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INVITED REVIEW

Toxicity assessment using neural organoids: innovative approaches and challenges

Si-Hyung Park[1] · **Woong Sun**[1]

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Abstract

Assessment of toxicity and efficacy in the nervous system is essential to ensure the safety of compounds and the efficacy of neurotherapeutics. Recently, technologies using neural organoids to mimic the structural and functional properties of human brain tissue have been developed to improve our understanding of human-specific brain development and to model neurodevelopmental disorders. This approach offers the potential for standardized toxicity testing and large-scale drug screening at the organ level. Here, we review recent advances in neural organoids and explore the possibility of establishing more accurate and efficient systems for toxicological screening applications. Our review provides insights into toxicity and efficacy assessment research using neural organoids.

Keywords Neural organoid · Neural toxicity and efficacy · Disease modeling · High-throughput screening

Introduction

Evaluating the toxicity and efficacy of candidate substances is essential in drug development, chemical management, and the assessment of the safety of cosmetics and food additives [1]. Identifying any potential harmful effects and efficacy before these substances are introduced to humans is crucial [2]. The central nervous system (CNS) is the most delicate and well-organized organ in the body, serving as the primary control center for coordinating various activities, including motor functions, sensory perception, learning and memory, and emotions. Accordingly, the CNS is particularly sensitive to damage during developmental stages, and such damage can have long-term effects on cognition, behavior, and motor function, making neurotoxicity assessments especially important [3].

The Developmental Neurotoxicity/In Vitro Battery (DNT/IVB) is an in vitro testing system designed to evaluate developmental neurotoxicity [4]. This system is primarily based on studies conducted with animal models and two-dimensional (2D) cell cultures, allowing for the efficient evaluation of the toxicity and efficacy of chemicals that affect nervous system development [5–7]. Traditionally, neurotoxicity assessments have relied on animal models such as rats, mice, and nonhuman primates [8]. However, due to interspecies differences, data derived from these models may not be directly applicable to humans [9]. Furthermore, efforts are underway to reduce the use of laboratory animals and conduct more ethical toxicological research through alternative experimental methods [4]. Particularly in the United States and Europe, movements to regulate or cease animal testing are accelerating, leading to increased attention on the potential use of in vitro models that can mimic the structure, microenvironment, and physiological functionality of human tissues and organs [10]. In cellular-level research, it is possible to observe the real-time onset and progression of diseases outside the body, making it a key application area for stem cell research [11]. However, replicating the heterogeneity and interactions of various cells within actual tissues, as well as the microenvironment of tissues excluding cells, remains challenging [12].

The advent of human pluripotent stem cells (hPSCs), which include human embryonic stem cells (hESCs) and human-induced pluripotent stem cells (hiPSCs), offers a new dimension for modeling the human brain, studying neurological disorders, and assessing neural toxicity [13, 14]. Brain organoids, three-dimensional (3D) multicellular aggregates

✉ Woong Sun
woongsun@korea.ac.kr

[1] Department of Anatomy, Brain Korea 21 Plus Program for Biomedical Science, College of Medicine, Korea University, 73, Goryeodae-ro, Seongbuk-gu, Seoul 02841, Republic of Korea

derived from hPSCs, can partially replicate the structural features of the brain, making them promising models for neurotoxicant screening [15]. Recently, neural organoids, which exhibit a high degree of histological and functional similarities with human brain, have been proposed as bridging models that link toxic responses at the cellular level to the organismal level (Fig. 1).

This review summarizes the advancements in brain organoid technology, explores their application in neural toxicity assessments, and discusses the limitations and potential improvements of current organoid models.

Current status of neural organoid technology

Fundamentals of various neural organoid culture techniques

The technique for generating organoids to construct 3D tissue cultures that resemble different brain regions is well established. Neural organoids can be generated by two distinct methods: unguided methods, which do not target their characterization to specific brain regions, and guided methods, which target inducers to specific regions. The unguided method results in a more or less random mixture of parts that have features of different brain regions, while the guided method is characterized by a higher similarity but limited to specific brain regions and less randomness [16]. While there are clear advantages and disadvantages to each method, the guided method is preferred for toxicity studies because it requires less variation between batches to achieve statistical significance. The actual embryonic development process is continuous and occurs through interactions between various neural and non-neural tissues, resulting in a complex interaction of various factors in space and time, but it is very difficult to replicate the entire process in vitro, so guided cultures are designed to replicate the most critical processes by replacing them with discrete steps. Typical steps include (1) induction of neuroepithelial cells from embryonic stem cells, (2) patterning or regional specification, and (3) neuronal maturation, and by optimizing the type and combination of inducers, treatment duration, and concentration, neural organoids that mimic various brain regions are produced (Fig. 2A).

