Commentary

Human cerebral organoids and neural 3D tissues in basic research, and their application to study neurological diseases

Theresa S P Rothenbücher*,1 & Alberto Martínez-Serrano*,1

1Department of Molecular Biology, Univ. Autónoma de Madrid; & Department of Molecular Neuropathology, Center of Molecular Biology Severo Ochoa (CBMSO, UAM-CSIC), Nicolás Cabrera 1, 28049 Madrid, Spain
*Authors for correspondence: amserrano@cbm.csic.es

Research with neural tissue in 3D is receiving great attention recently, since 3D offers a more physiological setting than traditional 2D cultures. In this commentary we overview and compare cerebral organoids and other 3D aggregated cell cultures. Last we briefly summarize examples of neurological diseases, mostly neurodevelopmental, where organoids are starting to be of help to understand the disease etiology, and in some cases to design therapies.

In vitro studies of neural tissue development, physiology, biochemistry, and molecular and cell biology are presently understood to be more relevant if experimental procedures are conducted in 3D as compared with 2D (monolayer cultures). The rationale behind this notion resides in the mutual interaction of cells in 3D, the possibility for the cells/tissue to generate its own extracellular matrix, and the cells' capacity to establish 3D projections and multiple interactions, and that this would yield tissue constructs and organoids that better resemble the *in vivo* developmental processes, and the organization of functional, mature tissues, as well as the physiology and pathophysiology of neurodevelopmental processes, possibly including aging.

The 3D human neural tissue culture dates back to decades ago, early 1990s, when Weiss and collaborators described the generation of human neurospheres (spheroids), comprised of human neural stem cells, co-existing with differentiated cells [1]. The neurosphere culture method rapidly expanded and was extensively used, until presently. Interestingly, by that time and for many years, the focus was on how to proliferate the recently identified neural stem cells, rather than on the cytoarchitecture of the (putatively) self-organizing neural tissues in neurospheres. As a matter of interest, at that time the term 'neural stem' was not even widely accepted and therefore it was important to demonstrate their successful proliferation in culture. Consequently, neurosphere cultures were rendered as an experimental tool to proliferate the few neural stem cells that remained in such stem cell stage. Later on, the capacity to generate neurospheres, was used in clonogenic assays, to identify neural stem cells in a given neural cell preparation. But little, if any, attention was paid to the 3D organization of developing neural tissue in the 3D neurospheres.

With the availability of pluripotent stem cells (PSCs) by the end of the 90s, a renewed interest in 3D cultures arose. The trigger for this revival was the PSCs' characteristic of pluripotency (understood as the capacity to give rise to cell derivatives of different embryonic germinal layers, that is, endoderm, mesoderm and ectoderm). The new angle was to let the cells develop into different tissues, after providing them with the appropriate developmental signals. Subsequently, during the last decade, different groups managed the development of organ-like tissues in 3D cultures of PSCs, that resembled some of the histological features, tissue architecture and some basic functionality of their *in vivo* counterparts (e.g., intestine, retina, cerebral cortex, . . . ; [2–5]).

More recently, we have seen an exponential rise of published studies using 3D human neural cell cultures, ranging from simple cell aggregates (lacking tissue cytoarchitecture, cellular self-organization), to other rather organized tissues generated from embryoid bodies. It is important to highlight at this point that organoids are much more complex than simple 3D aggregates of cells. An organoid is generated through a series of complex developmental decisions and continuous processes, combined with a progressive restriction of proliferation of the

progenitors responsible for the generation of such organ-like structure. Therefore, the successful *in vitro* organ-like development is the result of a highly complex-combinatorial, time-restricted contribution of signals and events. In other words, it is the re-action of the developing tissue to the provided cues, resulting into decreased proliferation while simultaneously and progressively integrating developmental signals, which prompt migration, differentiation and maturation that together render an organ-like histological structure. All of these processes are similar to the events during embryonic development *in vivo*. The 3D cellular aggregates, on the other hand, are more simple to generate and less likely to integrate any development signals or to show cellular self-organization. However, they constitute good models for the quick assessment of cell biology in 3D (see an example on Alzheimer's disease, below).

This is what we can conclude from our own work with both types of 3D tissues (Supplementary Figure 1), where we have compared 3D cell aggregates with real cerebral organoids, finding that their properties are essentially different, and therefore, their potential uses become different. In brief, real organoids recapitulate development and maturation, possibly aging, and are therefore useful to study biological processes related to these events, whereas 3D aggregates provide simple means to evaluate very basic questions in 3D, like neuron generation or neuron/glia differentiation and survival, constituting a good instrumental tool, but not recapitulating nerve tissue development.

