Molecules and Cells

Minireview

Neural Organoids, a Versatile Model for Neuroscience

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Three-dimensional cultures of human neural tissue/organ-like structures *in vitro* can be achieved by mimicking the developmental processes occurring *in vivo*. Rapid progress in the field of neural organoids has fueled the hope (and hype) for improved understanding of brain development and functions, modeling of neural diseases, discovery of new drugs, and supply of surrogate sources of transplantation. In this short review, we summarize the state-of-the-art applications of this fascinating tool in various research fields and discuss the reality of the technique hoping that the current limitations will soon be overcome by the efforts of ingenious researchers.

Keywords: central nervous system, human pluripotent stem cells, *in vitro* modeling, neurodevelopment, neurological disorders, organoid

INTRODUCTION

The advent of new techniques often drives new discoveries that can change our concepts in science. It is likely that three-dimensional (3D) culture of brain-like organoids is one such technique. After the first report on the 3D culture of neural cell aggregates that exhibit some aspects of brain histoarchitecture (Eiraku et al., 2008), there has been an explosion of technical improvements in culture methods, and new discoveries have been made using these techniques (Kadoshima et al., 2013; Lancaster et al., 2013; Nakano et al., 2012; Qian et al., 2016; Warmflash et al., 2014). These 3D cultures of neural cell aggregates, collectively called neural organoids (NOs), are formed by recapitulating the developmental processes and organization of the developing human brain *in vitro*. It is still unclear how 3D culture of human pluripotent stem cells (hPSCs) resulted in strikingly different and *in vivo*-like consequences compared to the two-dimensional (2D) monolayer culture (Costa et al., 2016; Duval et al., 2017). However, it is widely speculated that position-dependent cellular signaling and increased signal tone by compact cellular communications provide favorable conditions for *in vivo*-like developmental progress (Kapałczyńska et al., 2018). The ability of hPSCs to produce the brain *in vivo* or NOs *in vitro* is associated with their self-organization property, defined as a cell's ability to organize specialized morphology and histo-architecture (Brassard and Lutolf, 2019; Eiraku et al., 2008; Kadoshima et al., 2013). These processes often coincide with cell type specification, and the separation of these two events is difficult to achieve *in vivo*. In this respect, NOs provide a unique experimental model to segregate cell differentiation and morphogenesis processes and to explore neural development and disease processes beyond the currently available levels. NOs have been successfully used to supplement or replace animal models and to address the unique features of human nervous system development (Amin and Paşca, 2018; Di Lullo and Kriegstein, 2017; Kim et al., 2020; Koo et al., 2019). This review elaborates on the usefulness and potential

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of NOs, for promoting their use in research.

NEURAL ORGANOIDS FOR DEVELOPMENTAL STUDIES

NOs are produced by mimicking developmental processes, and our knowledge about neural development is the most important resource for the establishment of protocols for the region-specific NO production. Typically, protocols for NO generation consist of 3-4 steps, each mimicking the continuous but conceptually segregated *in vivo* developmental cascades (Fig. 1). hPSCs are first induced into neuroepithelia-like cell populations (neural induction step) by applying factors known to determine neural fates. Induction of anterior and posterior neural parts occurs through different developmental processes; anterior parts are induced by the conventional 'dual SMAD inhibition' procedure (Chambers et al., 2009), whereas caudal neural induction requires transient neuromesodermal fate specification (Denham et al., 2015; Gouti et al., 2014; Lippmann et al., 2015). After initial neural induction, 3D neural cell clusters can be further specified into specific domains depending on the applied regional morphogen (regional specification step). Because local signals pointing to specific brain regions have been identified *in vivo*, many region-specific NO protocols have been successfully established by screening efficient recipes of these factors. These specified NOs can then be cultured in the appropriate conditions for neural differentiation, long-term maturation, and advanced neural development (neural differentiation/maturation step; summary in Table 1).

