Induction of inverted morphology in brain organoids by vertical-mixing bioreactors

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Organoid technology provides an opportunity to generate brain-like structures by recapitulating developmental steps in the manner of self-organization. Here we examined the vertical-mixing effect on brain organoid structures using bioreactors and established inverted brain organoids. The organoids generated by vertical mixing showed neurons that migrated from the outer periphery to the inner core of organoids, in contrast to orbital mixing. Computational analysis of flow dynamics clarified that, by comparison with orbital mixing, vertical mixing maintained the high turbulent energy around organoids, and continuously kept inter-organoid distances by dispersing and adding uniform rheological force on organoids. To uncover the mechanisms of the inverted structure, we investigated the direction of primary cilia, a cellular mechanosensor. Primary cilia of neural progenitors by vertical mixing were aligned in a multidirectional manner, and those by orbital mixing in a bidirectional manner. Single-cell RNA sequencing revealed that neurons of inverted brain organoids presented a GABAergic character of the ventral forebrain. These results suggest that controlling fluid dynamics by biomechanical engineering can direct stem cell differentiation of brain organoids, and that inverted brain organoids will be applicable for studying human brain development and disorders in the future.
Clariﬁcing the principles regarding how brain cells arise and assemble tissue is important for understanding brain development and disease mechanisms. Model animals and genomic studies help us to analyze brain structure and evolution. However, there has been no method to study the hominid brain directly due to a lack of appropriate approaches. Brain organoid technology using human induced pluripotent stem cells (iPSCs) enables us to use a brain-like structure for research, and provides many insights into the development and diseases of human brain.12 Brain organoids from human iPSCs retain these outstanding advantages in preparing various portions of brain and discrete cell types with neuronal circuitry, and a variety of brain organoid protocols have been developed. However, contributing factors to self-organization-driven formation of brain organoids are still not fully understood, and little is known about the effect of mechanical forces except by orbital mixing on the formation of brain organoids from human iPSCs.

Organoids are cultured as three-dimensional cell aggregates in floating condition using shakers or bioreactors,1,15, and various mechanical stimuli including shear stress and turbulent energy, as well as energy dissipation affect cell differentiation and organoid formation. There have been some studies regarding the effects of mechanical forces on organoid development.16–18 In the present study, we analyzed the effect of mechanical forces by vertical mixing on the formation of brain organoids. We used a vertical mixing bioreactor, which mixes culture medium with minimized shear stress, and maintains a uniform and stable environment by continuous monitoring of culture conditions, including stable temperature, pH, and dissolved oxygen concentration. As a consequence, the brain organoids generated by vertical mixing showed an inverted structure with neurons localizing at the center of organoids and covering outside by neural progenitors. This result proved that mechanical forces by biomechanical engineering contributed to human iPSC-derived brain organoid structures with enlarged and homogeneous areas of neurons inside inverted brain organoids, applicable for studying human brain development and disorders.

Results

Generation of inverted brain organoids by vertical mixing. We generated brain organoids using orbital shakers for orbital mixing or reciprocal vertical bioreactors for vertical mixing (Fig. 1a, Supplementary Fig. 1). The structure of organoids after two months of culture by vertical mixing showed a different structure compared with that of organoids by orbital mixing. By orbital mixing, many neural tube-like structures were generated with SOX2-positive cells surrounded by MAP2-positive neurons. These structures were randomly distributed in brain organoids, showing an inside-out order with the neuron layer in peripheral regions and the neural progenitor layer in non-parallel regions of organoids (Fig. 1b). In contrast, brain organoids generated by vertical mixing showed inverted structure, with the neural progenitor layer in the peripheral region and the neuronal layer in the central region of organoids (Fig. 1c). These inverted brain organoids contained a uniform area consisting of neurons in the middle of the structure. In vertical-mixing organoids, the area of SOX2-positive cells in the peripheral region, which is defined as within 100 μm from the edge of brain organoids, was significantly larger compared to that in orbital-mixing organoids (Fig. 1d).

