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Contents

Monthly Volume 15 Number 6 June 26, 2023

REVIEW

MINIREVIEWS

ORIGINAL ARTICLE

Basic Study

SYSTEMATIC REVIEWS

World Journal of Stem Cells

Monthly Volume 15 Number 6 June 26, 2023

Contents

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REVIEW

Neural lineage differentiation of human pluripotent stem cells: Advances in disease modeling

Yuan-Wei Yan, Eddie S Qian, Lauren E Woodard, Julie Bejoy

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Abstract

Brain diseases affect 1 in 6 people worldwide. These diseases range from acute neurological conditions such as stroke to chronic neurodegenerative disorders such as Alzheimer's disease. Recent advancements in tissue-engineered brain disease models have overcome many of the different shortcomings associated with the various animal models, tissue culture models, and epidemiologic patient data that are commonly used to study brain disease. One innovative method by which to model human neurological disease is *via* the directed differentiation of human pluripotent stem cells (hPSCs) to neural lineages including neurons, astrocytes, and oligodendrocytes. Three-dimensional models such as brain organoids have also been derived from hPSCs, offering more physiological relevance due to their incorporation of various cell types. As such, brain organoids can better model the pathophysiology of neural diseases observed in patients. In this review, we will emphasize recent developments in hPSC-based tissue culture models of neurological disorders and how they are being used to create neural disease models.

Key Words: Induced pluripotent stem cells; Astrocytes; Oligodendrocytes; Microglia; Brain organoids; Assembloids

Core Tip: This review discusses recent advances in the field of disease modeling using human-induced pluripotent stem cell-derived neural cell types as well as organoids. It also discusses challenges that exist with current approaches, in addition to considerations for possible improvements that will further advance the field of disease modeling.

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INTRODUCTION

Human pluripotent stem cells (hPSCs) include both human embryonic stem cells (hESCs) and human-induced PSCs (hiPSCs). Current *in vitro* disease models that use hiPSCs begin with skin or blood cells that have been reprogramed with the four transcriptional elements octamer-binding transcription factor 4, SRY-box 2, Krüppel-like factor 4, and MYC[1]. Through differentiation, these hPSCs are the starting material to create models for different organs including the brain[2], kidney[3], liver[4,5], lung[6], and pancreas[7]. These models are able to simulate a "disease-in-a-dish", mimicking different disease phenotypes *in vitro*[8,9]. Both genetically modified hiPSCs and patient-derived hiPSCs can generate disease models[10-12]. These models are advantageous because of their accessibility, quick processing, and species-specific human attributes. Patient-derived hiPSCs can also be used to test personalized medicine approaches to effectively model gene mutations and chromosomal abnormalities. To study neurological diseases, scientists have generated multiple neural cell types from hPSCs including neurons[13], astrocytes[14], and oligodendrocytes (OLs)[15]. In the past decade, advancements in disease modeling and tissue engineering have also led to the "brain organoid" model[16]. Brain organoids are self-assembled structures that resemble the fetal human brain and are composed of progenitor, neuronal, and glial cells. A related system is the spheroid, a circular aggregate of cells that may reflect biological properties of an organ system but ultimately lacks structural complexity. Perhaps the most cutting-edge form of modeling technology in stem-cell research is the assembloid, which are three-dimensional (3D) structures made by fusing and integrating two or more cell types or organoids from different organ culture protocols[17]. These assembloids can model the organ crosstalk interactions that occur across physiological systems in the human body.

In this review, we discuss recent advancements in the field of disease modeling using hiPSC-derived neural cell types as well as organoids. We also discuss challenges that exist with current approaches, in addition to considerations for possible improvements that will further advance the field of disease modeling.

NEURAL CELL TYPE DIFFERENTIATION FROM HPSCS

Neural progenitors

The development of the mammalian brain initially occurs at the gastrula stage when the ectoderm differentiates to form the neural tube. This process is called neural induction, wherein neural tube cells become neural progenitors[18,19]. These progenitors subsequently give rise to specific neuronal subtypes along the rostral-caudal axis and dorsal-ventral axis[20]. Protocols for neural progenitor differentiation from hPSCs have been developed that reflect this neural induction principle (Table 1). In 2001, the first protocol of neural progenitor differentiation from hPSCs was developed using the embryoid body (EB) method, which was a combined two-dimensional (2D) monolayer and 3D suspension culture [21]. Without extrinsic factors, the EB method mainly derived dorsal forebrain cortical neurons. In 2008, advances in the EB method eventually gave rise to a complete 3D culture system called the serum-free floating culture of EB-like aggregates with quick aggregation (SFEBq)[22]. The SFEBq method generated neural tissues with self-organized structure using hPSCs, paving the way for the development of more complex systems such as brain spheroids and organoids. Following this advance, in 2009 the dual SMAD inhibition method was developed, which successfully directed over 80% of hESCs to induce neural differentiation[23]. The dual SMAD inhibition method was initially intended for 2D monolayer culture by inhibiting the bone morphogenetic protein (BMP) and transforming growth factor beta (TGF-β) signaling pathways, but it has been widely applied to 3D culture for neural progenitor differentiation

Table 1 Comparison of methods for neural induction from human pluripotent stem cells

