Development of Brain Organoids with Genome-Edited iPSC-Derived Brain Cells

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2.1 Human-Cell Based Cerebral Organoids for Advanced Brain Research

Why do we need a proxy of the human brain? The brain is the most complex organ in the human body. Brain development is an extraordinary sequence of events and regulated by spatial and temporal factors. Impairments during this patterning process cause dysfunctions of the brain. Studies of human material in vivo are limited, due to scarce availability of the tissue. Therefore, the analysis of the human brain is mostly restricted to post-mortem and fetal tissue. These time points are a snapshot of either disease-related changes or early developmental processes, respectively.

The study of animal brains, mostly rodents, led to important insights into brain development and understanding of evolutionary similarities between species. Nevertheless, there are major interspecies differences between mice and men. One of these obvious differences is a 50-fold larger brain size and gyrification, the folding of the cerebral cortex in humans. There are limits to modeling human physiology and metabolic processes in animal models.[1] For example, in 80% of neurodevelopmental disease the underlying pathological process still remains unknown.[2,3]

[1] Kuzawa et al. (2014), "Metabolic costs and evolutionary implications of human brain development".

[2] Mariani et al. (2015), "FOXG1-dependent dysregulation of GABA/glutamate neuron differentiation in autism spectrum disorders".

[3] Coe et al. (2012), "The genetic variability and commonality of neurodevelopmental disease".

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Scientists strive to overcome these limitations of animal models and tissue accessibility by designing new human in vitro models. The goal is to understand human pathogenic variants and disease processes and their complex impact on human disease. A first step forward in human disease modeling was the discovery that in vitro human embryonic stem cells (hESC) can be differentiated into neurons. While hESC raise ethic concerns, a major breakthrough was the discovery of reprogramming. Researchers in 2007 demonstrated the capability of somatic cells to be reprogrammed back into a state of pluripotency, to induced pluripotent stem cells (iPSC).[4] Similar to hESC cells, human-derived iPSC (hiPSC) can be differentiated into neural lineages. Both can be used not only for two-dimensional (2D) neuronal cultures but also are able to form 3D cell aggregates, the so-called brain organoids. Those hiPSC-derived human cerebral organoids serve as an alternative platform to animal models by resembling more closely the human brain and as a new tool for disease investigation, drug discovery, and personalized treatment.

Section 2.2 will provide basic insights into the technology of generating hiPSC (2.2.1) and hiPSC-derived 3D cerebral organoids (2.2.2). Section 2.3 will recapitulate the profile and characteristics of hiPSC-derived cerebral organoids by looking at key developmental events of the cerebral organoid's structure (2.3.1) regarding cytoarchitecture and cell diversity (2.3.1.1) and maturation and circuit formation (2.3.1.2). Part 2.4 will question if human cerebral organoids are human brains in a dish and will discuss the current biological limitations.

2.2 Generation of Human Cerebral Organoids

2.2.1 The Use of Pluripotent Stem Cells

The groundwork for in vitro modeling of human brain cells came from the capability of differentiating them from hPSC inspired by developmental processes. The first human in vitro model was established by Thomson et al. 1998, who cultured hESC from human embryos starting at the blastocyst stage.[5] ESC have the unique property of pluripotency. Pluripotency implies the unlimited capacity of self-renewal and the ability to differentiate into the three primary germlayers (ecto-, endo-, and mesoderm) and further into somatic or finally differentiated types of cells. The usage of hESC not only led to tremendous advances in understanding human development, drug development, and cell therapy, it also raised enormous ethical concerns.[6]

In 2007, scientists from Japan succeeded to generate hiPSC from specific skin cells, the fibroblasts. They overexpressed specific transcription factors, the so-called Yamanaka factors, which jumpstarted endogenous transcription factors for

[4] Takahashi et al. (2007), "Induction of pluripotent stem cells from adult human fibroblasts by defined factors".

[5] Thomson et al. (1998), "Embryonic stem cell lines derived from human blastocysts".

[6] de Wert and Mummery (2003), "Human embryonic stem cells: research, ethics and policy".

