A. & Knoblich, J. A. Generation of cerebral organoids from human pluripotent stem cells. Nat. Protoc. 9, 2329–2340 (2014).
5. Camp, J. G. et al. Human cerebral organoids recapitulate gene expression programs of fetal neocortex development. Proc. Natl Acad. Sci. U. S. A. 112, 15672–15677 (2015).
6. Kadoshima, T. et al. Self-organization of axial polarity, inside-out layer pattern, and species-specific progenitor dynamics in human ES cell-derived neocortex. Proc. Natl Acad. Sci. U. S. A. 110, 20284–20289 (2013).
7. Asaka, M. et al. Functional cortical neurons and astrocytes from human pluripotent stem cells in 3D culture. Nature 518, 671–678 (2015).
8. Qian, X. Y. et al. Brain-region-specific organoids using mini-bioreactors for modeling zivk exposure. Cell 165, 1238–1254 (2016).
9. Muguruma, K., Nishiyama, A., Kawakami, H., Hashimoto, K. & Sasai, Y. Self-organization of polarized cerebellar tissue in 3D culture of human pluripotent stem cells. Cell Rep 10, 537–550 (2015).
10. Jo, F. et al. Midbrain-like organoids from human pluripotent stem cells contain functional dopaminergic and neuromelanin-producing neurons. Cell Stem Cell 19, 248–257 (2016).
11. Yoo, H. et al. Self-formation of functional adenohypophysis in three-dimensional culture. Nature 480, 57–62 (2011).
12. Sakaguchi, H. et al. Generation of functional hippocampal neurons from self-organizing human embryonic stem cell-derived dorsomedial telencephalic tissue. Nat. Commun. 6, 8896 (2015).
13. Birey, F. et al. Assembly of functionally integrated human forebrain spheroids. Nature 545, 54 (2017).
14. Xiang, Y. et al. Fusion of regionally specified hPSC-derived organoids models human brain development and interneuron migration. Cell Stem Cell 21, 383–398 e387 (2017).
15. Bagley, J. A., Reumann, D., Bian, S., Levi-Strauss, J. & Knoblich, J. A. Fused cerebral organoids model interactions between brain regions. Nat. Methods 14, 743–751 (2017).
16. Xiang, Y. et al. hESC-derived thalamic organoids form reciprocal projections when fused with cortical organoids. Cell Stem Cell 24, 487–497 (2019).
17. Cederquist, G. Y. et al. Specification of positional identity in forebrain organoids. Nat. Biotechnol. 37, 438–444 (2019).
18. Kavuri, S. D. et al. Cerebral organoids in the air-liquid interface generate diverse nerve tracts with functional output. Nat. Neurosci. 22, 669–679 (2019).
19. Wang, Y., Wang, L., Guo, Y., Zhu, Y. & Qin, J. Engineering cell-derived 3D brain organoids in a perfusable organ-on-a-chip system. RSC Adv. 8, 1677–1685 (2018).
23. Berger, E. et al. Millifluiddic culture improves human midbrain organoid vitality and differentiation. Lab Chip 18, 3172–3183 (2018).
24. Mansour, A. A. et al. In vivo model of functional and vascularized human brain organoids. Nat. Biotechnol. 36, 432–441 (2018).
25. Faissner, A. & Reinhard, J. The extracellular matrix compartment of neural stem and glial progenitor cells. Glia 63, 1330–1349 (2015).
26. Yin, X. et al. Engineering stem cell organoids. Cell Stem Cell 18, 25–38 (2016).
27. Kim, S., Cho, A.-N., Min, S., Kim, S. & Cho, S.-W. Organoids for advanced therapeutics and disease models. Adv. Ther. 2, 1800887 (2018).
28. Barros, C. S., Franco, S. J. & Muller, U. Extracellular matrix functions in the nervous system. Cold Spring Harb. Perspect. Biol. 3, a005108 (2011).
29. Bartsch, S. et al. Expression of tenascin in the developing and adult cerebral cortex. J. Neurosci. 12, 736–749 (1992).
30. Maeda, N. Proteospondins and neuronal migration in the cerebral cortex during development and disease. Front. Neurosci. 9, 98 (2015).
31. Xu, J. et al. The extracellular matrix glycoprotein tenasin-R regulates neurogenesis during development and in the adult dentate gyrus of mice. J. Cell Sci. 127, 641–652 (2014).
32. Naba, A. et al. The matrisome: in silico definition and in vivo characterization by proteomics of normal and tumor extracellular matrices. Mol. Cell. Proteom. 11, M111.014647 (2012).
33. Naba, A., Hoersch, S. & Hynes, R. O. Towards de novo identification of extracellular matrix proteins and their roles in development and evolution of primate neocortex. Prog. Brain Res. 185, 379–397 (2013).
34. Kim, M.-S. et al. A draft map of the human proteome. Mol. Cell. Proteom. 3, 739–813 (2004).
35. Kadowaki, M. et al. N-cadherin mediates cortical organization in the mouse brain. Lab Chip 15, 3172–3182 (2015).
36. Robinson, S., Li, Q., Dechant, A. & Cohen, M. L. Neonatal loss of gamma-glutamylcysteine ligase catalytic subunit (γ-GCLC) impairs mitochondrial respiratory function and leads to hypoxic-ischemic encephalopathy. Free Radic. Biol. Med. 64, 362–369 (2013).
37. Jovanov Milošević, N., Judaš, M., Aronica, E. & Kostovic, I. Neural ECM in laminar organization and connectivity development in healthy and diseased human brain. Prog. Brain Res. 214, 139–178 (2014).
38. Li, Y. et al. Induction of expansion and folding in human cerebral organoids. Cell Stem Cell 20, 385–396 (2017).