During the maturation period, NO exhibits many features of histogenesis including migration, neurogenesis, and laminar formation. Studies on cerebral organoids have demonstrated the formation of human-specific outer subventricular zone neurogenic niches, highlighting that hPSC-derived NOs recapitulate the human-specific features of brain development (Bershteyn et al., 2017; Kadoshima et al., 2013; Lancaster et al., 2013; Qian et al., 2016). An extended NO culture can reproduce gliogenesis and myelination, which mainly occur during the postnatal stages (Bershteyn et al., 2017; James et al., 2021; Madhavan et al., 2018; Shaker et al., 2021). However, the occurrence of these postnatal events does not directly indicate that the NO reaches mature stages resembling the postnatal brain. Transcriptome or epigenome analyses suggest that an approximately 10-month culture is required to obtain perinatal features, even though gliogenesis is evident by 2-4 months of culture (Amiri et al., 2018; Gordon et al., 2021; Yoon et al., 2019). These discrepancies might be caused by the protocols for NO production, asynchronized developmental progress in NOs, and different experimental sensitivities/methodologies. Considering that later brain development is more influenced by nearby structures and cell migration across embryonic brain regions, and that neural circuit formation with other brain regions is essential for later brain development (Tau and Peterson, 2010; Valiente

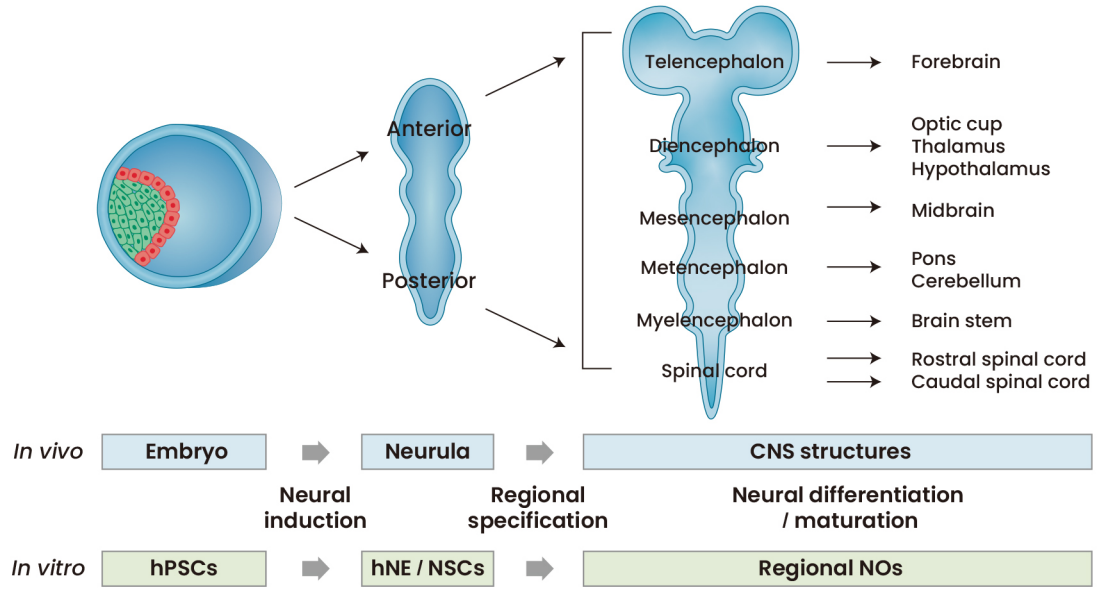

Fig. 1. The strategy to generate NOs. The procedures for generating region-specific NOs have been established based on the process of neural development *in vivo*. In the first stage, hPSCs are induced to differentiate into neural lineage under appropriate culture condition along the anterior-posterior axis. To derive the anterior neural parts, the "dual SMAD inhibition" via small molecules is well-defined. Upon Wnt activation with other signaling (fibroblast growth factor [FGF] activation and/or transforming growth factor β [TGFβ] inhibition), hPSCs are derived to the posterior neural parts. Next, hNE/NSCs sequentially exposed various morphogens for regional specification in accordance with *in vivo* developmental program. After the specification is completed, each NO is provided with the defined medium containing nutrients for differentiation/maturation of the region-specific cell types. Detailed culture conditions for the region-specific NOs are summarized in Table 1. CNS, central nervous system; hNE/NSCs, human neuroepithelial cell/neural stem cells.