Next, we investigated the layer formation of the cerebral cortex by analyzing cortical neuron markers. Inverted layer formation was observed in vertical-mixing organoids in comparison to those in orbital-mixing organoids (Fig. 1e, f). Alteration of the apical-basal order of layer formation was revealed by immunostaining with anti-N-CADHERIN, a marker for apical membrane, antibody (Fig. 1g, h). We investigated the effect of Matrigel for organoid structure by removing the Matrigel embed step for the orbital organoids, and found that the removal of Matrigel did not promote the generation of inverted structure (Supplementary Fig. 2a). Therefore, we consider that vertical mixing mainly contributes to the production of inverted brain structure, although removal of Matrigel may contribute to the inversion. These findings suggested that brain organoids by vertical mixing exhibited a special structure with an inverted inside-out pattern of layer formation, referred to as inverted brain organoids (Fig. 1i). We also found that inverted organoids were generated when orbital mixing was initiated on Day 15 following vertical mixing (Supplementary Fig. 2b), suggesting that the initiation of vertical mixing at an early stage of differentiation would be crucial for the formation of inverted organoids.

Functional analysis of inverted brain organoids. To investigate the functional maturation of inverted brain organoids, we evaluated neuronal activity by electrophysiological analysis. Inverted brain organoids dissociated to clumps on Day 56 were cultured on MEA chips for 6 additional weeks (Fig. 2a), and their spontaneous extracellular field activity was recorded. A representative phase-contrast image of a sample is presented (Fig. 2b). Spontaneous firing and synchronized burst firing were detected by recording, which indicated that the organoids harbored neuronal networks. To evaluate their synaptic functions, the response to compounds including GABA receptor antagonist and glutamate receptor antagonists were investigated. GABA receptor antagonist, picrotoxin (PTX), increased the spike frequency and array-wide spike detection rate (ASDR), and a selective N-methyl-D-aspartate (NMDA) receptor antagonist, D-2-amino-5-phosphonopentanoate (AP-5), and AMPA/kainate glutamate receptor antagonist, 6-Cyano-7-nitroquinoxaline-2,3-dione (CNQX), inhibited the firing (Fig. 2c, d). These data suggest that inverted brain organoids exhibit functional maturity.

Computational simulation of fluid dynamics. To understand the mechanism of the inversion of the brain organoid structure, we implemented computational fluid dynamic (CFD) analysis both for orbital mixing and vertical mixing. Using CFD analysis, flow velocity, shear stress, strain rate, vorticity, turbulent energy, and energy dissipation were calculated in orbital mixing and three conditions of vertical mixing (45, 60, and 75 mm/s) (Fig. 3a). The turbulent energy presented a higher magnitude in vertical mixing than in orbital mixing, suggesting that the difference in turbulent energy may contribute to and be a positive factor for the formation of inverted brain organoids. We also calculated the velocity of each organoid using a solid–liquid mixed-phase flow analysis. The dispersion state in the orbital shaker is not equal, with movement of spheres along the container wall and toward the center (Fig. 3b, upper panel, Supplementary Video 1). Meanwhile, the organoids in vertical mixing are evenly dispersed throughout the entire culture tank (Fig. 3b, lower panel, Supplementary Video 2). Such flow characteristics and dispersion action might have a positive effect on the inverted brain structure by vertical mixing. Moreover, the discrete phase model (DPM) showed the difference in velocity of the organoids itself and the drag force applied to organoids between orbital mixing and vertical mixing. Analysis of three-dimensional flow velocity showed that organoids in orbital mixing rarely moved in the Z direction, while organoids in vertical mixing showed movement in the Z direction as well as X and Y directions (Fig. 3c). These results suggest that the formation of the inverted brain organoids obtained in this study might be due to the dispersive and leveling action of the organoids produced by vertical mixing and the low resistance that is applied to the movement of
the organoids themselves. Besides, the turbulent energy of vertical mixing is higher than that of orbital mixing, suggesting the necessity of appropriate stimulation that the cells receive from the fluid in the induction of differentiation.

**Direction of primary in inverted brain organoids.** To reveal the link between fluid dynamics and cellular mechanosensing in vertical and orbital mixing, we performed immunohistochemical analysis of the primary cilium, the special sensory organelle in eukaryote cells, which receives signals from the environment and transduces them to the cell\(^1\)\(^9\),\(^2\)\(^0\) and plays an important role in control proliferation, migration and neural patterning\(^1\)\(^9\),\(^2\)\(^0\).