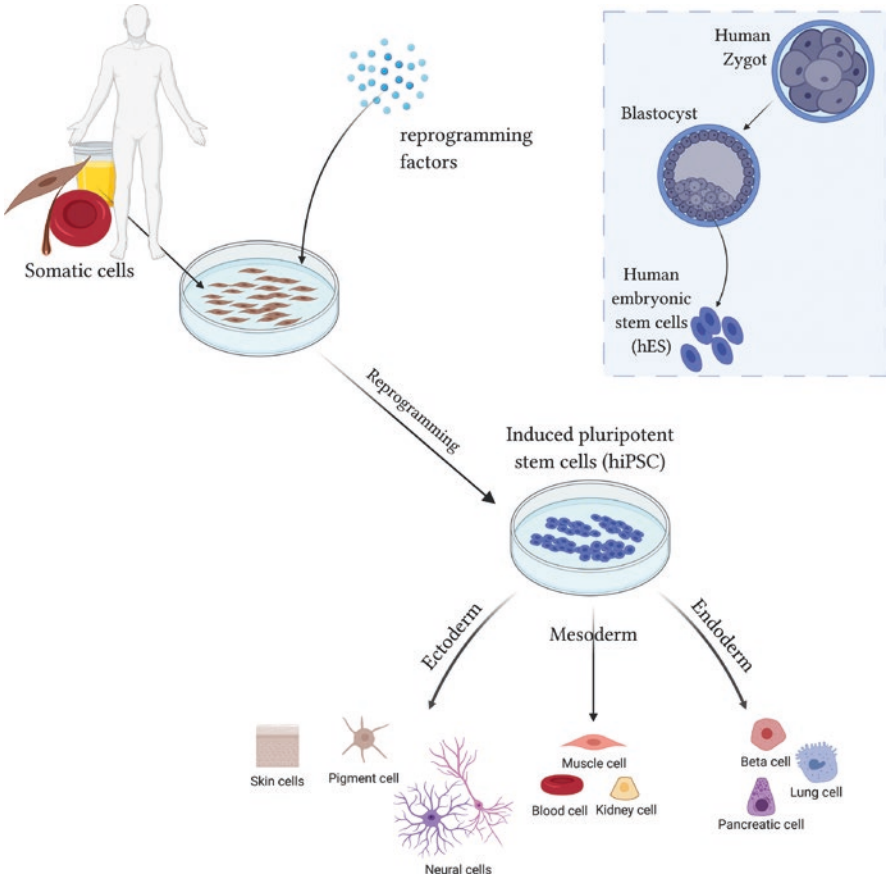

Fig. 2.1 Somatic cells (e.g. blood, urine, hair, or fibroblasts) from humans are reprogrammed by specific reprogramming factors into human induced pluripotent stem cells (hiPSC). hiPSC have the unique property to differentiate into all cell types from the three primary germ layers ecto-, endo-, and mesoderm. For differentiation into neural cells, e.g. neurons, hiPSC are guided to differentiate towards the ectodermal, more precise the neuroectodermal lineage. Neural cells are used in a bi-dimensional (2D) culture model of the human brain. Upper right box: Human embryonic stem cells (hESC) can be harvested from the inner cell mass of the blastocyst stage of the embryo at an early stage post-fertilization. Created with Biorender.com

pluripotency.[7] The fibroblasts could therefore be returned to a state of pluripotency, which is why the reprogrammed fibroblasts were called hiPSC. Moreover, similar to hESC, hiPSC can be differentiated into neural cells (Fig. 2.1). Neuronal differentiation is achieved by sequential application of exogenous growth factors, which first turn the hiPSC into a neural progenitor cell (an intermediate state), before it then

[7] Takahashi et al. (2007), "Induction of pluripotent stem cells from adult human fibroblasts by defined factors".

develops into a somatic brain cell (mostly neurons and support cells such as astrocytes).

On the one hand, the use of hiPSC has overcome significant ethical concerns raised by the use of human embryos to harvest hESC from the blastocyst stage (see Fig. 2.1 upper right box). Additionally, hiPSC-models serve as a complementary model to the rodent in vivo models and thus reduce the amount of animal testing. hiPSC-derived cells and their derivatives are an almost unlimited supply of human stem cells. hiPSC can be bio-banked and patient-specific hiPSC are readily available for disease modeling and drug screening. 2D modeling of brain disease using hiPSC-derived patient models led to new insights into neurodevelopmental, neuropsychiatric, and neurodegenerative diseases.[8][9] However, the human brain is not a layer of cells but a complex 3D structure.