Table 1. Summary of the region-specific NO generation procedures

NO type	Neural induction	Regional specification	Neural differentiation/maturation	Reference
Cerebral organoid	- (N2 medium)	-	-	Lancaster et al., 2013
Cortical organoid	DKK-1 (Wnt inhibitor) LeftyA (TGFβ inhibitor) BMPRIA-Fc	-	-	Eiraku et al., 2008
Cortical organoid	IWP1e (Wnt inhibitor) SB431542 (TGFβ inhibitor)	40% O_2 FBS 10% Matrigel (1% in medium)	Matrigel (2% in medium)	Kadoshima et al., 2013
Forebrain organoid	Dorsomorphin (BMP inhibitor) A83-01 (TGFβ inhibitor)	WNT3A CHIR99021 (GSK3 inhibitor) SB431542 (TGFβ inhibitor)	BDNF, GDNF (neurotrophic factors) TGFβ Ascorbic acid (vitamin C) cAMP	Qian et al., 2016
Forebrain organoid	Dorsomorphin (BMP inhibitor) SB431542 (TGFβ inhibitor)	FGF2 EGF	BDNF, NT3 (neurotrophic factors)	Paşca et al., 2015
Ventral forebrain organoid	-	IWP2 (Wnt inhibitor) SAG (SHH agonist)	Matrigel (1% in medium)	Bagley et al., 2017
Subpallium spheroid (ventral forebrain)	Dorsomorphin (BMP inhibitor) SB431542 (TGFβ inhibitor)	FGF2 EGF IWP2 (Wnt inhibitor) SAG (SHH agonist)	BDNF, NT3 (neurotrophic factors)	Birey et al., 2017
Choroid plexus organoid	-	CHIR99021 (GSK3 inhibitor) BMP4	Matrigel (2% in medium)	Pellegrini et al., 2020
Hippocampus organoid	IWP1e (Wnt inhibitor) SB431542 (TGFβ inhibitor)	CHIR99021 (GSK3 inhibitor) BMP4	40% O_2	Sakaguchi et al., 2015
Optic cup organoid	IWP1e (Wnt inhibitor)	CHIR99021 (GSK3 inhibitor) SAG (SHH agonist) FBS	-	Nakano et al., 2012
Thalamus organoid	SB431542 (TGFβ inhibitor) LDN193189 (BMP Inhibitor)	BMP7 PD325901 (MEK-ERK inhibitor)	BDNF (neurotrophic factors) Ascorbic acid (vitamin C)	Xiang et al., 2019
Hypothalamus organoid	SB431542 (TGFβ inhibitor) LDN193189 (BMP Inhibitor) Thioglycerol	WNT3A Purmorphamine (SHH agonist)	FGF2 CNTF	Qian et al., 2016
Midbrain organoid	SB431542 (TGFβ inhibitor) Noggin (BMP inhibitor) CHIR99021 (GSK3 inhibitor)	FGF8 SHH	BDNF, GDNF (neurotrophic factors) Ascorbic acid (vitamin C) cAMP	Jo et al., 2016
Midbrain organoid	SB431542 (TGFβ inhibitor) LDN193189 (BMP Inhibitor) SHH, purmorphamine (SHH agonist) FGF8	LDN193189 (BMP Inhibitor) CHIR99021 (GSK3 inhibitor) SHH, purmorphamine (SHH agonist) FGF8	BDNF, GDNF (neurotrophic factors) TGFβ Ascorbic acid (vitamin C) cAMP	Qian et al., 2016
Midbrain organoid	Dorsomorphin (BMP inhibitor) A83-01 (TGFβ inhibitor) IWP2 (Wnt inhibitor) CHIR99021 (GSK3 inhibitor)	FGF8 SAG (SHH agonist) Laminin	BDNF, GDNF (neurotrophic factors) Ascorbic acid (vitamin C) cAMP	Kwak et al., 2020

Table 1. Continued

NO type	Neural induction	Regional specification	Neural differentiation/maturation	Reference
Cerebellum organoid	SB431542 (TGFβ inhibitor)	FGF2 FGF19 SDF1	SDF1	Muguruma et al., 2015
Brainstem organoid	Dorsomorphin (BMP inhibitor) SB431542 (TGFβ inhibitor)	Transferrin Insulin Progesterone	BDNF, GDNF, NT-3 (neurotrophic factors) Ascorbic acid (vitamin C) cAMP	Eura et al., 2020
Spinal cord organoid	CHIR99021 (GSK3 inhibitor) LDN193189 (BMP Inhibitor)	Retinoic acid Purmorphamine (SHH agonist)	BDNF, GDNF (neurotrophic factors) Ascorbic acid (vitamin C)	Hor et al., 2018
Spinal cord organoid	CHIR99021 (GSK3 inhibitor) SB431542 (TGFβ inhibitor) FGF2	Retinoic acid +/- SAG (SHH agonist) +/- BMP4	BDNF, GDNF (neurotrophic factors)	Ogura et al., 2018
Spinal cord organoid	CHIR99021 (GSK3 inhibitor) SB431542 (TGFβ inhibitor)	FGF2	Retinoic acid	Lee et al., 2020