39. Matsui, T. K. et al. Six-month cultured cerebral organoids from human ES cells contain mature neural circuitry. Nat. Neurosci. 21, 1077–1084 (2018).
40. Susaki, E. A. et al. Whole-brain imaging with single-cell resolution using a genetically encoded calcium indicator. Science 349, 12770–12775 (2015).
41. Cakir, R. et al. Engineering of human brain organoids with a functional vascular-like system. Nat. Methods 16, 1169–1175 (2019).
42. Wang, Y., Wang, L., Zhu, Y. & Qin, J. Human brain organoid-on-a-chip model to predict prenatal nicotine exposure. Lab Chip 18, 851–860 (2018).
43. Neo, H. I. et al. Thermo-responsive polymeric nanoparticles for enhancing neuronal differentiation of human induced pluripotent stem cells. Nanomedicine 11, 1861–1869 (2015).
44. Giandomenico, S. L., Sutcliffe, M. & Lancaster, M. A. Generation and long-term culture of advanced cerebral organoids for studying later stages of neural development. Nat. Protoc. 16, 579–802 (2021).
45. Ashburner, M. et al. Gene ontology: tool for the unification of biology. The Gene Ontology Consortium. Nat. Genet. 25, 25–29 (2000).
46. The Gene Ontology resource. enriching a GO mind. Nucleic Acids Res. 49, D335–D344 (2021).
47. Mi, H., Muruganujan, A., Ebert, D., Huang, X. & Thomas, P. D. PANTHER version 14: more genomes, a new PANTHER GO-slim and improvements in enrichment analysis tools. Nucleic Acids Res. 47, D419–D426 (2019).
48. Uhlén, M. et al. Tissue-based map of the human proteome. Science 347, 1246419 (2015).
49. Susaki, E. et al. Whole-brain imaging with single-cell resolution using chemical cocktails and computational analysis. Cell 157, 726–739 (2014).
50. Robinson, S., Li, Q., Dechant, A. & Cohen, M. L. Neonatal loss of gamma-glutamylcysteine ligase catalytic subunit (γ-GCLC) impairs mitochondrial respiratory function and leads to hypoxic-ischemic encephalopathy. Free Radic. Biol. Med. 64, 362–369 (2013).
51. Kadowaki, M. et al. N-cadherin mediates cortical organization in the mouse brain. Dev. Biol. 304, 22–33 (2007).
52. Fietz, S. A. et al. OSVZ progenitors of human and ferret neocortex are epithelial-like and expand by integrin signaling. Nat. Neurosci. 13, 690–699 (2010).
53. Bajpai, U., Allemann, E. & Doecke, E. Development of a nanoprecipitation method intended for the entrapment of hydrophobic drugs into nanoparticles. Eur. J. Pharm. Sci. 24, 67–75 (2005).
54. Ashburner, M. et al. Gene ontology: tool for the unification of biology. The Gene Ontology Consortium. Nat. Genet. 25, 25–29 (2000).
55. The Gene Ontology resource. enriching a GO mind. Nucleic Acids Res. 49, D335–D344 (2021).
56. Mi, H., Muruganujan, A., Ebert, D., Huang, X. & Thomas, P. D. PANTHER version 14: more genomes, a new PANTHER GO-slim and improvements in enrichment analysis tools. Nucleic Acids Res. 47, D419–D426 (2019).
57. Susaki, E. et al. Whole-brain imaging with single-cell resolution using chemical cocktails and computational analysis. Cell 157, 726–739 (2014).
58. Robinson, S., Li, Q., Dechant, A. & Cohen, M. L. Neonatal loss of gamma-glutamylcysteine ligase catalytic subunit (γ-GCLC) impairs mitochondrial respiratory function and leads to hypoxic-ischemic encephalopathy. Free Radic. Biol. Med. 64, 362–369 (2013).
59. Kadowaki, M. et al. N-cadherin mediates cortical organization in the mouse brain. Dev. Biol. 304, 22–33 (2007).
60. Fietz, S. A. et al. OSVZ progenitors of human and ferret neocortex are epithelial-like and expand by integrin signaling. Nat. Neurosci. 13, 690–699 (2010).
61. Robinson, S., Li, Q., Dechant, A. & Cohen, M. L. Neonatal loss of gamma-glutamylcysteine ligase catalytic subunit (γ-GCLC) impairs mitochondrial respiratory function and leads to hypoxic-ischemic encephalopathy. Free Radic. Biol. Med. 64, 362–369 (2013).
62. Kadowaki, M. et al. N-cadherin mediates cortical organization in the mouse brain. Dev. Biol. 304, 22–33 (2007).
63. Fietz, S. A. et al. OSVZ progenitors of human and ferret neocortex are epithelial-like and expand by integrin signaling. Nat. Neurosci. 13, 690–699 (2010).
64. Robinson, S., Li, Q., Dechant, A. & Cohen, M. L. Neonatal loss of gamma-glutamylcysteine ligase catalytic subunit (γ-GCLC) impairs mitochondrial respiratory function and leads to hypoxic-ischemic encephalopathy. Free Radic. Biol. Med. 64, 362–369 (2013).
Acknowledgements
This work was supported by grants from the National Research Foundation of Korea (NRF) (2016M3C9A4921712, 2017M3C7A1047659, 2018M3A9H1021382) funded by the Ministry of Science and ICT (MSIT), Republic of Korea. This work was also supported by Samsung Research Funding & Incubation Center of Samsung Electronics (Project Number SRFC-TC2003-03) and the Institute for Basic Science (IBS-R026-D1). This work was also supported by Singapore National Medical Research Council Open-Fund Individual Research Grant (NMRC/OFIRG/0050/2017) and Singapore National Research Foundation Competitive Research Programme (NRF-CRP17-2017-04).