Since dorsal forebrain is known to be induced by the most default pathway [17, 18], dual SMAD inhibition (transforming growth factor-β (TGF-β)/bone morphogenetic protein (BMP)) is sufficient for inducing forebrain fate [19–21]. Recent reports have demonstrated that under these induction conditions, the wingless-related integration site (WNT) signal, which is required for posterior regionalization is

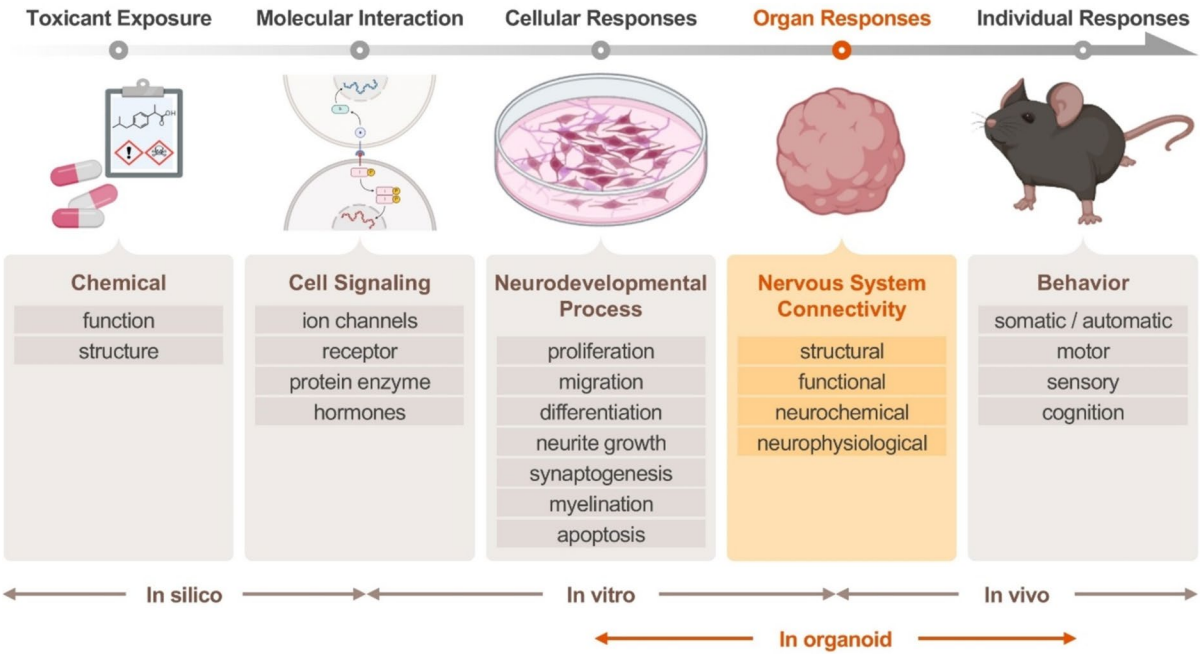

Fig. 1 Adverse Outcome Pathway (AOP) for Developmental Neurotoxicity. After toxicant exposure, molecular interactions, cellular responses, and individual responses are performed in silico, in vitro, and in vivo, respectively. Neural organoids, which show high similarity to human systems, are proposed as a bridging model at the organ response level that links cellular and individual toxicity reactivity. In addition, neural organoids are expected to demonstrate excellent predictive power compared to toxicity tests using experimental animals due to their high genetic and physiological similarity to humans