BDNF, brain-derived neurotrophic factor; BMP, bone morphogenetic protein; CNTF, ciliary neurotrophic factor; cAMP, cyclic adenosine monophosphate; EGF, epidermal growth factor; FBS, fetal bovine serum; FGF, fibroblast growth factor; GDNF, glial cell-derived neurotrophic factor; GSK3, glycogen synthase kinase-3; NT3, neurotrophin-3; SAG, smoothened agonist; SDF1, stromal cell-derived factor 1; SHH, sonic hedgehog; TGFβ, transforming growth factor-beta.

and Marín, 2010), regionally isolated NOs cannot replicate this interaction-based neural development. Further, non-neural lineage cell populations, such as blood vessels and microglia (Adams and Eichmann, 2010; Lenz and Nelson, 2018), also play important roles in brain development, which cannot be generated by simple NO induction. Accordingly, many approaches to include multiple components in NO have successfully demonstrated that some aspects of these insufficiencies can be overcome by complex technologies such as fusion, assembly, connection, and polarization (Fig. 2).

Cumulative results have demonstrated that NOs faithfully replicate many aspects of the developmental progress of the human brain. Thus, NOs provide a great opportunity to glimpse human brain development *in vitro* with many destructive technologies. However, *in vitro* NO growth is not completely the same as brain development *in vivo*, and observations from the NO model cannot provide direct evidence that the same events occur during human development. In this respect, to identify novel developmental processes of the human brain, human specimen studies need to be combined with NO-based observations, increasing the need for access to the embryonic human brain using innovative imaging methods or embryonic human brain banks (Kretzschmar, 2009; Lee et al., 2016; Ueda et al., 2020).

NEURAL ORGANOIDS FOR DISEASE MODELING AND DRUG SCREENING

Modeling human brain diseases using NO technology is one of the major goals that researchers pursue. Considering that NOs are produced via a developmental program for organogenesis, modeling developmental defects were immediately addressed upon the advent of the NO technique. Observation of known developmental defects with specific genetic mutations in NO cultures is considered *per se* as efficient validation method of the NO culture system. Therefore, modeling of developmental defects and establishment of NO culture methods have emerged inseparably in most early studies (Birey et al., 2017; Lancaster et al., 2013; Li et al., 2017; Qian et al., 2016). Further, as early developmental defects are closely associated with morphological defects, histological analyses are the major tools used to evaluate them. Therefore, defects in early morphogenesis, neurogenesis, and cell migration can be addressed by the morphology, size, and cellular composition of NOs. For instance, early neurulation-related cellular specification is closely associated with tube morphogenesis, and the NO model has been successfully used for modeling tube morphogenesis (Karzbrun et al., 2021; Lee et al., 2020). Similarly, microcephalic phenotypes in human genetic mutations are not faithfully replicated in mouse models, whereas NO-based assays can successfully replicate these phenotypes (Lancaster et al., 2013; Qian et al., 2016; Zhang et al., 2019). Considering that many rare genetic mutations that cause developmental brain disorders have been identified by state-of-the-art whole-genome screening, combinations of gene editing and NO techniques have great potential to address these rare genetic diseases. A list of the successful studies of these types of developmental disease modeling is presented in Table 2.