Author contributions
A.N.C. and Y.A. designed and performed the experiments and analyzed the data. Y.J. and J.K. (Junghoon Kim) analyzed the data. Y.J. and S.W.C. designed and supervised the study, and wrote the paper with contributions from all authors. J.K. (Jihun Kim) designed and analyzed microfluidic device. Y.S.C. and J.S.L. prepared BEM. W.Y.C. analyzed RNA-sequencing data. D.J.K. and D.Y.K. performed 3D organoid imaging. G.E.C. and W.Y. performed organoid patch clamp. Y.S.C. and S.H.J. performed proteomic analysis. J.Y.K. and N.C. analyzed oxygen levels. J.K. (Jihun Kim) and H.C.K. provided human brain tissues. Y.G.K., E.C., Y.J.K., and H.S.J. advised the analysis and helped data interpretation.

Competing interests
S.W.C., A.N.C., Y.J., and J.S.L. are co-inventors on patent applications (Korean Patent 10-2021-0037583, US patent 16/448,954, and EP patent 19181495.3) related to decellularized brain matrix for brain organoid culture. The remaining authors declare no competing interests.

Additional information
Supplementary information The online version contains supplementary material available at https://doi.org/10.1038/s41467-021-24775-5.

Correspondence and requests for materials should be addressed to S.-W.C.

Peer review information Nature Communications thanks Arti Ahluwalia and the other anonymous reviewer(s) for their contribution to the peer review of this work.

Reprints and permission information is available at http://www.nature.com/reprints

Publisher’s note Springer Nature remains neutral with regard to jurisdictional claims in published maps and institutional affiliations.

Open Access This article is licensed under a Creative Commons Attribution 4.0 International License, which permits use, sharing, adaptation, distribution and reproduction in any medium or format, as long as you give appropriate credit to the original author(s) and the source, provide a link to the Creative Commons license, and indicate if changes were made. The images or other third party material in this article are included in the article’s Creative Commons license, unless indicated otherwise in a credit line to the material. If material is not included in the article’s Creative Commons license and your intended use is not permitted by statutory regulation or exceeds the permitted use, you will need to obtain permission directly from the copyright holder. To view a copy of this license, visit http://creativecommons.org/licenses/by/4.0/.

© The Author(s) 2021