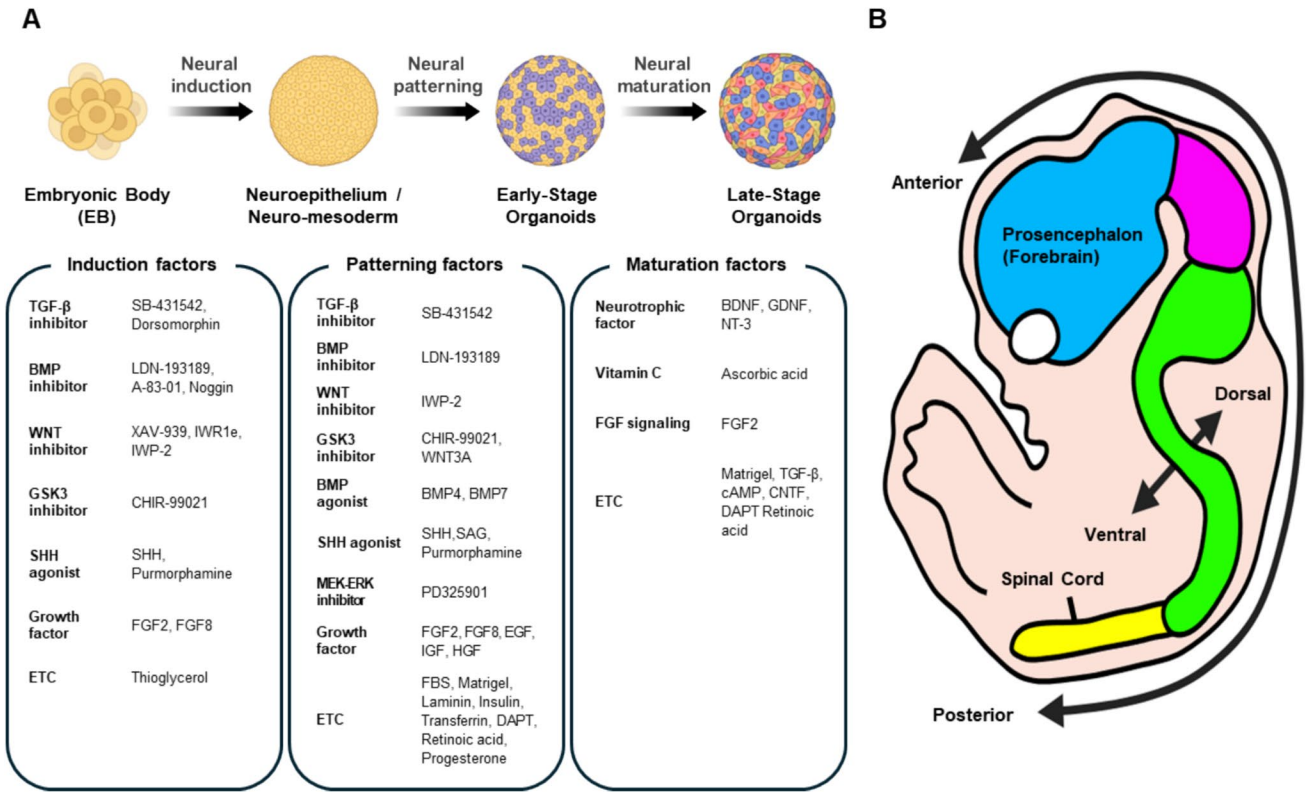

Fig. 2 Stepwise Culture Method for Generating Regionally-Specified Neural Organoids. **a** Organoids are produced by aggregating human pluripotent stem cells or induced pluripotent stem cells into embryoid body (EB). First, the EB is treated with a combination of induction factors for neural induction, typically leading to the formation of neuroepithelium or neuro-mesoderm. Next, neural patterning can be achieved by applying a combination of patterning factors to facilitate more sophisticated regionalization. Finally, neural maturation is to be conducted by incorporating maturation factors to produce neural organoids. **b** Schematic of anterior–posterior and dorsal–ventral axes during embryonic development

spontaneously generated, and thus inhibition of WNT signaling allows for a more stable induction of forebrain fate [22, 23]. Despite some differences in culture conditions, this process can be generally considered as neuroepithelial induction. Based on this, factors that regulate fibroblast growth factor (FGF), WNT, and retinoic acid (RA) signaling can be used in combination with bFGF during the posterior patterning and specification steps to more precisely specify posterior structures [24, 25]. Importantly, the concentrations of FGF, WNT, RA signaling factors concentrations roughly determine the area along the anterior–posterior axis of the nervous system [26, 27]. On the other hand, the most caudal part of the nervous system undergoes a different developmental pathway from neuroectodermal progenitors than the anterior part, which proceeds through neuroectodermal induction, so the lower spinal cord organoids cannot be made using neuroectodermal induction. Therefore, it is necessary to use an early induction method that induces neuro-mesodermal progenitor (NMP) rather than dual SMAD inhibition [28, 29]. Since NMP can give rise to both neural and mesodermal tissues, depending on the induction method and culture conditions, it is possible to induce spinal cord organoids [30–34] or even complex organoids such as neuro-muscular organoids [35–37].

The nervous system can be divided based on the anterior–posterior (A–P) and dorsal–ventral (D–V) axes (Fig. 2B). The D–V axis of the nervous system is mainly specified by activation of the dorsal inducer BMP and ventral inducer sonic hedgehog (SHH) signaling pathways. Therefore, further treatment of these factors can refine the regionalization along the A-P axis. For example, since the forebrain induction conditions mentioned above result in a predominantly dorsalization by default, treatment with SHH to induce ventralization results in neuronal induction characteristic of the subpallium, where inhibitory neurons are predominantly generated [38]. Based on this induction method, there have been successful instances where neural organoids that more precisely mimic the forebrain were created by fusing together dorsally and ventrally induced organoids [38, 39]. Spinal cord organoids can be ventralized to enrich them with motor neurons [30, 40], successfully modeling motor neuron-related diseases, such as amyotrophic

lateral sclerosis (ALS), by promoting a motor neuron-rich environment [41].

Through the appropriate combination of these A–P and D–V regionalization factors, it is possible to produce organoids that mimic various brain regions. For example, hippocampal organoids are produced by first inducing anterior neuroepithelial cells through dual SMAD inhibition (TGF-β/WNT), then inducing posterior dorsal region characterization through treatment with BMP4 and CHIR, and then producing them through the neuronal maturation stage [42, 43]. The generation of thalamic/hypothalamic organoids are started from similarly induced neuroepithelial cells, then treated with SHH signaling and appropriate additional factors (PD0325901, BMP7/WNT3a) for elaborate region characterization [20, 44–46]. The midbrain organoids are also produced through the elaboration and neuronal maturation process through the FGF and SHH signaling pathways to induce neuroectodermal differentiation toward a floor plate through the treatment and concentration of WNT activators after neuroepithelial cell induction, and then patterning to a mesencephalic fate [20, 47, 48]. In this way, each brain region can be induced with the appropriate combination of inducers to specify the desired region, and incubation with maturation factors such as brain-derived neurotrophic factor (BDNF) and neurotrophin-3 (NT-3) induces neuronal maturation (Table 1). The increasingly complex neural circuits created by long-term culture of organoids will provide an important source for contributing to the study of neural function and development.