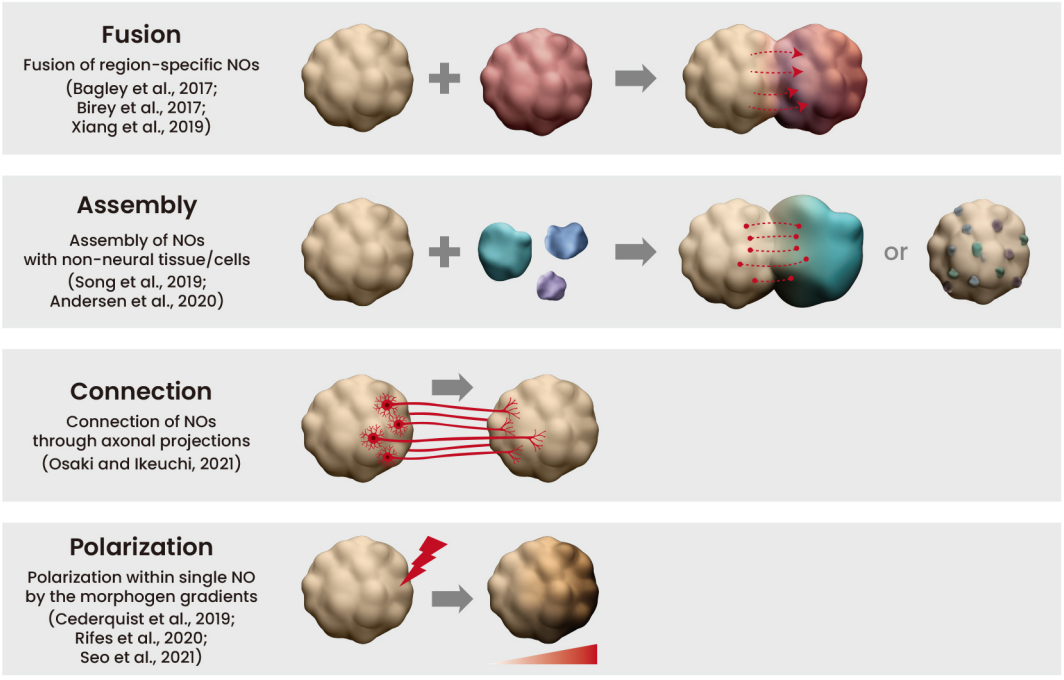

Fig. 2. Complex NO models. Fusion is defined as a hybrid among region-specific NOs. This approach is considered a way to investigate the interaction of different regional NOs, and can be easily obtained by positioning two or more NOs close to each other. It is suitable for exploring cellular migration or innervation of nerve fibers. Assembly is defined as a connection or co-culture of NO and non-neural tissue/cells. Upon the developmental program, tissues derived from mesoderm/endoderm that cannot be simultaneously induced from neural induction protocol are generated separately and then mixed with NO. For examples, the assembloid with microglia is provided as a model to observe neuro-immune responses within NOs. Connection technology provides a system capable of building a sophisticated neural network between NOs. To establish axonal connectivity, microdevices are required to allow directional axon outgrowths. Polarized NOs represent enhanced patterning features by the external stimulation. Polarization can be achieved by latest technologies such as inducible focal gene expression, microfluidic gradients, or micropatterning.

Defects in later developmental programs often do not exhibit overt morphological changes; therefore, more precise analyses with physiological tools are required. Methods for detecting the physiological responses of NOs have been widely used only recently (Osaki and Ikeuchi, 2021; Trujillo et al., 2019; Zafeiriou et al., 2020), and the physiological defects in these models are only beginning to be reported (Andersen et al., 2020; Birey et al., 2017; Mariani et al., 2015; Samarasinghe et al., 2021; Ye et al., 2017). For instance, epileptic episodes can be recognized by altered neuronal burst activity and local field potentials. Accordingly, using these physiological tools, Rett's syndrome and Schizophrenia were successfully modeled using NOs derived from patient iPS cells (Samarasinghe et al., 2021; Ye et al., 2017). The use of NOs for psychiatric diseases is more challenging because these pathologies are known to be associated with neural circuit formation, synaptic plasticity, and other sophisticated abnormalities without gross signs of structural changes (Das et al., 2020; McTeague et al., 2017; Van Spronsen and Hoogenraad, 2010). Many brain regions are involved in the pathogenesis of psychiatric diseases, and region-specific NOs may have limited potential to exhibit these circuit-dependent pathologies. Furthermore, psychiatric diseases are often modeled in experimental animals exhibiting similar behavioral defects. However, the current NO models have limited complexity compared to multiregional neural circuits *in vivo* and have no readout systems comparable to animal behavior. Therefore, these limitations impede the use of NO in modelling these types of diseases.

NOs have also been successfully used to model neurodegenerative diseases. NOs produced from iPSCs of patients with Alzheimer's disease (AD)-related mutations exhibit AD-like symptoms, such as amyloid-beta deposits (Jack et al., 2010; Murphy and LeVine, 2010). The NO-based Parkinson's disease model has also been reported to exhibit Lewy body-like inclusions and alpha-synuclein aggregations (Jo et al., 2021). It is unclear why these late-onset symptoms in aged human patients were observed in the NOs, which replicate a much younger aged brain. It is plausible that the current 'standard' cultures of NO may be pro-degenerative owing to non-humanized media and the lack of sufficient signals/factors for healthy growth, which is presumably supplied from the blood or other parts of the body *in vivo*. Supporting this, NOs have been reported to exhibit increased transcriptome signatures associated with oxidative stress compared to stage-matched embryonic brains (Bhaduri et al., 2020). Alter-