The primary cilium marker ARL13B, a small G protein localized in the cilia membrane, in SOX2-positive neural progenitors was investigated. Neuroepithelial cells lining the ventricle of
Fig. 1 Brain organoid generated by vertical mixing showed inverted structure in comparison with brain organoid generated by orbital mixing. 

- Schematic diagram of conditions used to induce brain organoid by orbital mixing (upper schema) and by vertical mixing (lower schema).
- Immunostaining for neural progenitor (SOX2, magenta) and neuron (MAP2, green) in brain organoid generated by orbital mixing (b) or vertical mixing (c) on Day 56.
- Quantification of SOX2-positive area in the peripheral region of brain organoid on Day 56. The peripheral region was defined as 100 µm inside from the edge of brain organoid. Brain organoid from vertical mixing showed higher percentage of SOX2-positive area in peripheral region in comparison with brain organoid from orbital mixing. Data represent mean ± SD (n = 6 for brain organoids by orbital mixing, n = 7 for brain organoids by vertical mixing). Difference between the two conditions was analyzed by Student’s two-tailed t-test (P < 0.0001).
- Immunostaining for markers of neural progenitors (SOX2, blue), intermediate neural progenitors (TBR2, gray), and cortical neurons (CTIP2, red; MAP2, green) in brain organoids by orbital mixing (e) or vertical mixing (f) on Day 56.
- Immunostaining for markers of ventricular neuroepithelial cells (N-CADHERIN: N-CAD, gray), neural progenitors (SOX2, magenta), and cortical neurons (MAP2, green) in brain organoids by orbital mixing (g) or vertical mixing (h) on Day 56.
- Note the apical side of organoids generated by orbital mixing is located inside organoids, while that of organoids generated by vertical mixing is located at the surface of organoids.

We focused on the cilia-related signaling pathway in the population of SOX2-positive cells, based on our findings that vertical mixing might facilitate the promotion of primary cilia in neural progenitors. Similar cell clusters were de

Gene expression analysis of inverted brain organoids by single-cell RNA sequencing. To analyze the cell types in inverted brain organoids, we performed single-cell RNA sequencing (scRNA-seq) of three organoids generated by orbital mixing and three organoids generated by vertical mixing on Day 90 of culture. Two thousand cells from each organoid were targeted, and in total 12,000 cells were analyzed. At first, we aligned and co-clustered the cells from organoids in both orbital and vertical mixing by Uniform Manifold Approximation and Projection (UMAP) algorithm and compared the expression of cells from each organoid (Fig. 5a). UMAP is the conventional dimensionality reduction of the data matrix of gene expressions in each cell. In the above process, cell clusters were defined using K-means clustering on principal component analysis (PCA) space, and the number of clusters was decided using the elbow method. Organoids in both orbital and vertical mixing enriched neuron marker-positive cells, and detailed distributions in UMAP were presented in Fig. 5b. Inverted brain organoids also presented primary cilia to the lumen of neural tube-like structures (Fig. 4a, Supplementary Fig. 3a, b). On the other hand, these cells in vertical-mixing organoids possess primary cilia toward the outer space of organoids (Fig. 4b, Supplementary Fig. 3c, d). We analyzed the direction of the primary cilia, calculated as the cilia angle between the cilia body and migration direction of neural progenitors, detected by co-staining with pericentrin, a protein localized at the centrosome (Fig. 4c, Supplementary Fig. 3e, f). While the primary cilia in neural progenitors of orbital-mixing organoids showed mainly two directions, those of vertical-mixing organoids showed random distribution in various directions (Fig. 4d). These data suggested that fluid dynamics in the bioreactors have a great impact on the inside-out or outside-in structure of iPSC-derived brain organoids via the direction of primary cilia in neural progenitor cells.