The factors required for development often act sequentially and redundantly, so that different combinations can lead to broadly similar, if not identical, regionalization. These characteristics make standardization of neural organoid production protocols a very difficult issue, as different

Table 1 Summary of the procedures and relevant factors for generating regional-specific organoids

Region	Guided/Unguided	Neural induction	Neural patterning	Neural maturation	References
Cerebral organoid	Unguided	–	Matrigel	–	[17, 18]
Forebrain organoid	Guided	Dual SMAD inhibition [TGFβ/BMP] (Option) WNT inhibitor	TGFβ inhibitor GSK3 inhibitor FGF2 EGF	BDNF GDNF NT3 Ascorbic acid cAMP TGFβ	[19–23, 38]
Hippocampal organoid	Guided	Dual SMAD inhibition [TGFβ/WNT]	GSK inhibitor BMP agonist SHH agonist	BDNF GDNF	[42, 43]
Thalamic organoid	Guided	Dual SMAD inhibition [TGFβ/BMP]	MEK-ERK inhibitor BMP agonist	BDNF Ascorbic acid	[44]
Hypothalamic organoid	Guided	Dual SMAD inhibition [TGFβ/BMP]	SHH agonist WNT inhibition	BDNF GDNF Ascorbic acid cAMP DAPT FGF2 CNTF	[20, 45, 46]
Midbrain organoid	Guided	Dual SMAD inhibition [TGFβ/BMP] (Option) GSK3 inhibitor SHH agonist WNT inhibitor FGF8	SHH agonist BMP inhibitor GSK3 inhibitor FGF8 Laminin	BDNF GDNF Ascorbic acid cAMP TGFβ	[20, 47, 48]
Spinal cord organoid	Guided	GSK3 inhibitor (Selection) 1. TGFβ inhibitor 2. BMP inhibitor 3. FGF2	FGF2 Retinoic acid SHH agonist	BDNF GDNF Ascorbic acid Retinoic acid	[30–33, 41]
Neuro-muscular organoid	Guided	GSK3 inhibitor FGF2	FGF2 IGF HGF DAPT SHH agonist Retinoic acid	IGF HGF	[35–37]

protocols for inducing the same regional specificity can coexist. Given the diversity of cells present in the human fetal brain, region-specific neural organoids generated using different protocols are likely to represent different subset combinations of cells, and a very detailed analysis and characterization of this aspect is essential. Therefore, it is now established as a standard identification technique to perform single-cell transcriptome analysis of organoids for the unbiased characterization [49, 50]. However, in order to standardize protocols and perform batch quality control in the future, it will be necessary to construct more rapid and inexpensive biomarkers or PCR panels.

Advancements in neural organoid production and culture techniques for toxicity testing

Further advancements in neural organoid culture technology are needed for toxicity testing. This is being accomplished on two fronts: one is to make neural organoids more brain-like, and the other is to simplify the process for mass production to meet the needs of toxicity tests, especially for high-throughput screening (Fig. 3).

The brain relies on the interaction of neurons, microglia, and various non-neural cells, such as endothelial and meningeal cells, to maintain its function [51, 52]. These microglia and non-neural cells play crucial roles in inflammatory responses, blood–brain barrier (BBB) formation, and more, allowing for a more realistic representation of brain function. Most neural organoids are produced by inducing embryonic stem cells into the neural lineage, so they do not include microglia and vascular cells, which are derived from mesoderm. Therefore, the addition of these cells can provide greater precision and accuracy in studies of immune response, metabolic function, and drug delivery, and can make a significant contribution to disease modeling [53–55]. Because most neural organoids correspond to limited brain regions, rather than simulating the entire brain, they differ significantly from the characteristics of the brain, which functions in an integrated manner across many regions. Consequently, the challenge of producing and refining a brain-like model can be addressed by connecting multiple brain organoids representing different functional brain regions, collectively referred to as assembloids. Using assembloid technology, it is possible to recreate complex neural networks, enabling the construction of various large neural circuits responsible for memory and learning, such as the cortical-hippocampal circuit, the cortical-spinal circuit, and the reward circuits connecting the cerebrum and the striatum [44, 56, 57]. This approach facilitates modeling various psychiatric disorders induced by circuit disruptions. For example, it has been reported that it is possible to simulate the dorsal and ventral sides of the brain, or to induce the frontal and occipital parts of the brain, by implementing a concentration gradient by implementing an assembloids using a cell population that secretes SHH, which is responsible for dorsal induction, or an organoid that secretes FGF8 as an organizer center [26, 58, 59].