Method	Neural induction outcomes	Significance	Ref.
Embryoid bodies; selected neural rosettes; 2D and 3D culture	Neural tube-like rosettes stained with Nestin, Musashi-1 and NCAM; positive neuronal markers MAP2 and TUJ1 expression	First study of neural progenitor differentiation from hPSCs	Zhang *et al*[22], 2001
SFEBq aggregate; sorting cells; 3D culture	Self-organized structure with four distinct zones: ventricular, early and late cortical-plate, and Cajal-Retzius cell zones	Pure 3D culture, provides the basis for the brain organoid method	Eiraku *et al*[23], 2008
Dual SMAD inhibition; 2D monolayer culture	Complete neural conversion of > 80% of hESCs	Mostly wild used method; also enables neural induction in 3D culture	Chambers *et al*[24], 2009
Dual SMAD inhibition combined with retinoid signaling; 2D monolayer culture	More than 95% of hPSCs were PAX6 and OTX1/2 cortical progenitor cells in 15 d	Improved the dual SMAD inhibition protocol and higher neural induction efficiency	Shi *et al*[62], 2012
Cortical organoid/spheroid; 3D culture	Form layered structure tissues partially mimicking human cerebral cortex	Mostly brain-like tissue with some functions	Lancaster *et al*[17], 2013; Paşca *et al*[26], 2015; Qian *et al*[27], 2016
Dual SMAD inhibition combined with Wnt, FGF and Notch inhibition	Generate functional cortical neuron in 16 d	Improved the dual SMAD inhibition protocol and accelerated neural induction	Qi *et al*[28]

EB: Embryoid body; FGF: Fibroblast growth factor; hPSCs: Human pluripotent stem cells; MAP2: Microtubule-associated protein 2; NCAM: Neural cell adhesion molecule; OTX: Orthodenticle homeobox; PAX6: Paired box 6; SFEBq: Serum-free floating culture of EB-like aggregates with quick aggregation.

from hPSCs. It is worth noting that both the SFEBq and dual SMAD inhibition methods can enable the generation of cortical spheroids and organoids[24-26]. In 2017, Studer's group modified the dual SMAD inhibition protocol to also block mitogen-activated protein kinase (MAPK), fibroblast growth factor (FGF), and Notch signaling, thereby accelerating forebrain cortical neuron derivation[27]. Although these protocols yield primarily deep-layer cortical neurons, deriving upper layer cortical neurons such as L2/3 and L4 cells is still a challenge.

Astrocytes

Astrocytes are star-shaped populations of glial cells that help maintain homeostatic balance and support neuron growth within the central nervous system. There are two distinct groups of astrocytes: The highly branching protoplasmic astrocytes of the grey matter and the fibrous astrocytes found in the white matter that interact with OLs and axons[28]. Activated astrocytes can release neuroinflammatory cytokines and chemokines that mediate intercellular communication with microglia and invoke various neuroinflammatory responses. Similar to neurons, there are many subtypes of astrocytes depending on their location, morphology, molecular signature, and physiological function. The differentiation of glia cells from hPSCs usually takes more time and is more complicated than differentiating a neuron (Figure 1).

During brain development, astrocytes differentiate from radial glia or neural progenitors at the subventricular zone. It is currently unknown what signaling regulates the regional identity of astrocytes. The differentiation of astrocytes is usually initiated by inhibition of dual SMAD signaling using small molecules or by the EB method to generate neuroepithelial cells. Glial progenitors expressing nuclear factor IA (NFIA), S100β, and cluster of differentiation 44 (CD44) are derived from these neuroepithelial cells[29]. Ultimately, mature astrocytes are generated from radial glia by activating the signal transducer and activator of transcription 2 signaling pathway using ciliary neurotrophic factor. The most common marker for astrocytes is glial fibrillary acidic protein (GFAP)[30]. Mature astrocytes express aldehyde dehydrogenase family 1 member L1, aldolase C, glutamate transporter-1, and aquaporin 4[31]. In 2011, the first reported protocol for hPSC-derived astrocytes in a chemically defined system required long-term culture of up to 6 mo[29,32]. This protocol used the EB method and supported differentiation through the addition of the factors FGF2 and epidermal growth factor (EGF). To attenuate the culture time, shorter 4-6 wk long accelerated protocols for generating functional astrocytes through overexpression of the transcription factors SOX9 and NFIB were developed[33,34]. In 2017, the Pasca lab found a method to derive functional astrocytes using 3D cortical organoids. However, this protocol required up to 590 d, limiting its application[30]. The majority of recent studies use commercially available astrocyte differentiation medium to differentiate astrocytes from neural progenitor cells[35,36].

Astrocytopathies including Alexander disease[37,38], Aicardi-Goutières syndrome (AGS)[39], and vanishing white matter disease[40] can be effectively modeled with hiPSC-derived astrocytes[41]. Neurodegenerative diseases including Alzheimer's disease (AD)[36], Parkinson's disease (PD)[42], and Huntington's disease have also been modeled using similar methods. The familial presenilin-1 (*PS1*)