A revolution in the field was Madeline Lancaster's observation that hiPSC are able to create 3D brain structures through self-organization even with just little external support.[10] This was the first step of the generation of hiPSC-derived 3D cell clusters, the so-called human cerebral organoids. One example of an important insight gained through cerebral organoid research was the delineation of the interaction of Zika virus and microcephaly.[11] Moreover, this technique led to novel insights into understanding neuronal heterotopia in patients, for example, with rare neurodevelopmental variants in DCHS1 and FAT4.[12]

2.2.2 Self-Organization into 3D Cerebral Organoids

Cerebral organoids are complex 3D structures with heterogeneous tissues resembling various regions of the brain. They are produced much as other 3D multicellular structures resembling eye, gut, liver, or kidney. Knoblich and Lancaster pioneered the field by relying on the cell's intrinsic development programs and self-patterning ability to generate the so-called human whole-brain or human cerebral organoids.[13] The process is as follows: hiPSC are instructed to aggregate to little balls called Embryoid Bodies (hEBs). The floating hEBs (Fig. 2.2 Left) are then confronted with a specific medium composition, which forces the development of the neuroectoderm[14] layer (Fig. 2.2 Right). These neural aggregates are then placed

[8] Brennand et al. (2011), "Modelling schizophrenia using human induced pluripotent stem cells".

[9] Prots et al. (2018), "U-Synuclein oligomers induce early axonal dysfunction in human iPSC-based models of synucleinopathies".

[10] Lancaster et al. (2013), "Cerebral organoids model human brain development and microcephaly".

[11] Qian et al. (2017), "Using brain organoids to understand Zika virus-induced microcephaly".

[12] Klaus et al. (2019), "Altered neuronal migratory trajectories in human cerebral organoids derived from individuals with neuronal heterotopia".

[13] Lancaster et al. (2013), "Cerebral organoids model human brain development and microcephaly".

[14] The neuro-ectoderm layer consists of cells derived from the ectoderm, the formation of which is the first step in the development of the nervous system and in which the neural tube is developed in the embryo.

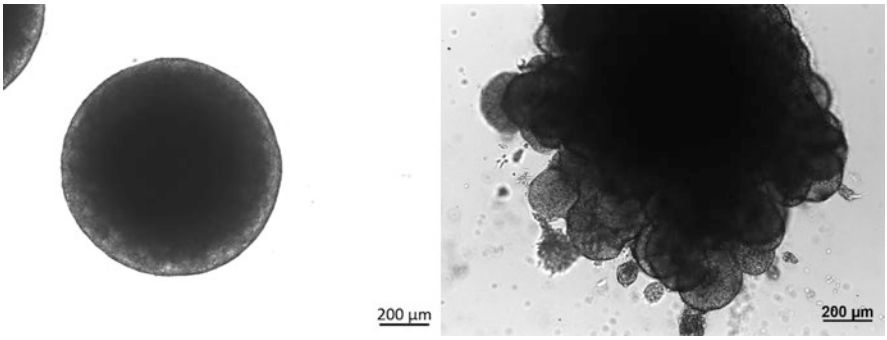

Fig. 2.2 **Left:** hiPSC aggregate to hEBs in floating condition. The formed hEBs are characterized by their circular shape. Image of 8 days in culture. **Right:** The neural induction leads to the forming of a neuroectodermal layer on the surface of the hEBs with bud formation. Image was taken after 15 days in culture. Source: Johanna Kaindl, Department of Stem Cell Biology, Erlangen

in gel droplets (Fig. 2.3). The gel as a matrix provides, on the one hand, both a 3D support and, on the other hand, regulates the proliferation, differentiation, distribution, and migration of neural progenitor cells.[15] While cerebral organoids can be generated by self-organization in a whole-brain organoid,[16] patterned organoids, containing different brain regions such as the forebrain, midbrain, and hindbrain, can be guided to form region-specific tissue of interest with specific sets of signaling molecules.[17,18]

2.3 Profile and Characteristics of Cerebral Organoids

To assess the cellular composition of cerebral organoids and ultimately to compare them to the human brain, sophisticated techniques are required. One of these widely used technologies is single-cell RNA-sequencing (scRNA-seq: see Box 2.1, 2.3). It can be used to analyze the genetic profile of every single cell independently. When cerebral organoids are studied at different time points of differentiation, developmental steps and fates of cell populations can be followed up. Ultimately, this technique enables researchers to study and compare the cellular composition and gene expression of organoids and human brains on a single cell level.[19]

[15] Long and Huttner (2019), "How the extracellular matrix shapes neural development".