To maintain neurons or organoids in cultures that closely mimic the actual in vivo environment and to rapidly acquire functional information, it is advantageous to integrate advanced culture techniques, such as microfluidic systems, with sophisticated measurement techniques. These integrated systems are collectively referred to as Microphysiology Systems (MPS), which are particularly valuable for simulating complex physiological structures like the BBB and for studying how drugs are delivered and distributed within the brain [60, 61]. This approach enables drug discovery and toxicity testing to obtain accurate data that reflects real-world physical conditions, and when combined with assembloids that integrate complex neural circuits, it facilitates even more sophisticated research. Although these high-precision models provide clear advantages, there are also barriers to integrating them with mass-production technologies due to the potential complexity of the process itself.

Automation through mass production and high-throughput screening (HTS) is becoming essential in drug discovery and toxicity screening. Traditional 2D cell cultures use 384 or 1,536 well plates, which allow for rapid testing of multiple drugs or concentrations simultaneously within a single plate, reducing reagent usage and increasing experimental efficiency [62]. Recently, organoids have also become increasingly suitable for HTS, with several microplate-compatible protocols developed for these systems [63, 64]. However, challenges still remain. Standardization of experimental methods is crucial for reducing variability between experiments and ensuring reproducibility. Currently, many organoid experiments are subject to various variables, such as different culture media compositions used in different laboratories, the presence or absence of Matrigel embedding, and the use of orbital shakers, yet no standardization methods are in place. Moreover, while many laboratories employ the embryoid body (EB) method for organoid production, this approach is limited by high intra- and inter-batch variability. To address these issues, alternative culture methods beyond EBs should be considered. Recent studies have proposed using micropattern technology to precisely regulate specific regions of organoids [33, 65], or employing a method of 2D neuronal induction followed by re-aggregation to produce organoids. Notably, re-aggregation technology has been shown to significantly reduce both inter- and intra-batch variability and enhance homogeneity, thus facilitating mass production and increasing efficiency [32, 35, 66]. Instead of growing embryonic stem cells in 3D aggregates to create organoids, re-aggregation technology isolates neuroepithelialized cells in 2D as single cells, counts them precisely to ensure uniformity, and then re-aggregates them