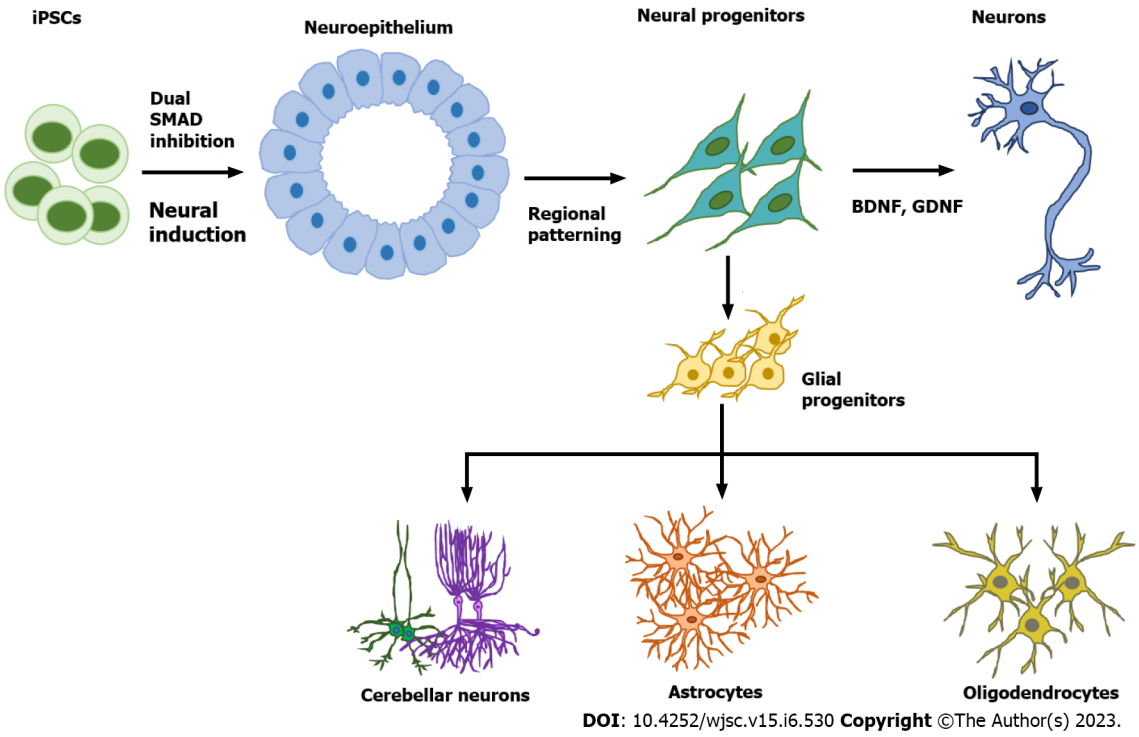

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Figure 1 Neural cell subtype differentiation from human pluripotent stem cells. The first step of neural cell differentiation is neural induction to generate neuroepithelial cells, usually by the dual SMAD inhibition method. Specific neural progenitors can be generated by tuning different signaling pathways such as Sonic Hedgehog, Wingless/integrated, retinoic acid, and bone morphogenetic protein. Neural progenitors can then be directed to become mature neurons through induction with neurotrophic factors such as brain-derived neurotrophic factor and glial cell line-derived neurotrophic factor or derived into glial progenitors through treatment with the growth factors fibroblast growth factor 2 and epidermal growth factor. Glial progenitors can give rise to either astrocytes or oligodendrocytes. BDNF: Brain-derived neurotrophic factor; GDNF: Glial cell line-derived neurotrophic factor.

mutation along with PD familial leucine-rich repeat kinase 2 (*LRRK2*) G2019S mutations were both modeled using hiPSC-derived astrocytes. The results showed the crucial role of astrocytes in the disease pathogenesis of AD and PD, respectively[36,42]. When co-cultured with neurons, astrocytes generated from Huntington's disease patient-derived hiPSCs displayed decreased electrophysiological activity and diminished neuroprotection consistent with Huntington's disease[43]. Regarding *in vitro* stroke modeling, ischemia-like conditions can be simulated by replacing normal oxygen (O_2)/carbon dioxide (CO_2) conditions with nitrogen (N_2)/CO_2 and subjecting cells to glucose deprivation[44,45]. However, cultures in 2D cannot effectively model stroke due logistical difficulties in restricting oxygenation as well as maintaining nutrition deprivation. However, Wevers *et al*[46] used neurovascular unit on-a-chip, which included a triculture of brain vascular cells, hiPSC-astrocytes, and hiPSC-neurons to model ischemic stroke. The study used antimycin-A, an inhibitor of complex III of the electron transport chain, to induce hypoxic conditions[45]. Modeling the motor neuron pathology linked to amyotrophic lateral sclerosis (ALS) was achieved using hiPSC-derived astrocytes from a patient who had the C9ORF72 mutation[46,47]. Recent studies also reported generation of ventral spinal cord-like astrocytes, which better reflect ALS pathophysiology[48]. Zika virus targeting of astrocytes has also been studied using hiPSC-derived astrocytes, which corroborated the reactive oxygen species imbalance, mitochondrial abnormalities, and DNA damage observed after Zika virus infection[49]. Astrocytes derived from hiPSCs are also beneficial in modeling neurodevelopment disorders including Down's syndrome[50-53], Rett syndrome[54-57], and schizophrenia[58,59]. Rare genetic diseases such as the lysosomal storage disorder Gaucher disease can be modeled using patient hiPSC-derived astrocytes[60,61] (Figure 1, Table 1).

Oligodendrocytes

Protocols to differentiate hiPSCs into pre-OL progenitors were first established in 2012[62]. Retinoic acid (RA) and purmorphamine, a small-molecule agonist of Sonic Hedgehog (Shh) signaling, were used to make pre-OL progenitors that express the markers oligodendrocyte transcription factor 2 (OLIG2) and NK2 homeobox 2. Pre-OL progenitors were then further differentiated into bipotential OL progenitor cells (OPCs) that expressed markers SOX10 and platelet-derived growth factor receptor alpha (PDGFRA) using PDGF-AA, triiodothyronine, and neurotrophin-3. The OPCs at this stage were further developed into either $O4^+$ and myelin basic protein-positive (MBP^+) human-induced OLs or $GFAP^+$ astrocytes. OPCs ameliorate neurological deterioration and support survival of *shiverer* mice after engraftment[62]. However, this protocol requires a lengthy 120-d culture period. Efficient and robust

generation of hiPSC-derived OPCs in 95 d has been achieved more recently[63,64]. Improved differentiation of myelinating OLs was obtained using brain extracellular matrix from decellularized human brain tissue[65]. Fast and efficient OL generation has additionally been achieved with SOX10 overexpression, either by introducing lentiviral vectors at the neuroepithelial stage or by direct transfection of hiPSCs prior to differentiation[66,67].

Shaker *et al*[68] published a 42-d protocol to derive organoids containing myelinating human OLs and astrocytes. Differentiated OLs that were produced using hiPSCs from primary progressive multiple sclerosis patients were found to be functional and supported *in vivo* myelination in *shiverer* mice[63]. Death of OLs is a hallmark of Pelizaeus-Merzbacher disease, an X-linked leukodystrophy caused by mutations in proteolipid protein 1 (PLP1)[63]. Human-induced OLs from individuals with PLP1 mutations have helped to identify important subgroups based on cell-intrinsic phenotypes and to elucidate the pathogenesis of various PLP1 mutations[15,68]. Involvement of OLs in neurodegenerative diseases, including AD and PD as well as multiple system atrophy[69], have also been studied using human-induced OLs.