[16] Lancaster et al. (2013), "Cerebral organoids model human brain development and microcephaly".

[17] Pasca et al. (2015), "Functional cortical neurons and astrocytes from human pluripotent stem cells in 3D culture".

[18] Sakaguchi et al. (2015), "Generation of functional hippocampal neurons from self-organizing human embryonic stem cell-derived dorsomedial telencephalic tissue".

[19] Quadrato and Arlotta (2017), "Present and future of modeling human brain development in 3D organoids".

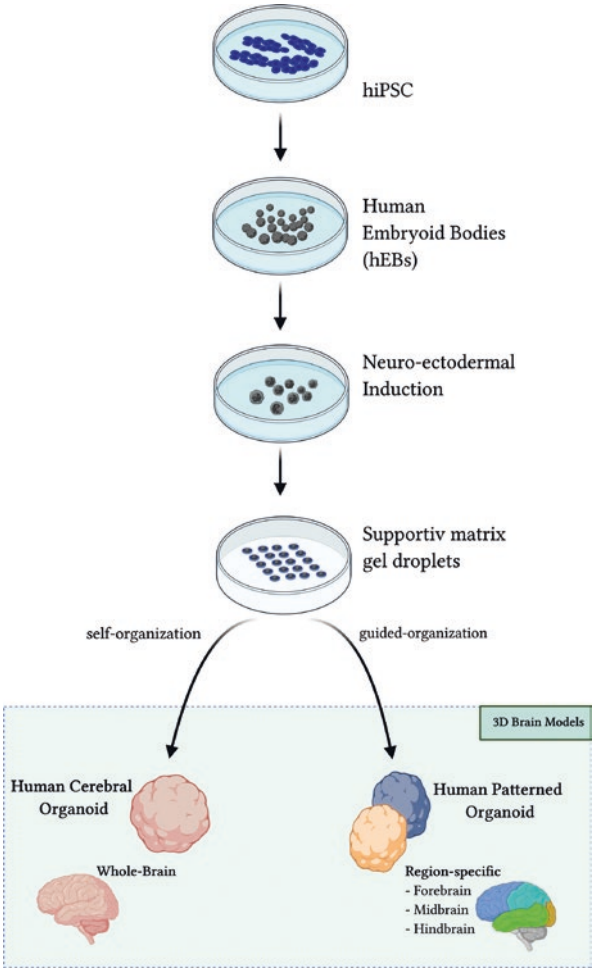

Fig. 2.3 Cerebral organoids as 3D brain models in vitro can be generated from human neural aggregates, the so-called human embryoid bodies (hEBs), which are aggregations of hiPSC induced by specific signaling molecules. The hEBs are cultivated in a specific medium which allows only the neuroectodermal layer to form further. Depending on the 3D brain model, these neuroectodermal aggregates are either placed in droplets of a gel, which serves as a supportive matrix or are cultured in a suspension. Cerebral organoids or whole-brain organoids are generated through self-organization whereas patterned organoids (e.g. forebrain, midbrain, hindbrain) are generated through patterning factors in a guided organization. Representation not true to scale. Created with Biorender.com

Box 2.1: Background to Single-Cell RNA-Sequencing

The technique of single-cell RNA-sequencing (scRNA-seq) is used to enable analytical approaches to characterize organoids. Whereas initially only a few cells could be extracted and analyzed, the number now exceeds more than 1.000.000. Even though there are many different techniques, they generally have the following steps in common: The physical extraction of viable single cells, the subsequent opening of the cells and extraction of the RNA (RNA, ribonucleic acid), to translate the RNA back into cDNA (cDNA, complementary deoxyribonucleic acid), and finally the generation of large sequencing data of the genome. Concerning the study of organoids, questions can be answered such as: which cell types and brain regions are found in the organoid? How

"mature" are the cells, what stage of development do they correspond to compared to the human brain? Which gene expression pattern do they show means which genes are controlling the developmental processes and in which subsets of cells are those genes expressed in. A small overview of possible questions that can provide a lot of information about the 3D model's properties.