Table 2. Studies on neurological disease using NOs

	Disease	NO type	Causes or risk factors	Major associated disease phenotype in NOs	Potential therapeutic approaches	Reference
Neurodevelopmental diseases (morphological defects)	Microcephaly	Cerebral organoid	CDK5RAP2 mutation	Overall smaller organoids Premature neural differentiation (decreased radial glial cells and increased neurons)	Overexpression of CDK5RAP2	Lancaster et al., 2013
	Zika virus-induced microcephaly	Forebrain organoid	Zika virus infection	Overall smaller organoids Reduced ventricular zone thickness and increased ventricular lumen size Increased cell apoptosis and suppressed proliferation of neural progenitors	-	Qian et al., 2016
	Miller-Dieker syndrome (lissencephaly)	Cerebral organoid	Deletions of chromosome 17 (17p13.3)	Mitotic defect in outer radial glial cells Increased apoptosis of neuroepithelial stem cells Defective neuronal migration of cortical neurons	Compensatory duplication of wild-type chromosome 17	Bershteyn et al., 2017
	Bosch-Boonstra-Schaaf optic atrophy syndrome (BBSOAS syndrome)	Cerebral organoid	NR2F1 haploinsufficiency	Delayed neurogenesis (increased progenitors and reduced neuronal differentiation)	-	Bertacchi et al., 2020
	Neural tube defect	Spinal cord organoid	Antiepileptic drug	Delayed progression of neural tube morphogenesis Abnormal morphology of neural tube	-	Lee et al., 2020
Neurodevelopmental diseases (physiological defects)	Autism spectrum disorder	Telencephalic organoid	Idiopathic ASD patient-derived hiPSC	Accelerated cell cycle and decreased cell cycle length during early stages Increased neuronal differentiation and synaptic formation Overproduction of ventral neural progenitors and GABAergic neurons Imbalance between Glutamatergic and GABAergic neurons	FOXG1 Knockdown	Mariani et al., 2015
	Timothy syndrome	Fused organoid (dorsal forebrain and ventral forebrain)	CACNA1C mutation	Defects in the saltatory movement of GABAergic interneurons	Nimodipine (LTCC blocker) Roscovitine (cyclin-dependent kinase inhibitor)	Birey et al., 2017

Table 2. Continued

	Disease	NO type	Causes or risk factors	Major associated disease phenotype in NOs	Potential therapeutic approaches	Reference
	Rett syndrome	Fused organoid (cerebral cortex and ganglionic eminence)	MECP2 mutation	Hyperexcitability and hypersynchrony Defects in the balance of excitatory and inhibitory synapses Aberrant neural oscillation	Pifithrin-a (TP53 target inhibitor)	Samarasinghe et al., 2021
	Schizophrenia	Forebrain organoid	DISC1 mutation	Delayed cell-cycle progression of radial glial cells Formation of the DISC1/Ndel1 complex	-	Ye et al., 2017
Neurodegenerative disease	Alzheimer's disease	Cortical organoid	APP duplication PSEN1 mutation	Increased Aβ aggregation Hyperphosphorylation of tau protein Abnormal endosome morphology and recycling	Compound E (γ-secretase inhibitor BACE-1 (β-secretase inhibitor)	Raja et al., 2016
	Alzheimer's disease	Cerebral organoid	APOE ε4	Enhanced cell apoptosis and decreased synaptic integrity Increased Aβ accumulation and phosphorylation of tau	Isogenic conversion of APOE4 to APOE3	Zhao et al., 2020
	Alzheimer's disease	Cerebral organoid	Sporadic AD patient-derived hiPSC APOE ε4	Increased Aβ and tau protein Localization of the Aβ plaques in extracellular space Hyperphosphorylation of tau in neurons	6 FDA-approved candidate drugs	Park et al., 2021
	Parkinson's disease	Midbrain organoid	LRRK2 mutation MPTP-induced neurotoxicity	Reduced dopaminergic differentiation and decreased neurite length Abnormal localization of α-synuclein Increased mitophagy and autophagy	GSK2578215A (LRRK2 kinase inhibitor) TXNIP knockdown	Kim et al., 2019