Once the difference between cell aggregates and organoids has been discussed, we will explore the potential that the currently available cerebral organoid models offer for the investigation of neurological diseases, deficits, and also for basic neurobiology research.

Origins of the cerebral organoid technology

After seminal studies by the groups of Sasai, Clevers, Knoblich and Pasca [2–5], the available technical knowledge was coaxed into published procedures for the generation of differentiated neural/neuronal clusters or neuronal regions in 3D in cerebral organoids [5–7]. These 3D tissue constructs showed some regions containing neural tissue with a cytoarchitecture reminiscent of their *in vivo* counterparts (mostly cortical tissue, since this is the default phenotype according to those protocols). Therefore, those 3D tissues were described as 'cerebral organoids' or 'cerebroids' (colloquially referred to as 'mini-brains'). However, the term mini-brain is profoundly misleading, since these 3D tissues do not organize themselves into discrete regions showing interconnections resembling the developing brain.

When examining the histology of the cerebral organoids in detail, the outcome was on the one hand new and exciting, thus promising. But on the other hand, it became clear that the current cerebroids have limitations and there still remain many challenges to overcome before having a real human brain model. Multiple reports described the appearance of neuralized tissue, with some cellular organization, but the whole composition of such 3D tissue constructs did not resemble or was not reminiscent of a developing brain. In contrast, the appearance of discrete, regionally specified brain regions was very limited, and subjected to a stochastic process. In some organoids there were neural tissues, while in others the neural tissue did not appear. In some successful, neuralized, cerebral organoids, some markers of regionally specified neurons appeared, randomly and without control by the investigator. In some cases, groups of cells expressing cortical or hypothalamic neurons appeared, while in many other cases those regions did not appear [2,5,7,8]. This scenario calls for a better engineering by the experts. Engineers collaborating with biologists. Recently, a simple scaffolding procedure (randomly arranged, floating poly(lactide-co-glycolide) fibers to which the embryoid bodies attached) has been described, partially enhancing the reproducibility of generating successful cerebroids containing cortex-like tissue from human PSCs [8].

Essentially, we are at a very initial stage in the research process to decipher how to generate cerebral organoids really recapitulating the development of the human brain. For this challenging task, it is obvious that some type of scaffolding, providing regional and time-resolved developmental cues, physical support, together with an artificial or biological vascularization/perfusion, is needed. For some applications, the external provision of microglial cells will definitely be a must, when dealing with physiopathological processes mediated by microglia. It is unclear at the moment how to combine different cell types during the generation of successfully developing cerebral organoids.

After recognizing the exceptional research opportunities that cerebroids offer, since they recapitulate the generation of some histologically organized brain tissue, we need to adopt a critical approach. Presently, a cerebral organoid is an almost spherical structure (isotropic, no orientation landmarks, no polarization or developmental axis), where different regions develop/appear randomly, with no recognizable (brain) organ-like patterns, showing only discrete regions resembling parts of the *in vivo* brain organ.

Initial attempts to provide the cerebroids with some kind of regional patterning have been reported (e.g., for midbrain, forebrain or hypothalamic identity [9–11]; or striatal and cortical [12]). Currently used procedures for

cerebroid generation use culture media and inductive/differentiation/maturation cocktails optimized for their default differentiation toward the generation of cortical tissue. The studies aiming at generating other brain identities used procedures originally developed in 2D tissue culture to provide the cells with the appropriate, chronologically ordered, development cues, applied to the cells in the form of soluble factors/morphogens dissolved in the culture medium. These studies described a 'bulk' patterning, bathing the developing organoids with cocktails that thus convert the whole structure into, for example, hypothalamic or midbrain identity. This approach resulted in a tissue construct that did not recapitulate neither brain organ development, nor the brain circuitry. Regional induction existed, but it was not a true spatially resolved or organized patterning. The whole organoid became one or another identity. For developmental studies, an imaginative, elegant, alternative to this situation was to promote the fusion of differently patterned cerebroids, to study cell migration and the interconnections they could develop when becoming physically in contact [12–14]. Obviously, this was an instrumental strategy to get different regions (organoids) physically interacting, but distant from a truly spatially resolved patterning approach in a single organoid.

Examples of diseases approached using the cerebroid technology

In spite of technical hurdles and reproducibility related difficulties inherent to the cerebroid model system, researchers have successfully managed the study of the ethiology, as well as potential therapies, for diseases of high personal and social impact and burden.