The following subsections will recapitulate key developmental features of cerebral organoids and deal with the achievements and limitations. We will specifically focus on cortex-like structures in human cerebral organoids since a large number of datasets are available from scRNA-seq analyses of the cortex.

2.3.1 Recapitulation of Key Developmental Events and Limitations

2.3.1.1 Cytoarchitecture and Cell Diversity

Cerebral organoids measure about four millimeters (Fig. 2.4) and are therefore much smaller than the human brain. Nevertheless, remarkable similarities can be discovered upon closer inspection of cytoarchitecture, cell types, and regional identity. The human brain develops from the neural tube, it then forms ventricles, which are cavities filled with fluid. These ventricles are developmentally important since they home a neural stem cell population. In the human developing brain, neural stem cells divide and generate neural progenitor cells that migrate from the proliferative zones around the vesicles towards the cortex. A special type of cells, the radial glial cells, plays a decisive role here, as they guide the neurons to their correct place in the six-layered cortex and contribute to the generation of glial cells[20] and neurons.

Ventricle-like structures also occur in cerebral organoids (Fig. 2.5). The ventricle-like structures in cerebral organoids also contain proliferative zones of neural progenitor cells. In organoids, a comparable migration of neural progenitor cells derived from proliferative zones can be observed. They also form a layered structure of neurons comparable to the layering in the cortex. Moreover, regional specific cell types, such as the above mentioned radial glial cells, emerge spontaneously in organoids, and they even emerge similar to the timeline in the fetal brain. Similar to human brain development, the neural progenitor cells of the cerebral organoid intrinsically control the generation of specific subsequent cell types.[21][22]

[20] Glia, also called neuroglia, are non-neuronal cells in the central nervous system that do not generate electrical impulses but make a decisive contribution to homeostasis, functionality, and protection of neurons.

[21] Kadoshima et al. (2013), "Self-organization of axial polarity, inside-out layer pattern, and species-specific progenitor dynamics in human ES cell–derived neocortex".

[22] Quadrato et al. (2017), "Cell diversity and network dynamics in photosensitive human brain organoids".

Fig. 2.4 Cerebral organoids grown from human stem cells. They are up to four millimeters in size and are cultivated as swimming cell clumps in laboratories. These 3D brain models develop similarly to a human brain. The cultivation of these organoids requires a regular supply of nutrient media and constant movement of the cell clumps. Organoids can currently be cultivated for up to a year. Image: Stem Cell Department, Universitätsklinikum Erlangen

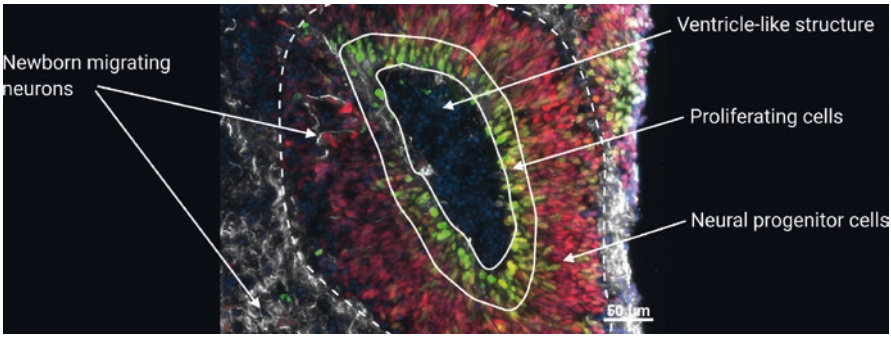

Fig. 2.5 Cross section of a 33-day-old human cerebral organoid, showing a ventricle-like structure (white dashed). In the center is a structural recess. In a thin zone, proliferating cells (green, solid line) are deposited, which are similar in arrangement and cell occurrence in the fetal brain. Neural progenitor cells can be found in the area above (red, between dashed and solid line), in which isolated migrating newborn neurons are shown (white). Source: Johanna Kaindl, Department of Stem Cell Biology, Erlangen

While major similarities in specific cell types were found between the human brain and cerebral organoids,[23] the relative numbers are different. The architecture of the neural tissue in cerebral organoids is an approximation. The human brain has more than 80 billion neurons and the same number of other cell types.[24] An organoid

[23] Quadrato and Arlotta (2017), "Present and future of modeling human brain development in 3D organoids".