OTHER CELL TYPES OF THE CENTRAL NERVOUS SYSTEM

Microglia

Although murine models have been the main tool for studying the genetics and function of microglia, there are important distinctions between murine microglia and human microglia when it comes to aging and associated diseases[70,71]. Historically, viable microglia cells have been obtained by extracting them from brain tumors or epileptic foci removed from surgery, but this procedure is logistically very challenging. These hurdles were reduced when multiple methods to differentiate microglia from hPSCs were developed[70-75]. Muffat *et al*[73] published the first protocol by producing microglia-like cells from regular and patient hESCs and hiPSCs. This method used serum-free neuroglial differentiation media, which contained various components with concentrations adjusted to biologically match human cerebrospinal fluid. Abud *et al*[76] described a two-step method to successfully derive microglia-like cells (iMGLs) from 10 different hiPSC lines in 5 wk. The transcriptome profile of the derived iMGLs was strikingly similar to that of both adult human and fetal microglia[75]. Most microglial directed differentiation protocols involve hematopoiesis[73,75,76]. Some reported studies use chemically-defined protocols to generate human microglia through the formation of myeloid progenitors in 30 d[77]. Ionized calcium binding adapter molecule 1, a protein that belongs to the calcium-binding protein family, is one of the main markers of microglia[78]. It is primarily involved in rearranging cytoskeleton and has been used as a marker for the 3D reconstruction of microglial cells[79,80]. Other general markers used for microglial identification are CD45 and CX3C motif chemokine receptor 1. In a recent study, Dräger *et al*[81] described an effective 8-d protocol for generating induced transcription factor microglia-like cells (iTF-Microglia) based on the inducible expression of six transcription factors (human MAF BZIP transcription factor B, CCAAT enhancer-binding protein, interferon regulatory factor (IRF8) PU.1, and IRF5).

The risk of developing late-onset AD is linked to several genes, including triggering receptor expressed on myeloid cells 2 (*TREM2*) and *CD33* expressed by microglia. Microglia accumulate around amyloid plaques during AD and exacerbate pathophysiology by secreting cytokines and chemokines that induce inflammation. Microglia that have been generated using hiPSCs can be effectively used to model neurological diseases *in vitro*[80,81]. Alternatively, microglia derived from patient hiPSCs have also been used for modeling neurodegenerative diseases. Recently, patient hiPSCs expressing the AD-linked R47Hhet *TREM2* variant was used to elucidate the signal transduction deficit observed during AD progression[82]. Another study using microglia derived from AD patient hiPSCs reported that dysregulated peroxisome proliferator-activated receptor gamma (PPARγ)/p38 MAPK signaling causes the phenotypic deficits observed in *TREM2* variants. The results of this study concluded that the activation of PPARγ/p38MAPK signaling can ameliorate metabolic deficits within these cells and consequently rescue critical microglial cellular functions such as β-amyloid phagocytosis[83].

BRAIN ORGANOID DEVELOPMENT FROM HPSCS

Protocols for producing brain organoids were derived from the EB and SFEBq methods for neural induction. The formation of brain organoids is based on the self-organization and self-renewal of stem cells to generate a mixed cell population in 3D suspension culture (Figure 2). In 2013, the first study on brain organoids was reported to generate whole brain tissues with regional specific structures using an EB-based culture involving Matrigel support in a spinning bioreactor[24]. The organoids were used to model microcephaly, a neurodevelopmental disease whose pathologic features are difficult to recapitulate using animal models. This was the first work done to generate brain-like tissue *in vitro* and apply them to study human pathological disorders. In the following years, a simpler method was

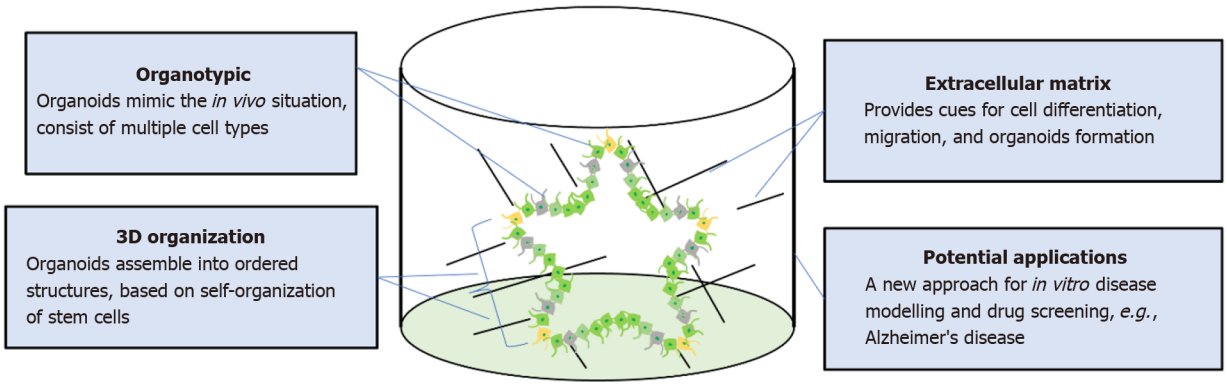

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Figure 2 Self-organization of brain organoids. Human brain organoids are generated based on the self-organizing properties of stem cells. Organoids usually contain multiple cell types including mature neurons and immature neural progenitors. The key to organoid regeneration is the extracellular matrix that is used to support stem cell growth and differentiation. Brain organoids have been widely utilized to model neurological pathology in disease such as Alzheimer's disease and microcephaly.

developed to generate cortical spheroids in 3D static culture without Matrigel and agitation[25]. This method first derived neural progenitors using dual SMAD inhibition and then induced regional specific patterning by supplementing culture with the growth factors FGF2 and EGF. The last step of this protocol extended cultivation of brain aggregates and replaced the growth factors with the neurotrophic factors brain-derived neurotrophic factor (BDNF) and glial cell line-derived neurotrophic factor (GDNF) for up to 3 mo. The generated cortical spheroids exhibited a cortical layer-like structure and were developmentally comparable with the human fetal cortex. Brain spheroids and organoids have been widely used to model human brain development and neurological diseases *in vitro* and have provided a promising platform for drug screening[84] (Table 2, Figure 2).