Enrichment of GABAergic neurons in inverted brain organoids. Immunostaining presented ventral neural progenitors on Day 56 and GABAergic neurons on Day 90 in both orbital mixing and vertical mixing, but with a predominant number in vertical mixing (Fig. 7a, b), consistent with the gene expression analysis (Figs. 5b, 6a, b, Supplementary Fig. 4a). Furthermore, we evaluated the generation of excitatory neurons and GABAergic neurons along the time axis in orbital mixing and vertical mixing, and found that vertical mixing might facilitate the promotion of GABAergic neuronal differentiation (Supplementary Fig. 5). These data suggested that the inverted brain organoid is a characteristic brain organoid that harbors a unique structure and special cell composition.

Disease modeling by inverted brain organoids. Several items of evidence have emerged to support the notion that alteration of GABAergic circuits contributes to Alzheimer’s disease (AD) pathogenesis by disrupting the overall network function. In order to utilize the inverted brain organoid for disease analysis, we generated inverted brain organoids from iPSCs derived from a healthy control subject and a familial AD patient carrying the deletion of E693 in APP protein, APP E693A. After 2 months of culture in vertical mixing, brain organoids showed the inverted structure with the expression of hippocampal maker PROX1, consistent with a single-cell RNA-seq (Fig. 8, Supplementary Fig. 4b). Aβ oligomer accumulation was observed in MAP2-positive neurons of AD brain organoids (Fig. 8), suggesting that the inverted brain organoids could be a disease model in vitro.

Discussion

Brain organoids are a self-organization of three-dimensional aggregates resembling brain structures generated from human
Fig. 2 Inverted organoids exhibited functional properties. **a** Timeline of the experiments. **b** A representative phase-contrast image of clumped organoids on MEA electrodes. **c** Quantification of spike frequency. Treatment with 100 μM PTX increased the spike frequency, and the addition of 50 μM AP-5 and 50 μM CNQX decreased it. ANOVA, p < 0.05, *post hoc p < 0.05, n = 3 organoids cultured on independent dishes. Pre: pre-treatment. **d** ASDR plots and individual raster plots for all 64 electrodes.
iPSCs. Although brain organoids recapitulate many key features of human brain development, the mechanism that controls the formation of brain organoids is still not fully understood. In this study, we produced inverted brain organoids that have a contradictory inside-out pattern of neural progenitors and neurons by the use of vertical mixing bioreactors. The fluid dynamics of the organoid and direction of a cellular mechanosensor, primary cilia, in the outside layer of neural progenitor cells were different between vertical and orbital mixing. scRNA-seq analysis revealed that the inverted brain organoids contained a neuronal area of GABAergic neurons, and it was applicable to the analysis of neurological diseases.

In order to determine the mechanism involved in the generation of inverted brain organoids, we analyzed the physical factors...
of fluid flow caused by vertical mixing. CFD analysis suggested that the turbulent energy of fluid flow might have a positive effect on the formation of inverted brain organoids. Moreover, the organoids in vertical mixing are equally dispersed throughout the culture tank due to the low drag force applied against them, suggesting that brain organoids in vertical mixing were floating freely in the low-stress flow of culture medium. Furthermore, the early cues may be imparted into iPSCs in the vertical mixing culture system that are retained long-term during their differentiation into brain organoids. On the other hand, the lack of extracellular matrix also might partially contribute to the inverted structure, although it was shown, in our study, that vertical mixing contributed to the generation of inverted brain structure. Previous studies used Matrigel, a source of many components of the extracellular matrix, as a scaffold for brain organoid growth and maturation. Nevertheless, the random distribution of the primary cilia direction and the alteration of cilia-related signaling detected by scRNA-seq in neural progenitors by vertical mixing suggest that fluid dynamics control the signaling pathway that regulates neural patterning. It is of interest that primary cilia activity is essential for the establishment of apical-basal polarity of the radial glial scaffold and that early neuroepithelial deletion of ciliary ARL13B led to the reversal of the apical-basal polarity of radial progenitors.