Table 2. Continued

Disease	NO type	Causes or risk factors	Major associated disease phenotype in NOs	Potential therapeutic approaches	Reference
Parkinson's disease	Midbrain organoid	GBA1 knockout + SNCA overexpression (dual perturbation)	Reduced dopaminergic differentiation Generation of Lewy body-like inclusions Accumulation of α-synuclein aggregates	-	Jo et al., 2021
Parkinson's disease	Midbrain organoid	DNAJC6 mutation	Reduced dopaminergic differentiation and neuron degeneration Increased neuronal firing frequency (stressful pacemaking) Aggregation of α-synuclein Mitochondrial and autolysosomal dysfunctions	Forced expression of DNAJC6 and LMX1A	Wulansari et al., 2021

AD, Alzheimer's disease; ASD, autism spectrum disorder; LTCC, L-type calcium channel.

natively, considering that the survival and growth of neurons are dependent on proper connectivity *in vivo*, the absence of appropriate neural connectivity in the NO model may be responsible for its pro-degenerative nature. Improvement of culture techniques and precise control of normal-like NO maturation are thus required for better modeling of neurodegenerative diseases.

In terms of disease modeling, there have been many significant attempts to upgrade NO culture systems suitable for high-throughput screening (HTS). At least two important features have been addressed and improved to achieve this goal. First, the variability of NOs in different batches and within batches should be precisely controlled. Notably, the NO induction response is highly dependent on batches. It is unclear what causes these differences, but it is predictable that the quality and/or condition of hPSCs may greatly affect the consequences of treatment. Further, the format of cell culture may affect the homogeneity of cellular responses regardless of batches. For instance, because cultured hPSCs in 3D appear to exhibit more variable responses to induction reagents depending on their relative position in the cluster, there have been attempts to induce hPSCs into neural-lineage cells in 2D (Lee et al., 2020; Renner et al., 2020). The resultant 'primed' neural-fate cells can then be dissociated and reaggregated into 3D spheroids with a precisely controlled number of cells. These 3D spheroids can also form a brain histoarchitecture as seen in 3D-initiated NOs. These approaches have obvious benefits in terms of quantifiability because better control of the initial cell number and conditions strongly contributes to the reduction of variations. Considering that this approach may sacrifice some aspects of the histoarchitectures in conventional NOs, it is especially suitable for producing NOs for brain regions where the histoarchitecture is less obvious or ignorable. Second, culture formation should become large-scale and suitable for automation. To achieve this goal, several culture platforms have been proposed using micro- or macro-fluidic designs. These include micro-spin culture systems and microcavity arrays (Brandenberg et al., 2020; Cho et al., 2021; Qian et al., 2016). With the combination of robotics, large-scale cultures and HTS can be achieved, and NOs can be used for drug screening or toxicology testing at the industrial front.

UNIQUE OPPORTUNITIES OFFERED BY THE NO MODEL

The NO model provides unique opportunities that cannot be addressed by other experimental models. These irreplaceable features are mainly derived from the maximal flexibility of the *in vitro* system (Fatehullah et al., 2016; Kim et al., 2020). Human-based studies are mostly observational due to difficulties in accessing manipulation or intervention; thus, they provide only correlational information obtained by non-experimental research methods. On the contrary, genetic modification, testing for unidentified drugs, or surgery to change pre-existing condition of biological systems are possible with animal models (Barré-Sinoussi and Montagutelli, 2015). Although they are valuable tools to identify the importance of genes at the organismic level and to implicate some aspects of human

diseases, they are limited by the constraints of biological systems. For instance, *in vivo* development of the brain is under the constraints of non-neural tissues surrounding it, and it is quite complicated to consider neural development separately from these non-neural components. In this respect, the *in vitro* growth of NOs from single cells enables maximal flexibility in the modulation of environmental and genetic factors. Thus, NO models provide important experimental approaches for addressing question that cannot be accessible otherwise. Some examples of ingeniously used NO models are as follows.

Analysis of morphogenesis with physical factors

Morphogenesis is controlled by multiple biochemical and physical factors such as cellular polarity, cell adhesion, and viscoelasticity (Von Dassow and Davidson, 2011; Zallen, 2007). Genetic mutation or treatment of small molecules that regulate biochemical pathways have been successfully used to elucidate the role of these multiple factors. However, these changes in *in vivo* models often affect both morphogenesis and cell differentiation, and the separation of two biological processes is complicated. Accordingly, the mechanism of morphogenesis has been less addressed. Furthermore, physical factors that may be important for controlling morphogenesis are difficult to evaluate *in vivo* and remain largely ignored. The advent of 3D culture of NOs opened up new possibilities for tackling this problem. For instance, the culture of NOs caged in a physically constrained microchamber resulted in cerebral gyrus-like folding (Karzbrun et al., 2018). The underlying mechanism appeared to be related to the surface-to-core differences in cell density and stiffness, and theoretical simulations supported these notions. Neural tube morphogenesis was also replicated in the NO model, either in the absence of extracellular matrix (ECM) or with the support of ECM/non-neural cells (Karzbrun et al., 2021; Lee et al., 2020). Thus, the physical factors underlying tissue morphogenesis will be more addressed based on the experimental alteration of physical factors and simulations using NO models.