Forebrain organoids

The areas of the brain originating from the telencephalon and diencephalon are referred to as the forebrain. The telencephalic region consists of the cerebral cortex and cerebellum, whereas the diencephalon includes the thalamus, hypothalamus, and pituitary glands. Self-organized cortical organoids or dorsal forebrain organoids were first reported using the SFEBq method. Multiple cortical layer tissues were generated through inhibition of TGF-β and Wnt signaling, resulting in dorsal-ventral patterning[22,85]. Lancaster *et al*[17] reported the first 3D culture system for deriving cortical organoids from hPSCs. Later, the Pasca lab generated more complex cortical spheroids and organoids from hPSCs, containing both neurons and astrocytes[25] (Figure 3). In following years, several groups attempted to develop protocols to derive cortical organoids from hPSCs. However, a common problem faced by many of these approaches was the presence of multiple ventricular subtypes within each organoid[26,86-91]. Cortical organoids derived from familial AD patient hiPSCs show increased levels of phosphorylated Tau and cytoplasmic neurofibrillary tangle-like deposits[92,93] (Table 1). Recent studies have shown that culture variations have an impact on the AD phenotypes seen in cerebral organoids and should be considered when using these models[94]. Cortical organoids were also used in stroke modeling to study the effects of oxygen-glucose deprivation (OGD), neuronal death that followed, and damaged neural networks[95]. These models use 2-8 h of OGD with O_2 (0.1%), CO_2 (5.0%), and N_2 (95.0%) gas levels and deoxygenated glucose-free medium to induce ischemia[95]. It has also been discovered that hypoxic conditions can reduce the number of progenitors and impair the differentiation of immature neurons during the development stage of brain organoids[96,97]. Ventral forebrain tissue-like medial ganglionic eminence (MGE) organoids are usually patterned by high Shh and low Wnt signals[85,98]. These MGE organoids contain diverse GABAergic interneurons subtypes including somatostatin, parvalbumin, calretinin, and calbindin. These MGE organoids were assembled to model the migration of human interneurons towards the cerebral cortex[98-100]. In 2020, the Pasca lab reported a method to generate striatal organoids expressing medium spiny neuron markers such as DARPP32 using activin A, IWP-2, and SR11237[101]. Brain organoid technology has also been utilized to generate organoids that can model other regions of forebrain tissue including thalamic organoids[102], hypothalamic organoids[103-105], and hippocampal organoids[106,107].

The hippocampus plays a significant role in learning, memory, and emotion. Hippocampal atrophy or hyperexcitability can cause neurological disorders such as schizophrenia and neurodegenerative diseases like AD. Hippocampal spheroids can be derived from hiPSCs using dual SMAD inhibition, Shh, and Wnt pathway inhibition followed with Wnt activation[107,108]. Commonly reported hippocampal markers include zinc finger and BTB domain-containing 20 and prospero homeobox 1. Hippocampal spheroids can be used to model AD pathology either by the exogenous addition of

Table 2 Comparison of methods for brain organoids generation from human pluripotent stem cells

Organoid type or brain region modeled	Method brief description	Model application	Ref.
EB-like aggregates; cerebral cortex	SFEBq, static suspension culture with cell sorting	Form self-organized structure mimicking the early cortiogenesis	Eiraku *et al*[23], 2008
Cerebral organoid; whole brain	Spinning bioreactor with Matrigel supporting	Form pyramidal identities with spatial separation mimicking the developing human brain at early stage; modeling microcephaly	Lancaster *et al*[17], 2013
Cortical neuroepithelium; cerebral cortex	Improved SFEBq, in 40% oxygen in Lumox plates	Inside-out layer pattern for human cortex	Kadoshima *et al*[86], 2013
Cortical spheroid; cerebral cortex	Static suspension culture with FGF-2 and EGF	Generated laminated cerebral cortex-like structure with some functions	Paşca *et al*[26], 2015
Cerebellar-plate-like neuroepithelium; cerebellum	Static suspension culture with FGF-19 and SDF-1	Mimicking the early development of human cerebellum	Muguruma *et al*[129], 2015
Telencephalic organoids; forebrain	Static suspension culture after neural rosettes isolation manually	Modeling autism spectrum disorder	Mariani *et al*[130], 2015
Dorsomedial telencephalic-like tissue; hippocampus	Improved SFEBq, in 40% oxygen	Modeling the development of human hippocampus	Sakaguchi *et al*[107], 2015
Forebrain organoids; cerebral cortex	Miniaturized spinning bioreactor	Zika virus exposure	Qian *et al*[27], 2016
Midbrain organoids; midbrain	Miniaturized spinning bioreactor	Midbrain organoids contained TH^+ cells	Qian *et al*[27], 2016
Hypothalamic organoids; hypothalamus	Miniaturized spinning bioreactor	Modeling early hypothalamus development	Qian *et al*[27], 2016
Midbrain organoids; midbrain	Static suspension culture on orbital shaker	Midbrain produced neuromelanin and dopamine	Jo *et al*[131], 2016
Pituitary organoid; anterior pituitary	Improved SFEBq	Formed pituitary placode with pituitary hormone-producing cells	Ozone *et al*[132], 2016
Cerebral organoid; cerebral cortex	Microfilament-engineered organoids under agitation	Formed polarized cortical plate and radial units	Lancaster *et al*[133], 2017
Cerebral organoid; whole brain	Spinning bioreactor with Matrigel supporting	Brain organoids formed spontaneously active neuronal networks	Quadrato *et al*[134], 2017
Brain assembloids; assembly dorsal and ventral forebrain organoids	Static suspension culture	Modelling migration of human interneurons and their functional integration into microcircuits using healthy and timothy syndrome cell line	Birey *et al*[99], 2017
Fused cerebral organoids; assembly dorsal and ventral forebrain organoids	Static suspension culture with Matrigel supporting on orbital shaker	Modelling migration of human interneurons in cerebral cortex	Birey *et al*[99], 2017
Fused cortical organoids and MGE organoids	Static suspension culture on orbital shaker	Modelling migration of human interneurons	Xiang *et al*[101], 2017
Neoplastic cerebral organoid	Static suspension culture with Matrigel supporting on orbital shaker	Modelling brain tumorigenesis	Bian *et al*[135], 2018
Granted brain organoids in mouse	Spinning bioreactor	Formed functional networks and blood vessels in the grafts	Mansour *et al*[136], 2018
Cortical spheroid	Static suspension culture	Modelling Alzheimer's disease	Yan *et al*[87], 2018
Cerebral organoids	Static suspension culture with Geltrex supporting on orbital shaker	Modelling Alzheimer's disease	Gonzalez *et al*[93], 2018
Neuromuscular organoid	Static suspension culture supporting on orbital shaker	Formed functional neuromuscular junctions and modelling myasthenia gravis	Faustion Martins *et al*[137], 2020
Section spherical organoid	Manually slicing forebrain organoids	Sliced organoids exhibited separated upper and deep cortical layer	Qian *et al*[90], 2020
Cortico-motor assembloids; assembly cortical spheroids, spinal spheroids, and skeletal muscle spheroids	Static suspension culture	Modeling cortical-motor circuits	Andersen *et al*[18], 2020
Cortico-striatal assembloids; assembly cortical spheroids and striatal spheroids	Static suspension culture	Modeling cortical-striatal circuits and 22q13.3 deletion syndrome	Miura *et al*[102], 2020
Air-liquid interface cerebral organoids	Slicing mature organoids and cultured in air-liquid interface not completely submerged in liquid	Formed network with functional output	Giandomenico *et al*[138], 2019