Our scRNA-seq data showed that inverted brain organoids have alterations in gene expression. Suspension cultures of iPSCs in the bioreactor have been shown to regulate not only cell aggregation, but also gene expression. This change might occur at the iPSCs stage and continue during the development of inverted brain organoids. We analyzed the characteristics of SOX2-positive cells from orbital mixing and vertical mixing by scRNA-seq and found that the gene expression patterns between them were different. SOX2-positive cells from vertical mixing exhibited the increased expressions of GABAergic progenitor markers DLX2 and NKX2.1, as well as the GABAergic neuron marker GAD2. These findings suggested that SOX2-positive cells from vertical mixing harbored different characteristics from those of orbital mixing. Although SOX2 is a marker for neural progenitors, its expression has also been described in differentiated neurons in various regions of the nervous system, and SOX2 expression in GABAergic neurons in mice is also reported. We consider that these references support our findings regarding the characteristics of SOX2-positive cells in organoids generated by vertical mixing. Furthermore, in the organoids generated by vertical mixing, the detection of GABAergic neurons by scRNA-seq with immunostaining is substantially increased in organoids generated by vertical mixing compared to that by orbital mixing. In the scRNA-seq data, we found altered gene expression that regulates cilia-related signaling in association with sonic hedgehog signaling in SOX2-positive cells. Previous studies have shown that abnormalities in cilia-related signaling can lead to changes in the migration of GABAergic neurons, suggesting an influence of cilia-related signaling in the development of GABAergic neurons. Therefore, we consider that alterations in cilia-related signaling with sonic hedgehog signaling activation may have contributed to the generation of GABAergic neurons. It is unclear whether the changes in cilia signaling-related genes are the cause or the result of the altered differentiation fate, but a previous report regarding a similar polarity of organoids shown in a midbrain organoid protocol with sonic hedgehog supported our findings.

In addition to the increase of GABAergic neurons, the results of single-cell analysis provided us with some insight regarding the fact that vertical mixing altered the composition of the cell.
population with cortical markers. There was a decrease in the number of deep cortical layer neurons in vertically mixed organoids as shown by the loss of TBR1 and SOX5. The relative increase in the number of GABAergic neurons resulted in a relative decrease in TBR1-positive cells, which are highly expressed in excitatory neurons\cite{38}, and SOX5-positive cells, which are specifically expressed in corticofugal neurons\cite{39}. On the other hand, the number of BCL11B-positive cells was retained, as they represent certain subtypes of inhibitory neurons\cite{40}. These alterations have adopted a ventral identity by vertical mixing. Taken together, the combination of physical factors might change the gene expression through the response of primary cilia, resulting in the changing neural patterning and differentiation fate in inverted brain organoids.

We analyzed a prominent feature of AD using inverted brain organoids. We are hopeful that these inverted brain organoids designed by controlling fluid dynamics will be a new model for studying human brain development and disorders.

**Methods**

**Ethics statement**. Generation and use of human iPSC were approved by the Ethics Committee of each institute. All methods were performed in accordance with the approved guidelines. Formal informed consent was obtained from a subject.
Maintenance of human iPSCs. Human iPSCs were maintained on a recombinant fragment of laminin-511 (iMatrix-511™, Nippi, Tokyo, Japan) by using StemFit AK02N medium (Ajinomoto, Tokyo, Japan). We used 201B7 iPSC line derived from fibroblasts of a healthy subject⁴¹ and APP1E111 iPSC line derived from fibroblasts of a patient with familial Alzheimer’s disease²⁹.

Generation of brain organoids by orbital mixing. iPSCs were cultured up to 80% confluence (typically 10 days after passage). iPSC colonies were dissociated into single cells after 4 min of incubation in 0.5 x Tryple Select/0.25 mM EDTA (Thermo Fisher Scientific, Waltham, MA) at 37 °C and suspended in StemFit

Fig. 6 Gene expressions of organoids analyzed by scRNA-seq were altered between orbital mixing and vertical mixing. a Heat map for brain lesion-related genes in total cells. b Heat map for genes altered in SOX2-positive cells.