Evolutionary studies

Owing to ethical and technical constraints on human research and the absence of an appropriate model, human evolution research has been almost impossible. Advances in biotechnology, including whole-genome sequencing, comparative genomic analysis, and the NO models, provide new opportunities to investigate human evolutionary studies (Mora-Bermúdez et al., 2016). At least two strategies have been reported for NOs as a model to explore human evolution, and comparison of NOs from human and other non-human primates has revealed that the emergence of human-specific cortical lamina features can be reproduced *in vitro* (Kanton et al., 2019; Pollen et al., 2019). Because invasive biological examinations are feasible with NO cultures, single-cell transcriptome analysis further provides potential biological cascades determining the evolution of human-specific features. For instance, experimental and comparative genomic research has reported the evaluation of the hominoid-type NOs regulated by evolutionary regulator gene, ZEB2 (Benito-Kwiecinski et al., 2021). The NOs derived from human PSC-manipulated ZEB2 was similar to the hominoids, showing less size expansion and atypical neural activity, compared to control human NOs. With more complete gene editing to achieve better homology with hominoid genotypes, these approaches can provide rich information on human brain evolution.

Complex organoids/assembloids

Since the cellular composition in NOs can be customized as desired *in vitro*, various genetically/environmentally distinguishable cell populations can be mixed to explore the specific contribution of specific cell populations during brain development or pathogenesis (Shi et al., 2020; Song et al., 2019; Wörsdörfer et al., 2019). Although mosaic animal models are available through genetic manipulation or cell transplantation approaches, the technological limitations of the utility are larger. On the contrary, the NO model provides greater freedom for experimental design. For instance, Rett syndrome was addressed using this strategy of mosaic fused organoids (Samarasinghe et al., 2021). hPSCs from Rett syndrome patients and control individuals were separated and used for producing cerebral organoids and ganglion eminence organoids, which provided inhibitory neurons to neural circuits in the cortical organoid. By reciprocal assembly of NOs from these different sources, it was discovered that epileptic symptoms in NOs from patients with Rett syndrome were strongly associated with ganglion eminence. In combination with cell-type-specific gene modulation, using cell populations from different sources and mosaic fused organoid/assembloid approaches will provide fruitful opportunities to tackle otherwise impossible questions.

OUTLOOK

NOs cannot replicate the brain accurately, and there are many limitations to be addressed. Some of these limitations might be overcome in the future, whereas others are too fundamental problems to solve them. By improving culture conditions and utilizing bioengineering techniques, NOs can be generated more similar to the human brain. Although most information on NO production has been borrowed from developmental studies, knowledge derived from NO-based studies will soon provide valuable insights into human brain development. Considering ethical principles, such as the risk-benefit ratio, it is very difficult to obtain normal human brain specimens. In this respect, even for disease modeling, the potential ability of NOs to replicate the normal human brain is valuable.

The flexibility of the experimental modulation of NO development may open up the so-called field of synthetic embryology. In combination with gene editing, synthetic embryology may serve as a tool for evolutionary biology, as synthetic biology utilizes the fundamental evolutionary concepts. This also means that synthetic neural networks can be produced based on artificial design, similar to the design and production of electrical circuits. These issues have never been addressed using either animal models or human studies; thus, such synthetic approaches will provide answers (and questions) about how neural circuits work and how they produce sophisticated

brain functions.

Finally, the importance of an industrial infrastructure for NOs cannot be emphasized. Considering the value of NOs as a disease model for basic research and drug development, enhancing the accessibility of NOs to the equivalent level of experimental animals will be key to maximizing the impact of NO techniques; for example, a biobank of organoids will facilitate NO-based research and development.

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AUTHOR CONTRIBUTIONS

W.S. conceived the project. W.S. and J.H.L. wrote the manuscript.

CONFLICT OF INTEREST

The authors have no potential conflicts of interest to disclose.

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