ASD: Autism spectrum disorder; EGF: Epidermal growth factor; FGF2: Fibroblast growth factor 2; hPSCs: Human pluripotent stem cells; SDF: Stromal cell-derived factor; SFEBq: Serum-free floating culture of EB-like aggregates with quick aggregation.

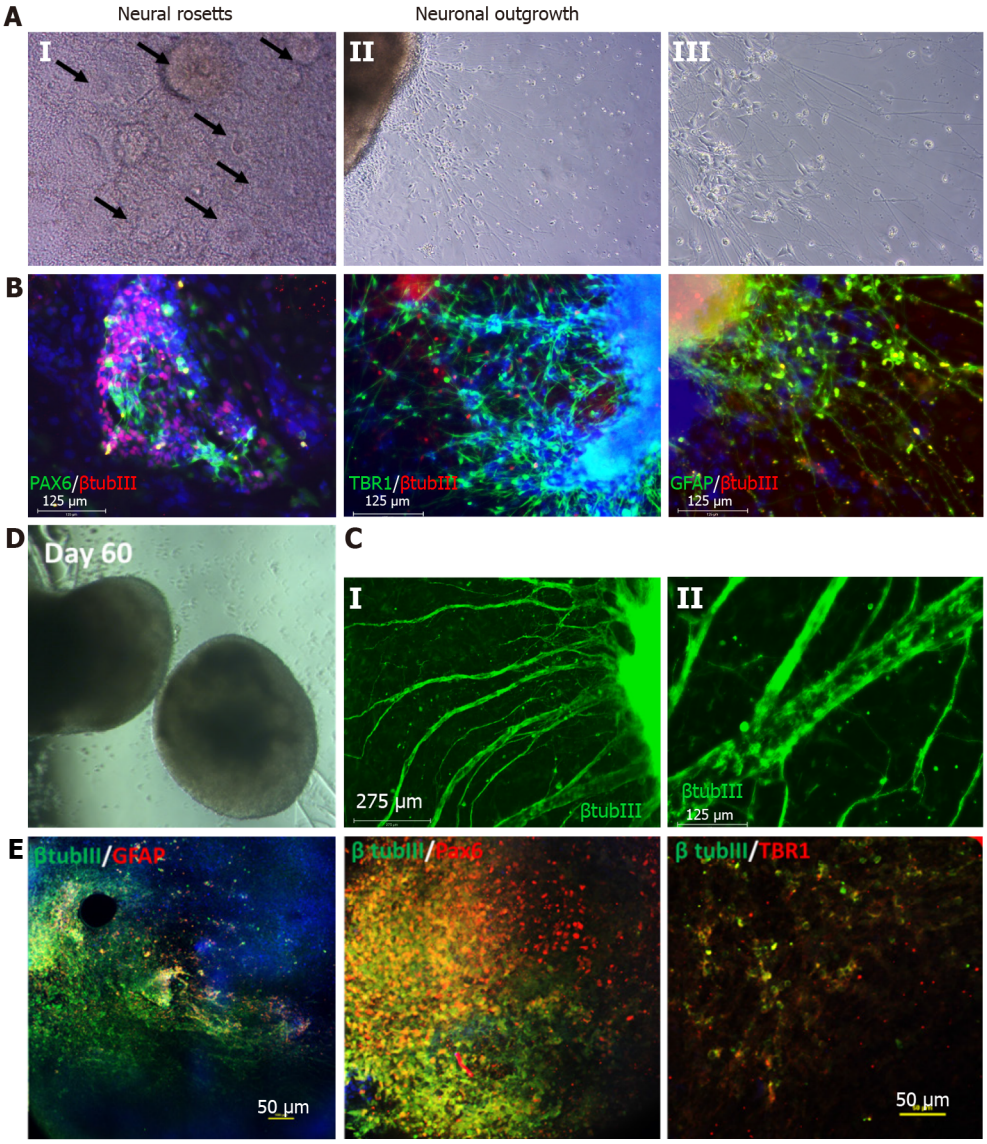

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Figure 3 Characterization of cortical organoids for neural and astrocyte marker expression. A: Brightfield images showing the neural rosettes and neuronal outgrowth from the organoids replated to an attachment plate at day 35 of differentiation; B: Resulting immunocytochemistry analysis of neural marker paired box 6 (PAX6), cortical deep layer VI marker T-box brain transcription factor 1 (TBR1), astrocyte marker glial fibrillary acidic protein (GFAP) co-stained with common neural marker β tubulin III, scale bar 125 μm; C: Immunostaining at later stage of the replating showing thick axon like extensions from the organoids, scale bar: 275 μm; D: Brightfield images of the day 60 cortical organoids; E: Confocal images of the day 60 organoids showing astrocyte marker GFAP, neural marker PAX6, cortical deep layer VI marker TBR1 co-stained with common neural marker β tubulin III, scale bar: 50 μm.

amyloid beta 42 oligomer[107,108] or by using amyloid precursor protein/PS1 variant hiPSCs. Current hippocampal organoids reflect the early stages of embryonic hippocampus development and successfully can create dentate gyrus granule and carbonic anhydrase 3 (CA3) pyramidal-like neurons, but are unable to produce CA1 pyramidal-like neurons.

Midbrain organoids

The protocol to differentiate human midbrain-like organoids (hMLOs) employs several molecules to mediate the differentiation of neuroepithelial cells. These factors include hBDNF, hGDNF, dibutyryl cyclic adenosine monophosphate, ascorbic acid, TGF-β3, and 1 purmorphamine[109,110]. The presence of dopamine transporter tyrosine hydroxylase as well as the expression of G-protein-regulated inward-rectifier potassium channel 2 are both characteristics of midbrain dopaminergic (mDA) neurons in hMLOs. Common midbrain genes including engrailed, nuclear receptor 4A2, LIM homeobox

transcription factor 1 beta (*LMX1B*), *LMX1A*, monoamine oxidase B, calbindin 1, tyrosine hydroxylase, catechol-O-methyltransferase, and dopa-decarboxylase have also been detected in these organoids. Additionally, neurons in hMLOs have been found to exhibit action potentials with large sag currents, indicating the existence of mDA neurons[111] (Figure 3).