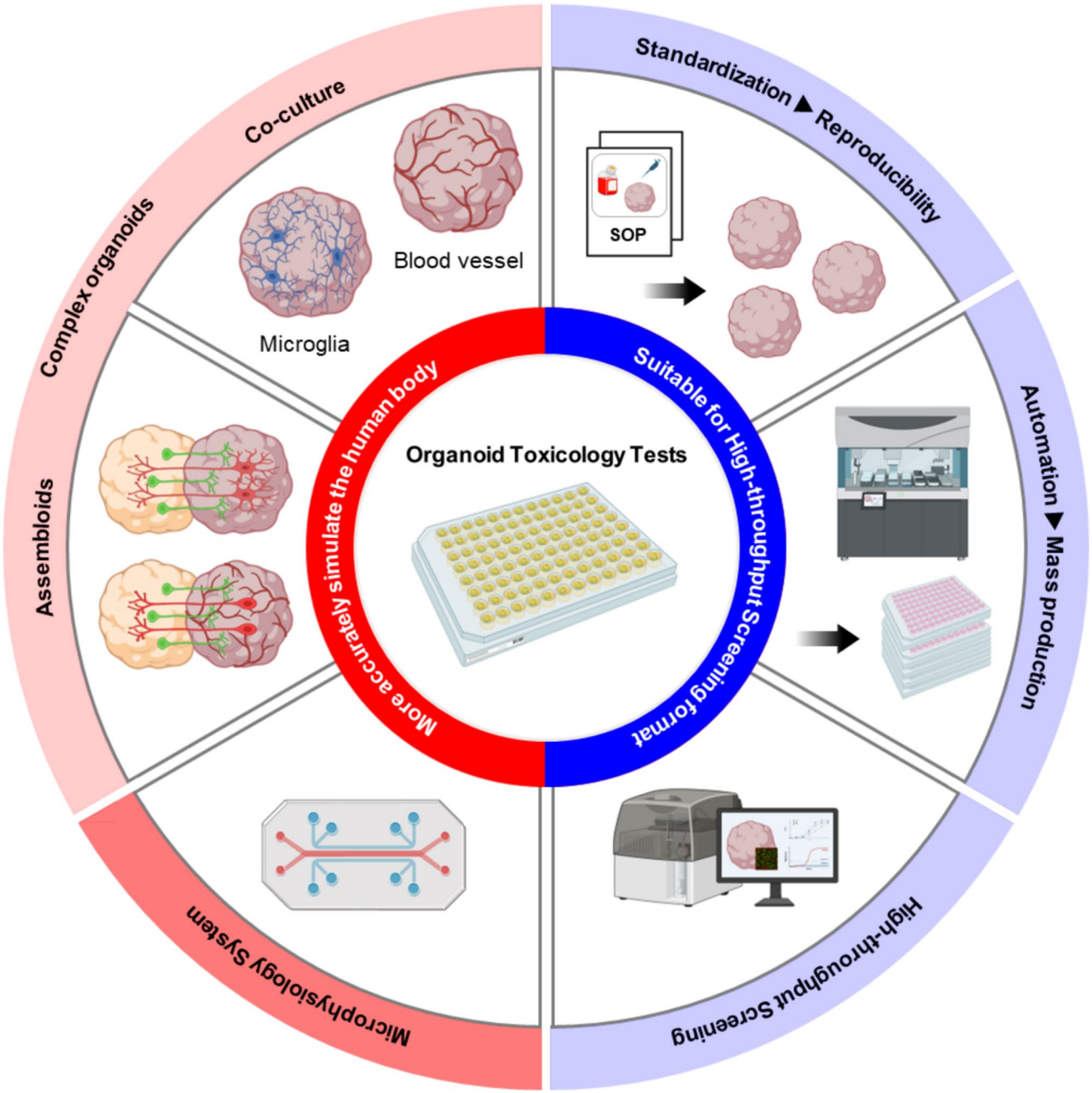

Fig. 3 Two Directions for Improving Neural Organoids Suitable for Toxicity Testing. In order to use organoids for toxicity testing, various technological advances are required. Research is underway to more accurately replicate the human body and to make organoids suitable for high-throughput screening (HTS) format. Numerous strategies are being investigated to improve human mimicry in the production of complex organoids, including assembloids, co-culture techniques, and the use of microphysiology systems. To develop organoids suitable for HTS, researchers are focusing on ensuring reproducibility through standardization, achieving large-scale production via automation, and performing HTS analyses

into organoids of consistent size. This approach has been found to help minimize size and differentiation differences among organoids. However, there is a risk that this method may hinder morphogenesis and reduce structural properties, necessitating a more comprehensive analysis of the characteristics of organoids produced in this manner.

In conclusion, the development of standardized technologies and automation technologies for organoids suitable for HTS automation is essential, as it enables large-scale drug screening and provides more efficient and accurate results in drug discovery and toxicity testing.

Toxicity and drug efficacy assessment using neural organoids

Neural organoids are well characterized during the early stages of development, making them suitable for toxicity assessment in neurodevelopment. While they have limitations in modeling late-onset diseases, such as degenerative brain disorders, there is active research focused on developing them as toxicity assessment platforms, leveraging the fact that early brain tissue is more sensitive to toxic environments compared to mature brain tissue. In this section, we will review the current state of toxicity assessment and validation using organoids.

Microcephaly

Microcephaly is a brain development disorder characterized by an abnormally small brain. It can be categorized into primary microcephaly and secondary microcephaly, depending on when the symptoms occur [67]. Primary microcephaly occurs during fetal development before birth, primarily resulting from reduced division or increased cell death of neural stem cells during early neurogenesis. In contrast, secondary microcephaly occurs after birth and is often caused by impairments in axonal growth or glial cell production that are observed during later developmental stages, frequently occurring alongside impaired corpus callosum formation.

Microcephaly is easily observable through simple microscopic techniques using neural organoids. It is modeled using cerebral organoids, which exhibit the same phenotype of reduced size or slowed growth. Many genetic brain developmental disorders are characterized by primary microcephaly, and in the early stages of establishing neural organoid culture technology, researchers observed size reductions in organoids resulting from genetic mutations in genes such as CDK5RAP2, ASPM, WDR62, and PTEN [17, 68–70]. These mutant organoids displayed an incomplete neuroepithelial architecture, evidenced by a reduced number of ventricular radial glia (vRG) and outer radial glia (oRG), which are precursor cells of the developing neuroepithelium. Notably, oRG cells are a specific type of neural stem cell found only in the human cerebral cortex, and such characteristics cannot be observed in animal models like mice, which demonstrates the importance of using human brain organoids to model microcephaly. In addition, organoid modeling of secondary microcephaly phenotypes in Rett syndrome (RTT) and developmental and epileptic encephalopathies (DEEs) has also been reported [71, 72]. In secondary microcephaly, developmental programs are affected at later stages, often resulting in growth retardation during the maturation or extended culture periods of the organoid models. Recently, a large-scale screening of microcephaly-causing gene variants was conducted using the CRISPR-lineage tracing at cellular resolution in heterogeneous tissue (CRISPR-LICHT) screening technique, which identified relevant gene networks, including IER3IP1 [73]. This research is expected to provide important insights for the identification of the cause of microcephaly, disease modelling through genetic manipulation, and drug target discovery.