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AK02N with 10 µM of Y-27632 (Nacalai Tesque, Inc., Kyoto, Japan) to count the number and viability.

iPSCs were transferred into an embryoid body (EB) formation medium (EB medium) consisting of DMEM/F12 (Thermo Fisher Scientific) with 20% Knockout Serum replacement (Thermo Fisher Scientific), 3% Fetal bovine serum (FBS) (Thermo Fisher Scientific), 1% Glutamax (Thermo Fisher Scientific), 1% non-essential amino acids mix (NEAA, Thermo Fisher Scientific), 1% penicillin/streptomycin (Thermo Fisher Scientific), 4 ng/ml basic FGF (Wako Chemicals, Osaka, Japan), and 50 µM Y-27632. Dissociated 9000 alive iPSCs in EB medium were disseminated into single wells of U-bottom 96-well plates (ultra-low attachment type, Nunclon™ Sphera™ microplates, 96U-Well Plate (174729), Thermo Fisher Scientific). The U-bottom 96-well plates were centrifuged at 200 × g for 3 min to make iPSCs aggregate quickly at the bottom of the well, and were kept in the incubator under a condition of 5% CO₂ at 37 °C. We defined the day of dissemination as Day 0. On Day 4, we replaced the medium with EB formation medium without basic FGF or Y-27632. On Day 6, we replaced the medium with neural induction medium consisting of DMEM/F12 with 1% N2 supplement (Thermo Fisher Scientific), 1% GlutaMAX, 1% NEAA, 1 µg/ml heparin (Nacalai Tesque, Inc.), and 1% penicillin/streptomycin. On Day 11, the outside of EBs became brighter and showed smooth edges. This appearance indicates that EBs contained radial organization. EBs were transferred into cold droplets of Matrigel™ (Corning, Corning, NY) on a sheet of Parafilm® M PM996, Bermis, WI) with small 3-mm dimples in a 10-cm petri dish and were incubated for 20 min in an incubator at 37 °C to allow Matrigel polymerization. After the polymerization step, the EB-Matrigel droplets were removed from the Parafilm sheet and transferred into Neuroepithelial expansion medium, which consisted of a

Fig. 7 Inverted brain organoid presenting GABAergic neurons. a Immunostaining with specific marker for ventral neural progenitor cells (NKX2.1, green) on Day 56. Organoids generated by vertical mixing exhibited NKX2.1-positive cells. Scale bars = 200 µm. b Immunostaining with specific markers for GABAergic neurons (GABA, red) and pan-neuron markers (MAP2, green) in brain organoid generated by orbital mixing and by vertical mixing on Day 90. Vertical mixing enriched GABA-positive cells. Scale bars = 50 µm.
1:1 mixture of DMEM/F12 and Neurobasal medium (Thermo Fisher Scientific) with 0.5% N2 supplement, 1% B-27 supplement without AO (Thermo Fisher Scientific), 1% GlutaMAX, 0.5% NEAA, insulin 2.5 µg/ml (9278, Sigma), 1% penicillin/streptomycin, and 0.1% Amphotericin B (Thermo Fisher Scientific). All 6-cm dishes were cultured in differentiation medium which consisting of 1:1 mixture of DMEM/F12 and Neurobasal medium with 0.5% N2 supplement, 1% B-27 supplement without AO (Thermo Fisher Scientific), 1% GlutaMAX, 0.5% NEAA, insulin 2.5 µg/ml, 1% penicillin/streptomycin, and 0.1% Amphotericin B. All 6-cm dishes were horizontally rotated on an orbital shaker (Cell Shaker, CS-LR 081704-000, Taitec, Saitama, Japan), equipped inside the incubator, at a rotating speed of 60 rpm. The culture medium was refreshed every 3 to 4 days.

From Day 40, EB-Matrigel droplets were cultured in differentiation medium with additional 1% Matrigel (growth factor reduced type, 354230, Corning). From Day 70, EB-Matrigel droplets were cultured in differentiation medium with additional 2% Matrigel (growth factor reduced type) and 2% B27 supplement without AO.

Culture brain organoids by vertical mixing. To establish vertical mixing under tight regulation of a stable temperature, pH, and dissolved O2 concentration, we utilized the vertically mixing bioreactor system HiD 4×4 (Satake Co. Ltd., Tokyo, Japan), with the controlling system of cultivation condition, S-BOX×02 (Satake) (Supplementary Fig. 1).

iPSCs were cultured up to 80% confluence (typically 10 days after passage). iPSCs cells were dissociated into single cells after 4 mm in culture and suspended in 0.5× Tryple Select/0.25 mM EDTA (Thermo Fisher Scientific) in a 37°C incubator, and suspended in StemFit AK02N with 50 µM of Y-27632 (Nacalai Tesque, Inc.) for number counting and viability determination.