According to single cell sequencing studies, hMLOs replicate early embryonic neurodevelopment and recapitulate disease characteristics[112,113]. However, the methods to generate midbrain organoids can take a significant amount of time, and can vary from batch to batch. To scale up the generation of midbrain organoids, Mohamed *et al*[114] recently published microfabricated disk technology using eNUVIO EB-Disks. Another study found that the use of recombinant spider-silk microfibers functionalized with full-length human laminin produced similar ventral midbrain organoids with lower inter-organoid variability[114,115]. Alternatively, an automated approach, termed automated midbrain organoids, was published by Renner *et al*[116] that produced high-throughput 3D midbrain organoids. The high-throughput production of hMLOs from hPSCs in spinner flasks was also reported using TH-TdTomato reporter hPSC lines as well[116].

As the second most prevalent neurodegenerative disease worldwide[117], PD is frequently studied using hPSC-derived hMLOs[87,111,112,115,118-120]. The disease is characterized by the loss of dopaminergic neurons in the substantia nigra and is mainly caused by mutations in glucocerebrosidase and LRRK2 genes in addition to α-synuclein (α-syn; *SNCA*) gene triplications[111,121,122]. The hMLOs generated from patients with these mutations display PD traits such as oligomeric and fibrillar α-syn aggregates, loss of mDA neurons, and Lewy body-like inclusions[111,118,119]. Since hPSCs can be edited using clustered regularly interspaced short palindromic repeats (CRISPR)/CRISPR-associated protein 9 technology, *SNCA* gene genome correction has been demonstrated to revert PD patient hPSCs back to wild-type phenotypes[119,123]. These hMLOs have also been successfully generated from hiPSCs carrying the *LRRK2*-G2019S mutation[124]. Biallelic pathogenic variations in the phosphatase and tensin homolog-induced kinase 1 gene that controls mitochondrial function is also connected to the etiology of PD[125]. Human Parkinsonism can also have a more direct cause such as the toxicity of some drugs including 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine, which has also been modeled using hMLOs[126-128] (Table 2)[129-138].

Hindbrain organoids

The medulla, pons, and cerebellum make up the hindbrain region, which is developed from the metencephalon and myelencephalon. Methods to generate hindbrain organoids commonly involve purmorphamine-mediated Shh signaling activation to convert neuroepithelial cells into ventral identity neurons. RA is used as a potent caudalizing agent to promote the fate of hindbrain cells instead of Wnt signaling activation, which is required for midbrain patterning[138]. Markers of hindbrain neurons include serotonergic neuron marker serotonin, human fifth Ewing variant, gastrulation brain homeobox 2, choline acetyltransferase (ChAT), and HB9. Due to their location, cerebellar neurons cannot be easily studied at the cellular or molecular level. Thus, using hiPSC-derived technologies is advantageous in this situation. The cerebellum can be divided into inhibitory GABAergic neurons known as Purkinje cells, which are derived from pancreas-specific transcription factor 1α progenitors, and excitatory glutamatergic neurons known as granule cells, which are descended from atonal homolog 1 (also known as MATH1) progenitors[139].