[24] Von Bartheld Christopher and Bahney (2016), "The search for true numbers of neurons and glial cells in the human brain: A review of 150 years of cell counting".

consists of around two to three million cells. Some cell populations are missing in cerebral organoids. One example here are microglia, "the innate immune system of the brain." Microglia have a different developmental origin from mesoderm, therefore, microglia cannot be generated simultaneously with the current protocols for cerebral organoids. Instead, they can be added in co-cultures with organoids if required. These experiments already showed that added microglia can migrate into the organoids.[25] Another important cell population, which is missing in cerebral organoids, are endothelial cells. They form the vasculature and enable the blood supply to the human brain. Since these are not emerging in the organoid protocols, and organoids do not have blood supply, but rather depend on permeability, the larger organoids can suffer from an insufficient supply of oxygen and nutrients. As a result, organoids can only be cultivated for a certain period, as they develop a necrotic nucleus during cultivation, which leads to cell death in the nucleus and the limitation of the organoid's size. A groundbreaking proof of concept study from the Gage laboratory transplanted cerebral organoids into a mouse brain. Interestingly, they observed that the mouse vasculature started perfusing the cerebral organoid and led to improved survivability.[26] Thus, the resulting limitations in size, tissue architecture complexity, and maturation are current disadvantages.

Moreover, meninges and skull, which define the limits of the human brain, are also missing. Cerebral organoids also lack a body axis, which in the embryo provides orientation and enables growth and inhibiting factors to have region-specific different influences. Therefore, cerebral organoids currently form brain-like regions but do not reflect the spatial arrangement. Further steps will be needed for the standardization of organoid development to standardize culture conditions and organoid handling, as well as their size and morphology.[27]

2.3.1.2 Maturation and Formation of Circuits

The human brain not only consists of billions of brain cells. More importantly, they are interconnected in different ways in a complex network. They communicate with each other via electrical signals, transfer chemical molecules via millions of synapses and render the brain the most complex organ of the body. However, how similar are organoids and the brain regarding functional properties? Transcriptional analyses, which compared the genetic expression of the in vitro cortical cells of cerebral organoids with those in vivo, concluded that the organoid's cells resemble those of primitive fetal brain during the second trimester of gestation.[28] When using isolated patterned organoids of specific regions, their connections are limited. Specifically, connections between brain regions, the so-called projection, are missing. Circuits are for example building the sensory system, where sensory information from the skin, such as heat, cold, or pressure, is transmitted via electrical

[25] Abud et al. (2017), "iPSC-derived human microglia-like cells to study neurological diseases".

[26] Mansour et al. (2018), "An in vivo model of functional and vascularized human brain organoids".

[27] Kanton et al. (2019), "Organoid single-cell genomic atlas uncovers human-specific features of brain development".

[28] Camp et al. (2015), "Human cerebral organoids recapitulate gene expression programs of fetal neocortex development".

impulses to the sensory cortex. This input can trigger a chain of reactions, including pain or motor movements. Organoids have no sensory input or motor output.

In 2017 the Arlotta laboratory showed that neuronal activity in organoids can be controlled by stimulation of photosensitive cells by light.[29] Another striking discovery came from the Muotri laboratory in 2019. When analyzing cerebral organoids using a multi-electrode system, they registered electrical activity indicative of network function in cerebral organoids. These network curves recorded from the cerebral organoids closely resembled electroencephalogram curves of premature babies which could be an indication for similarities between the stage of maturation and network function.[30] Due to the fact that not all regions are generated in a standardized way, we cannot speak of inter-regional cell interaction and communication, let alone of electrical impulse transmissions. Only very small local measurements were taken which cannot represent extensive inter-regional communication. To counteract this, two or more organoids from different regions can already be fused to the so-called assembloids in order to enable the organization of inter-regional connections.[31]

The aforementioned achievements are outstanding and raise the question of whether organoids will demonstrate many more similarities and functional properties of the human brain in the future.