Dissociated 2.5 × 106 cells in 250 ml of StemFit AK02N plus 50 µM of Y-27632 were dissociated into a single-use bottle (Supplementary Fig. 1) specialized for suspension culture under continuous vertical mixing of the HiD 4×4 system (Satake) at a setting of 60 mm/s speed and 15-mm stroke with continuous air flow of 30 ml/s. After 7 days of cultivation in StemFit AK02N medium, disseminated iPSCs formed spheres of homogeneous size (~100–150-µm diameter), and the medium was refreshed with EB formation medium as described above. We defined the day of medium refreshment as Day 0. On Day 6, we replaced the medium with Neural induction medium, as described above. On Day 17, we replaced the medium with Neuroepithelial expansion medium, as described above. On Day 25, we replaced the medium with Differentiation medium, also as described above. Sphere cultivation in the vertical mixing system was continued up to Day 90, with weekly medium change under tight regulation of pH 6.5–7.5, O2 concentration 20%, and temperature 32–34°C.

**Histological and immunohistochemical analysis.** Tissues were fixed in 4% paraformaldehyde for 15 min (1-mm organoids) or 30 min (3-mm organoids) at room temperature, followed by three times washing in PBS for 10 min. Tissues were allowed to sink in 30% sucrose overnight and were then embedded in O.C.T. compound (Sakura Finetek, Tokyo, Japan) and quickly frozen in liquid N2. Frozen tissues were cut into 12-µm slices by cryostat (CM1850, Leica Biosystems, Wetzlar, Germany) at −18 to −20°C. For immunohistochemistry, sections were permeabilized in 0.5% Triton-X100/ PBS (0.5% PBST) for 30 min at room temperature, and were then blocked in solution consisting of 1% PBST with 10% normal donkey or goat serum for 2 h at room temperature. Sections were then incubated with primary antibodies in blocking solution at 4°C overnight. These antibodies were used: anti-SOX2 (AB2018, R&D System, 1:1000), anti-β3-Tubulin (D1G9, Cell Signaling Technology, 1:500), anti-MAP2 (ab5392, Abcam, 1:3000), anti-TBR2 (ab23345, Abcam, 1:1000), anti-Pericentrin (ab28144, Abcam, 1:200), anti-SOX2 (14-9811-82, eBioscience, 1:400), anti-NKX2.1 (MAB5460, Merk, 1:500), anti-GABA (A2052, Sigma, 1:300), anti-VGLUT1 (135303, Synaptic Systems, 1:150), anti-CD31 (A2052, Sigma, 1:300), anti-APOE (14-9811-82, eBioscience, 1:400), anti-NKX2.1 (MAB5460, Merk, 1:500), anti-CD133 (ab28144, Abcam, 1:200), anti-Sox2 (ab2018, R&D System, 1:1000), anti-β3-Tubulin (D1G9, Cell Signaling Technology, 1:500), anti-MAP2 (ab5392, Abcam, 1:3000), anti-TBR2 (ab23345, Abcam, 1:1000), anti-Pericentrin (ab28144, Abcam, 1:200), anti-SOX2 (14-9811-82, eBioscience, 1:400), anti-NKX2.1 (MAB5460, Merk, 1:500), anti-GABA (A2052, Sigma, 1:300), anti-VGLUT1 (135303, Synaptic Systems, 1:150), anti-VGLUT1 (135303, Synaptic Systems, 1:150), anti-VGLUT1 (135303, Synaptic Systems, 1:150), anti-VGLUT1 (135303, Synaptic Systems, 1:150), anti-VGLUT1 (135303, Synaptic Systems, 1:150), anti-VGLUT1 (135303, Synaptic Systems, 1:150), anti-VGLUT1 (135303, Synaptic Systems, 1:150), anti-VGLUT1 (135303, Synaptic Systems, 1:150).
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