The first published granule cell differentiation protocol using hiPSCs involved several factors including FGF8B, Wnt proteins, BMPs, and RA. This method recapitulates anteroposterior and dorsoventral patterning and thereby induces *MATH1*-expressing mitotic neural progenitors, which can later be differentiated into cerebellar granule cells. Purkinje cells derived from hiPSCs initially had an immature phenotype, and thus needed to be co-cultured with mouse cerebellar granule cell precursors to allow for maturation[129,140]. However, cells made using this approach had substantial functional variability. Silva *et al*[141] recently published a protocol that generates mature cerebellar neurons without the need of such a co-culture system. This method involves stimulating the development of cerebellar precursors with FGF19, followed by self-organization and differentiation using stromal cell-derived factor 1 and BDNF/GDNF. Cerebellar neurons derived from hiPSCs are also helpful for modeling diseases, particularly cerebellar ataxia, a neurodegenerative disease that affects cerebellar neurons and eventually leads to motor incoordination. Cerebellar neurons derived from hiPSCs of either healthy human participants or ataxia patients were used in several recent studies to create an *in vitro* disease model. Spinocerebellar ataxia type 6 patient hiPSC-derived Purkinje cells have been used to model both thyroid hormone depletion-dependent degeneration and downregulation of the transcriptional targets TATA-Box Binding Protein Associated Factor 1 and BTG anti-proliferation factor 1, indicating their potential as a pathogenesis tool[141].

Recently, protocols for generating brain stem organoids have also been published, offering a new tool for evaluating the pathophysiology of disorders that impact the brainstem. Human brain stem organoids express the medullary marker ChAT, the pons marker hydroxylase, and the mature and functioning excitatory and inhibitory neuron markers vesicular glutamate transporter 1 and glutamic acid decarboxylase 67 in addition to various other relevant markers[142]. Both $OLIG2^+$ and MBP^+ OLs, as well as $S100^+$ astrocytes, are expressed in brain stem organoids[142].

Assembloids

Assembloids are systems that combine one type of spheroid or organoid with another type of spheroid or organoid. For example, assembloids can be produced by combining the dorsal and ventral forebrain, the cerebral cortex with the thalamus, or the cerebral cortex with any other non-neural cell type such as microglia, immunological cells, pericytes, and endothelial cells. The Pasca lab published the first assembloid study in 2017, in which human cortical spheroids were mixed with human subpallium spheroids[98]. The assembloids were created using human subpallium spheroids and cortical spheroids differentiated from hiPSCs from timothy syndrome (TS) patients with mutations in the α1c subunit of the L-type calcium channel (*CACNA1C*) gene. The two types of spheroids were combined in simple conical tubes and left undisturbed for 3 d to produce assembloids. The interneurons within the assembloids migrated, suggesting high potential for the study of certain aspects of migratory disorders such as TS. These patient-derived assembloids showed less effective interneuron movement, which was reversed by the administration of L-type calcium channel blockers.

Since then, many labs have sought to use assembloids to elucidate the interactions that occur between different physiological systems. The Knoblich lab reported the use of fused cerebral organoids that combined dorsal and ventral forebrain tissue cultures. They showed migration of C-X-C chemokine receptor type 4-dependent GABAergic interneurons from the ventral forebrain to the dorsal forebrain, which had a more MGE identity[143]. Assembloids of cortical organoids with integrated pericyte-like cells which express angiotensin-converting enzyme 2 have also been shown to enhance severe acute respiratory syndrome coronavirus 2 infection, suggesting the involvement of multiple cell types[100]. Another study employed cortico-striatal assembloids to recapitulate neurodevelopmental disorders that impair the cortico-striatal pathway, including schizophrenia, obsessive-compulsive disorder, and autism spectrum disorder[144-146]. These cortico-striatal assembloids were developed from patients with Phelan-McDermid Syndrome, a severe developmental disorder also known as 22q13.3DS. It is important to note that these patient-derived assembloids had a higher number of calcium spike events than striatal organoids, offering a better representation of altered neural activity. Interneuron migration has also been reported in a separate assembloids study that fused human MGE organoids with human cortical organoids[147].

CURRENT LIMITATIONS AND POTENTIAL ADVANCEMENTS

The extended culture times required by current methods to produce neural cell types as well as organoids restrict their application. Another consideration is the cell-line-to-cell-line and batch-to-batch variabilities of hiPSC differentiation. Therefore, accelerated protocols with less variable outcomes should be developed. For hiPSC-derived astrocytes, the major drawback is their lack of regional identity. Most protocols derive astrocytes with cortical identity, which may not be useful for modeling disease pathophysiology affecting the ventral part of the brain. Therefore, it is essential to employ experimental approaches that can produce astrocyte subtypes with the appropriate rostro-caudal and dorso-ventral identities. In the case of hiPSC-derived OLs, the lack of advanced OL disease models created using genetically-modified hiPSCs also limits their application. Finally, the lack of vascularization in current organoid and assembloid systems prevents the important study of cell-type crosstalk. Therefore, incorporating vasculature as well as reducing culture time would benefit multiple methods of neural lineage disease modeling.

CONCLUSION

Research in hPSCs has proven to be extremely helpful in creating disease models that can corroborate results gleaned from animal models and overcome their associated limitations. Distinct brain cell types can be produced using hPSCs including neurons, astrocytes, OLs, microglia, in addition to more advanced heterogeneous systems such as brain organoids. These systems have contributed to the development of models for neurological diseases such as AD, PD, and many others. Current models that employ hPSCs have certain shortcomings related to the absence of vasculature as well as microglia. However, developing research in the field of tissue engineering that use cocultures, organ-on-chip and assembloids may be able to get around these limitations in the years to come.

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FOOTNOTES

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Conflict-of-interest statement: Lauren E Woodard and Julie Bejoy have two patent applications submitted on "Accelerated protocol for deriving podocytes from hiPSCs" and "nephron progenitor exosomes" listed below; Inventors: Bejoy J and Woodard LE accelerated the protocol for the differentiation of podocytes from human pluripotent stem cells. Patent Application filed August 26, 2022. PCT/US2022/075447; Inventors: Bejoy J and Woodard LE Nephron progenitor exosomes, patent Application filed October 6, 2022. PCT/US2022/077692.

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