Most investigations have used brain organoids to model neurodevelopmental diseases like microcephaly [5,15], autism [16,17], Rett syndrome [18], Miller-Dieker syndrome [19,20], schizophrenia [21], Sandhoff disease [22] or Zika virus (ZIKV) infection involving neurodevelopment [9,15,23–27]. Also neurodegenerative diseases like Alzheimer's disease (AD) [28] and the potential for modeling Parkinson's disease [11] were approached. Importantly, disorders that manifest during embryonic/fetal development are more suitable for modeling in brain organoids, since brain organoids, at the present stage, recreate particularly well the early brain development.

There are several methods available to obtain brain organoids recapitulating disease features. First, adult cells (e.g., skin) can be taken from patients and reprogrammed into induced PSCs (iPSCs). Furthermore, it is possible to introduce known mutations that are causally related of a disease into healthy cells. Also, in case of Zika virus for example, a viral infection of the organoid is performed to study modifications induced by the disease. In parallel to these disease models, organoids that were generated with healthy cell lines act as control for a comparison. Recent studies are briefly summarized below to illustrate how brain organoids may be useful tools in the field of neurological disorders.

In the case of microcephaly, Lancaster *et al.* were able to model some patterns of the disease. They observed premature neuronal differentiation that caused a reduction of early progenitors and led to an overall reduced size of the neuronal tissue compared with the (healthy) control organoids [29]. Gabriel *et al.* however, studied microcephaly, which was induced by Zika viral infection [15]. They observed very similar manifestations, which strengthen the reliability of modeling disease in brain organoids.

Several other studies investigated different strains of the Zika virus [9,15,23–27]. They all confirmed that a ZIKV infected brain organoid has a reduced size similar to microcephaly and shows increased apoptosis. Moreover, Li *et al.* highlight folding defects that were visible in ZIKV organoids [23], and Qian *et al.* reported a decreased neuronal layer thickness and an enlarged lumen [9]. Janssens *et al.* revealed that an African Zika strain modifies DNA methylation in developing neurons by disturbing the activity of DNA methyltransferase [27]. Yoon *et al.* identified the NS2A protein as responsible for reduced proliferation of radial glia cells in a Zika-infected brain by reducing adherens junctions [26]. Dang *et al.* found out that Zika virus activates the Toll-like receptor 3, which is part of the innate immune response, causing apoptosis and at the same time dysregulation of genes that steer neurogenesis, axon guidance and differentiation [25]. Taken together, these studies emphasize the utility of brain organoids to reveal new aspects of a human brain disease, which is the baseline on the way toward finding a therapy.

Mariani *et al.* studied glutamate neuron differentiation in brain organoids generated from cells of patients with autism spectrum disorder (ASD) [16]. They found, that in ASD the *FOXG1* gene is strongly expressed, which leads to an overproduction of inhibitory neurons (GABA neurons). The amount of glutamate neurons however, was not increased, which causes an imbalance between excitation and inhibition. Mariani *et al.* concluded that the *FOXG1* gene is thus a molecular signature of ASD and could represent a future target in ASD therapy. Wang *et al.* produced telencephalic organoids with a mutation in the *CHD8* gene to study its impact in autism and schizophrenia [17]. They found that *CHD8* regulates the expression of the genes *AUTS2* and *TCF4* involved in ASD and schizophrenia,

respectively. They highlight the possibility of identifying a drug target with the combined knowledge obtained from their and other studies.

Stachowiak *et al.* modeled schizophrenia with cerebroids and investigated the role of FGFR1 [21]. The patient-derived organoids showed a very different architecture compared with the control. Among others they displayed an abnormal premature subcortical neurogenesis, which was apparent by a disruption of the developmental strata. Ki67 positive (cycling) cells showed an increased proliferation by migrating from the ventricular zone into the intermediate and cortical zone, which led to a reduction of mature neurons in the outer zone. They concluded that these cells have failed to exit the cell cycle and consequently to differentiate. Due to this, the cortical neuronal cells have a very low level of nuclear FGFR1, which is known to induce a dysregulation of downstream gene expression crucial for neurogenesis. This would lead to cortical malformation during brain development of schizophrenia patients. Stachowiak *et al.* state that changes in interneuron directionality could be the reason for disrupted cortical interactions in schizophrenia [21]. Complementary, Ye *et al.* discovered with their patient-derived schizophrenia-forebrain organoids (in parallel to *in vivo* studies), that a C-terminal mutation of DISC1 disrupts the interaction with Ndel1 [30]. They ascertained that this leads to a dysregulation of neuronal stem cell population in the ventricular zone during forebrain development.