2.4 Can We Talk of Human Brains in a Dish?

While cerebral organoids are a major advance in human disease modeling in neuroscience and specifically an important bio-assay tool for a research focus on early brain development, the limitations need to be taken into account and still pose great hurdles for science. Therefore, from a biological point of view, one cannot speak of "brains in a dish." Why? Cerebral organoids currently do not represent an exact model of the human brain. The smaller size, the less organized shape, and the lack of some functionally important cell types such as vascular cells or microglia are obvious differences. Functionally, differences in neural network activities and the lack of sensory inputs limit the current cerebral organoids to an immature in vitro model. Thus, at this point, an exact imitation of brain development and function is still a future perspective.

Time to speculate about characteristics and functional properties that cerebral organoids will be able to mimic in the future: technical innovations promise more complex and functional organoids. First, standardizing the protocols of organoid generation concerning efficiency and replicability is still ahead. This implies improving oxygen and nutrient supply, to allow the generation of more mature or aging organoids and thus to examine functional characteristics of neurodegeneration. By

[29] Quadrato et al. (2017), "Cell diversity and network dynamics in photosensitive human brain organoids".

[30] Trujillo et al. (2019), "Complex oscillatory waves emerging from cortical organoids model early human brain network development".

[31] Birey et al. (2017), "Assembly of functionally integrated human forebrain spheroids".

simulating a polarity and proper diffusion of the specific signal factors in a gradient similar to that in the brain, researchers hope to enable a correct arrangement of the expressed brain regions in the organoids and to recreate regional networks.

New techniques such as CRISPR/Cas9 genome editing improved the options to selectively study the effect of single pathogenic variants. Since the scRNA-seq technique has the restriction of missing some localization information, new techniques that also allow visualization of the respective cell at its original spot are the next step. To provide organoids with a blood supply, attempts have been made to implant organoids in mice. A vascularization and survival of the organoids were achieved.[32] The next step along this journey will be to implant endothelial cells and provide blood supply in specific chambers. The combination of region-specific organoids in assembloids in a functional system could enable the simulation of the interconnectivity of different tissues. The Pasca laboratory has already developed a working model of the motor system, linking the motor cortex to muscles in a tripartite circle. For this, Pasca brought organoid models for motor cortex, spinal connections, and skeletal muscles together. The individual models fused on their own to assembloids, showing contractions of the muscle cells.[33]

Cerebral organoids are a great platform and already boost research into neurodevelopmental and brain affecting diseases (Box 2.2: Studying SARS-CoV-2 Infection in Cerebral Organoids). But the more similar the organoid and the brain become, the louder the question of whether they also have a sense of consciousness. Although not every new finding will lead to new biological or translational insights, some results might be game-changing enabling the development of even more complex and voluminous cerebral organoids. In this regard, ethical discourse is important and legal and ethical guidance provided to scientists is crucial. Cerebral organoid research raises many difficult questions and concerns not only scientists, but also researchers, state organs, pharmaceutical companies, and the general public. In cooperation and common endeavors, the difficult obstacles and questions can be worked out and, at the same time, it will be possible to enable new and groundbreaking insights into the human brain.

Box 2.2: Studying SARS-CoV-2 Infection in Cerebral Organoids

Numerous clinical reports show neurological symptoms in patients with SARS-CoV-2 infection. However, it is still unclear whether the virus directly affects and damages neurons. To investigate whether a SARS-CoV-2 infection of the human brain is a reason for symptoms of neurological anomalies, researchers make use of human cerebral organoids by infecting them with the virus. The aim is to find out which cell types the virus attacks, what potential the virus has to cause further neurological defects and, ultimately, how

[32] Mansour et al. (2018), "An in vivo model of functional and vascularized human brain organoids".

[33] Andersen et al. (2020), "Generation of Functional Human 3D Cortico-Motor Assembloids".

cerebral organoids can contribute to the development of therapy in the SARS-CoV-2 pandemic. Cerebral organoids can serve as a promising, viable, and safe test system to study direct neurotoxic effects resulting from a SARS-CoV-2 infection, as well as other viruses.

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