The ability of organoid models to effectively replicate the genetic causes of microcephaly suggests that the risk of inducing microcephaly from toxicants or pathogens can be assessed. A prime example is a study that modelled microcephaly using Zika virus infection [20, 74, 75]. This research successfully recreated the process by which a fetus with microcephaly is born when a pregnant woman is infected with the Zika virus. This allowed for the confirmation that the Zika virus induces the death of neural stem cells and leads to microcephaly, while also identifying the molecular mechanisms involved. In addition, various toxicants known to be risk factors for microcephaly in humans, such as ethanol (EtOH), acrylamide, valproic acid (VPA), and cadmium, have also been reported to inhibit the growth of organoids, demonstrating that not only genetic variation but also toxicity caused by pathogens and toxicants can be investigated in organoids [76–79]. As microcephaly, especially primary microcephaly, is caused by disturbances in early development, the current characterization of organoids, which mimic early brain development well, and the relative simplicity of their analysis as size differences, are expected to be exploited rapidly.

Neural tube defects

Neural tube defects (NTDs) are among the most common congenital malformations, affecting approximately 300,000 fetuses worldwide each year. The neural tube is an early developmental nerve tissue that serves as the foundation for the central nervous system, forming the brain and spinal cord. NTDs occur due to an abnormal folding process that prevents the neural tube from closing properly. The process of neural tube formation in humans begins as early as the third week of gestation, making it challenging to observe in vivo, and discrepancies exist between studies using animal models and clinical reports in humans. Moreover, there are significant differences in the timing of neural tube formation between mice and humans [80], which has limited research into the pathogenesis and prevention of NTDs.

Recently, a study was reported that utilized spinal cord organoids to model NTDs [32, 81]. In this study, the research team established a method to fabricate spinal organoids that mimic neural tube formation, verified their similarity to real

spinal cord tissue through histological, transcriptomic, and electrophysiological analyses, and evaluated the toxicity of NTDs caused by treatment with multiple anticonvulsant drugs, including VPA. In particular, VPA is a well-established clinical risk factor for NTDs, and in this study, VPA was detected as the highest risk factor for NTDs [82]. Transcriptomic analysis revealed that VPA increases the expression of tight junction-related genes, providing insights into the pathogenesis of NTDs [81]. Because the list for genetic factors affecting NTD in humans is also rapidly increasing, this system will also be useful for testing the genetic factors affecting NTD in humans, especially by combination with iPSC or gene editing technologies.

On the other hand, the model is composed of neural tissue only, which means that NTDs that originate from disorders in non-neural tissue cannot be tested. Therefore, a more embryonic-like model may be useful. To address these issues, a micropatterned model of neural tube formation has been reported [83]. This model can reproduce more sophisticated neural tube formation processes, including interactions between neural and non-neural tissues, using micropatterns. Transcriptomic analyses have identified specific molecular and cellular processes involved in neural tube development, and the ROCK-inhibitor, Novobiocin, and VPA have been used to confirm NTDs. Among them, structural analysis showed that Novobiocin significantly reduced fibronectin synthesized in non-neural tissues, and reported that this process can be used to build models to examine NTDs originating from disorders of non-neural tissues and to understand pathogenesis.

Migration defects, axon projection failure and circuit defects

After most neurons are created during fetal development, they migrate to a specific location, become established, and begin the process of forming a neural network. Neurons also connect to other neurons via axons, which transmit neural signals. Axon projection failure occurs when an axon fails to grow properly or fails to connect to its target cell, which disrupts the transmission of neural signals. These defects interfere with the formation of neural networks, severely affecting sensory, motor, and cognitive functions. Circuit defects caused by these various processes do not cause significant changes in brain structure at the macroscopic level, but they do cause functional changes. For example, when the balance of signaling between excitatory and inhibitory neurons is disrupted, neural circuits become over-active or, conversely, under-active. This can lead to a variety of neurodevelopmental disorders such as epilepsy, autism spectrum disorder (ASD), and various inherited rare diseases such as RTT and Angelman Syndrome. In addition, exposure to hazardous substances during development can cause developmental abnormalities in neural circuits. As these developmental toxicities are very difficult to study in vitro cell culture models, there is no alternative model other than experimental animal models. Therefore, it is of great significance to establish a neurocircuit abnormality model using organoids to test for circuit toxicity.

Since circuit abnormalities by late developmental neurotoxicity are predicted by changes in neuronal activity rather than morphological changes in organoids, it is not enough to examine morphological abnormalities to model or establish assays for toxicity testing. Thus, it is also necessary to measure changes in neuronal activity. Calcium imaging and electrodes have been used to measure neural signaling. Calcium imaging indirectly detects neuronal signaling changes by expressing fluorescent calcium probes or calcium-sensing proteins such as GCAMP in organoids and measuring the fluorescence changes. Because imaging-based calcium signal analysis does not measure depolarization of neurons, but rather the resulting changes in intracellular calcium concentration, the signal changes slowly and cannot measure rapid firing changes in individual neurons. Nevertheless, because the analysis is based on imaging, it is possible to measure changes in neuronal activity by targeting individual neurons, and under the right optical conditions, it is easy to perform network analysis. The use of methods that directly measure electrical signals using electrodes is expanding [84–86]. In particular, multiple electrode arrays (MEA) technology is being applied more actively because it can acquire 3D network activity information from organoids depending on the arrangement of electrodes. In addition, it is important that MEA can measure Local Field Potential (LFP) in addition to measuring neural firing rate through neural spike measurement. LFP is an analogous measurement to Electro Encephalo Graphy (EEG), which is actively used in clinical practice, LFP in organoids can be compared with a large amount of EEG accumulated from clinical results. For example, by analyzing the LFP patterns of signals derived from organoids through AI, it is possible to identify the maturation stage of organoids, viral infections, disease-related characteristics, etc. [87, 88].