Raja *et al.* succeeded in modeling disease patterns of Alzheimer's – like amyloid aggregation, hyperphosphorylated tau protein and endosome abnormalities in cerebral organoids. Also, they successfully tested the efficacy of β- and γ-secretase inhibitors to reduce amyloid and tau pathology in Alzheimer patient-derived brain organoids [28]. Interestingly, an alternative model did not use human PSCs, but a *v-myc* immortalized cell line of ventral mesencephalic origin (ReNcell VM), to generate 3D tissue constructs for modeling AD [31].

A step toward modeling Parkinson's disease in brain organoids was done by Monzel *et al.* [11] and Jo *et al.* [10], who were able to generate brain organoids with midbrain identity, which contain not only dopaminergic neurons, but also show electrophysiological activity and neuromelanin accumulation. It will be interesting in the future to understand how dopaminergic neurons develop, and age, in Parkinson's disease brain organoids.

All these studies show that brain organoids – even if the experimental model is far from being optimized – have great potential for the study of some neurological diseases. Mostly, as described above, neurodevelopmental conditions lead to a disease. However, the usefulness of cerebral organoids for neurodegenerative conditions (e.g., AD, Parkinson's disease and others) faces many challenges. As a main challenge stands the translation of findings obtained in an *in vitro* system – cultured *in vitro* for many months – to reproduce the actual events occurring during the progression of a chronic degenerative disease in the human brain. Nevertheless, two aspects are of crucial importance: brain organoids from human PSCs provide the investigator with human tissues for research, and their development in 3D, expected to better recreate human brain biology. Especially important will be to exploit the use of brain organoids in the context of the 3Rs concept related to progressively reduce the use of animals in research. Human cerebral tissue will accelerate and foster successful research into the actual molecular and cellular key events leading to human disease, while reducing the unnecessary use of animals in research, a long time awaited goal in neurobiology research.

Attempts to enhance reproducibility, & improving the experimental model

In order to develop improved cerebral organoids for neurobiology/neurological research, it needs to be solved how to prospectively, provide spatially resolved regional patterning and specification cues in a whole cerebroid. Optimally, neurological research needs of cerebroids in which discrete brain regions could be prospectively generated at desired spatial locations being meaningfully organized, spatially resolved, to study for instance how projection and communication between different developing regions are established, modulated, remodeled, and how they survive and degenerate.

One of the possible solutions to this hurdle will be to engineer scaffolds with biocompatible materials for the development of cerebroids with improved organization. Such scaffold should provide perfusion/vascularization, as well as spatially distinct developmental cues, and overall, allow for the development of a self-organizing cerebroid. Optimally, such scaffold should also have electrically conducting properties for the stimulation and monitoring (bio-sensing) of the developing cells.

Next, the use of genuine (self-organized, nonpatterned) cerebral organoids as a model system for any type of systematic study, is hampered from a very low reproducibility rate. As described above, the generation of neural tissue in a cerebral organoid occurs stochastically. Moreover, the different neuronal types in the same organoid are generated almost randomly and most importantly, without control by the investigator. However, since the

development of an organoid resides in the self-organization and self-development capacities of the tissue, any interference with the 'self-drive' may result in an impaired development of the organoid, a difficulty that should be carefully considered.

While recognizing the interest of bulk-patterned organoids (very similar to patterned neurospheres) for some limited studies, the ideal scenario would be to generate an organoid with prospectively generated brain region identities at different locations, and that will show some type of communication/projection between different brain regions, spatially separated. For instance, to study cortico-striatal or nigro-striatal projections, and many others.

Basic requirements would be to enlarge the tissue constructs; to provide the developing organoid with a supportive (perfusable) scaffold, which would both provide physical and physiological support, and; providing spatially separated, regional developmental cues, for instance by hydrogels deposited at different locations in the scaffolds, loaded with different developmental cue molecules.

Research in this line includes attempts to provide the organoids with vascularization [32,33] and blood–brain barrier [34] in various ways. We expect seeing exciting developments in these research lines, which will help to exploit the full potential of cerebral organoids.

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Ethics statement

The work developed in the research group was approved by the local UAM Ethics Committee, and authorized by the Local Government of Comunidad Autónoma de Madrid, Consejería de Sanidad, after a positive report by the 'Comisión de Garantías', from the Health Institute Carlos-III.

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