Given that current organoids mainly show a maturation stage similar to that of the fetal brain, and that most of them simulate narrow brain regions, which are not well representative of the characteristics of broader brain circuits, it is still challenging to model developmental disorders or toxicity assessment in later brain development. Therefore, rather than targeting unknown drugs or genetic alterations, studies have been conducted to examine whether predictable outcomes in brain organoids can be achieved using known disease-related genetic alterations. Examples of organoid studies include ASD, RTT, Timothy's disease (TS), and Noonan's disease (NS) [38, 71, 89–91]. Most of the neurotoxicity studies in organoids using various substances have not focused

on neuronal activities yet. In the case of EtOH, studies have been extended to explore the changes in neuronal activities in organoids [92]. In addition to affecting cell proliferation, cell cycle, and apoptosis in the cortical organoids, EtOH exposure inhibited synaptogenesis, consistent with neuropathology reported in Fetal Alcohol Spectrum Disorder patients. Furthermore, MEA analysis confirmed that EtOH leads to abnormal neuronal circuitry, resulting in deleterious effects on neuronal network formation and activity. This is in contrast to the seizure tendencies seen in animal models. However, these observations were made in the hippocampal region of the animal model, whereas in the organoid model they were made in the cortex, limiting direct comparisons. Nevertheless, these findings suggest that the activity of neuronal circuits may be altered by substance treatment, which may lead to abnormalities in neuronal circuits.

Future directions

Toxicity studies using neural organoids are a powerful tool to reproduce and analyze in vitro the toxic responses that may occur during early stages of neurodevelopment [93], leading to a more precise and efficient system for drug development and toxicity assessment. In vitro assays have already been established in animal and cell cultures to measure changes in proliferation, differentiation, apoptosis, migration, neurite outgrowth, synaptogenesis and neuronal network formation, and to date, approximately 476 chemical test results are listed in the OECD in vitro developmental neurotoxicology test [4]. Organoids act as a bridge in these studies and are becoming increasingly important as a platform for evaluating drug effects in an in vivo-like environment. By reflecting complex cellular interactions and neural network functions, organoid-based assays can predict drug toxicity responses with greater precision than conventional single-cell assays, which can help identify toxic mechanisms in neurodevelopment and contribute to drug development.

The mass production of organoids with highly reproducible manner is essential for drug development and toxicity assessment. However, current neural organoids suffer from a lack of standardization, including variability in culture methods, inconsistencies in results, reproducibility issues, and the inability to determine how modifications of a specific gene affect areas beyond the target region [94]. To address these standardization challenges, many researchers are working on optimizing the care and maintenance of initial cells, media composition, culture conditions, and metrics collection [15, 95–97]. However, there is still a lack of standardized metrics for organoid growth, maturation, and quality control. Resolving these issues will enable the establishment of reproducibility and batch-to-batch consistency in organoids through standardized culture methods, as well as the development of an automated system for organoid production and analysis suitable for HTS. Automation of culture and assay evaluation is particularly important given the need to use the same culture conditions and systems for both drug development and toxicity assessment in drug treatment. This approach will reduce inter-assay variability and facilitate the comparison of the efficacy of new drugs and their toxic effects under standardized conditions.

Furthermore, it is essential to distinguish clearly between false positive and false negative results in toxicity assays to improve their predictive accuracy [98]. Toxicity testing requires high predictive precision, especially as false positives can lead to unnecessary drug development costs, while false negatives risk missing the potential dangers of toxic substances [99]. To address this, systematic validation with neural organoid models is critical, and assays that more accurately mimic real-world biological responses need to be developed. To enhance prediction accuracy and differentiate between false positives and false negatives in organoid-based systems, it is effective to employ multidimensional assays, including assessments of cell viability and function, transcriptome analysis, and evaluations of neural network formation. Such comprehensive assays will provide a more reliable prediction model, increasing their applicability in drug development and safety evaluation.

The nervous system performs complex functions not only through local neural circuits of different brain regions but also through the formation of distant neural networks between larger brain regions [100]. However, current research on neural organoids has focused on producing organoids from specific brain regions. This limitation restricts the ability to identify potential side effects or compensatory effects that may occur in connected brain areas outside the targeted region during drug development. To address this, assembloids are being created by directly combining brain organoids from different regions, yet they have yet to effectively simulate the complexity of brain circuits. Overcoming these limitations will significantly contribute to the development of toxicity assessment methods that better mimic late-stage brain development.

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Declarations

Conflict of interest The authors declare no conflicts of interest.

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