Review

Human mini brains and spinal cords in a dish: Modeling strategies, current challenges, and prospective advances

Journal of Tissue Engineering
Volume 13: 1–19

DOI: 10.1177/20417314221113391
journals.sagepub.com/home/tej
SAGE

Simeon Kofman, Neha Mohan, Xiaohuan Sun, Larisa Ibric, Emanuela Piermarini and Liang Qiang

Abstract

Engineered three-dimensional (3D) *in vitro* and *ex vivo* neural tissues, also known as "mini brains and spinal cords in a dish," can be derived from different types of human stem cells via several differentiation protocols. In general, human mini brains are micro-scale physiological systems consisting of mixed populations of neural progenitor cells, glial cells, and neurons that may represent key features of human brain anatomy and function. To date, these specialized 3D tissue structures can be characterized into spheroids, organoids, assembloids, organ-on-a-chip and their various combinations based on generation procedures and cellular components. These 3D CNS models incorporate complex cell-cell interactions and play an essential role in bridging the gap between two-dimensional human neuroglial cultures and animal models. Indeed, they provide an innovative platform for disease modeling and therapeutic cell replacement, especially shedding light on the potential to realize personalized medicine for neurological disorders when combined with the revolutionary human induced pluripotent stem cell technology. In this review, we highlight human 3D CNS models developed from a variety of experimental strategies, emphasize their advances and remaining challenges, evaluate their state-of-the-art applications in recapitulating crucial phenotypic aspects of many CNS diseases, and discuss the role of contemporary technologies in the prospective improvement of their composition, consistency, complexity, and maturation.

Keywords

human pluripotent stem cell, mini brain, mini spinal cord, neurosphere, organoid, assembloid, organ-on-a-chip, biomaterial

Received: 14 April 2022; accepted: 27 June 2022

Introduction

The field of neuroscience has proven to be one of the more mystical fields in the realm of science and with that has attracted some of the most brilliant minds. Research spanning subdisciplines of cellular and molecular neuroscience, systems neuroscience, cognitive and behavioral neuroscience, and computational neuroscience has gifted the world profound knowledge of the anatomy and physiology of the human brain. Furthermore, researchers immersing themselves in translational and clinical studies have been continuously pursuing innovative model systems to achieve valiant leaps into addressing some of the most complex central nervous system (CNS) diseases through study and trial for effective therapies, yet many of these disorders remain currently untreatable. While the field is rooted in the knowledge and insight that researchers bring with them, progress can only go forward as far as its tools and technologies will take it. There has been a continuous demand to minimize the gap between animal and human models in both basic and translational neuroscience for many years.[1]

Department of Neurobiology and Anatomy, Drexel University College of Medicine, Philadelphia, PA, USA

Corresponding author:
Liang Qiang, Department of Neurobiology and Anatomy, Drexel University, College of Medicine, 2900 Queen Lane, Philadelphia, PA 19129, USA.
Email: lq24@drexel.edu

While animal models provide an *in vivo* system that is more complete for studying neural development, disease progression, and treatment efficacy, there are several key differences between animal models and humans that begin to manifest themselves on the most important scale: the patient. Therapies for a range of CNS disorders which were validated in animal models have often failed in human trials.[2] Therefore, more relevant human models, especially those generated from patient tissue, will significantly empower researchers to address human-specific disease mechanisms in a cost-effective and scalable manner. In addition, these human models will help shape novel cell transplantation strategies aimed at replenishing lost or damaged cells in CNS injuries and disorders.[3]

Limited access to functional human brain tissues impedes a comprehensive understanding of brain development, disease mechanisms, and advancement of therapeutics.[4,5] Thus, for years, neurobiologists have turned to a variety of *in vitro* and *ex vivo* approaches to study the complexity of the human brain and its molecular processes.[4,5] However, the application of model organisms in the study of the human brain has not been fully informative due to remarkable differences between human brain development and that of other species. To date, most attempts at applying human cells or tissues to model CNS disorders have been based on two-dimensional (2D) cell cultures that do not include many aspects of the complex physiological environment seen in the CNS. These cultures do not always accurately represent how cells grow in a human brain and how they may be affected by CNS disease and injury. Thus, the desire to develop a system that can closely recapitulate the crosstalk between various cell types in the human brain has led to the employment of three-dimensional (3D) cultures based on human material. 3D tissue cultures have been in development since the early 20th century, as researchers for decades have realized their potential in disease modeling and drug development.[6] 3D human CNS tissue cultures, also known as human mini brains and spinal cords, are a subset of 3D models that usually retain a higher degree of structural complexity and maintain physiological homeostasis for prolonged durations. Some of them also contain various cell types that influence intricate cell-cell interactions, an element essential for the regulation of cell differentiation and metabolization. Ultimately, decades of experimental effort along with a continuously growing understanding of human CNS development have enabled progressive advancements in the generation of human mini brains resembling human brain structures. The field has greatly benefited from the use of these 3D brain technologies in studying cell-cell interactions and the assembly of neuronal circuits. These new types of *in vitro* human brain models serve as alternatives to existing animal models and close the gap in recreating human specific physiology and pathology of CNS development and disorders.

One of the biggest challenges for obtaining robust human 3D CNS models has been the relative scarcity of pertinent foundational cellular components used in their production. Human pluripotent stem cells (hPSCs), especially human induced pluripotent stem cells (hiPSCs), have proved to be a milestone in the biomedical community. Through various reprograming and gene modification methods, researchers can use hiPSCs with specific genetic profiles as the building blocks to grow any somatic tissue or organ *in vitro*.[7] From a neuroscience perspective, a great deal of work to generate human mini brains and spinal cords has been done on the front end in developing tuned differentiation protocols that mimic developmental cues to pattern different cell types and regions of the CNS. However, to date, there is still a great deal to be discovered and improved in engineering *in vitro* human mini brain models. In this review, we characterize and summarize the three main types of 3D CNS models: *neurospheres, CNS organoids*, and *CNS assembloids*, based on their production strategies and structural complexity (Figure 1). We also describe the emergence and role of revolutionary *organ-on-a-chip* systems in modeling specific elements of the CNS. We explain how these diverse but related structures differ from each other, what specific purpose each of them aims to achieve, and where each can be improved. In addition, we discuss some of the supporting technology that may be used in concert with these 3D CNS models to help introduce critical physiological cues and support appropriate culturing environments. Ultimately, this review hopes to create some consistency in the field and inspire researchers to continue to bring creativity to the design and development of *in vitro* human mini brain and spinal cord modeling platforms.

Neurospheres: Simplified human CNS spheroids

Spheroids typically serve as an umbrella term for cultured 3D sphere-like tissue structures. The multifunctionality of spheroid cultures with relatively homogenous cellular components allows them to be used as rudimentary 3D cellular models. A variety of cells, including hepatocytes, stem cells, tumor cells, and neural cells have been applied to generate spheroids with restricted cell types.[8] 3D human neural spheroids, commonly known as neurospheres, were initially developed as 3D cellular structures that were comprised of neural precursors or post-mitotic neurons derived from 2D cultures.[9,10] The early 1990s saw the first culture of neurospheres derived from the periventricular area encompassing the subventricular zone in adult mice.[9] In turn, adult neural stem cells emerged from the niche study of adult neurogenesis and the *in vitro* study of multipotent precursors from the adult brain.[10] Since then, human neurospheres have been differentiated

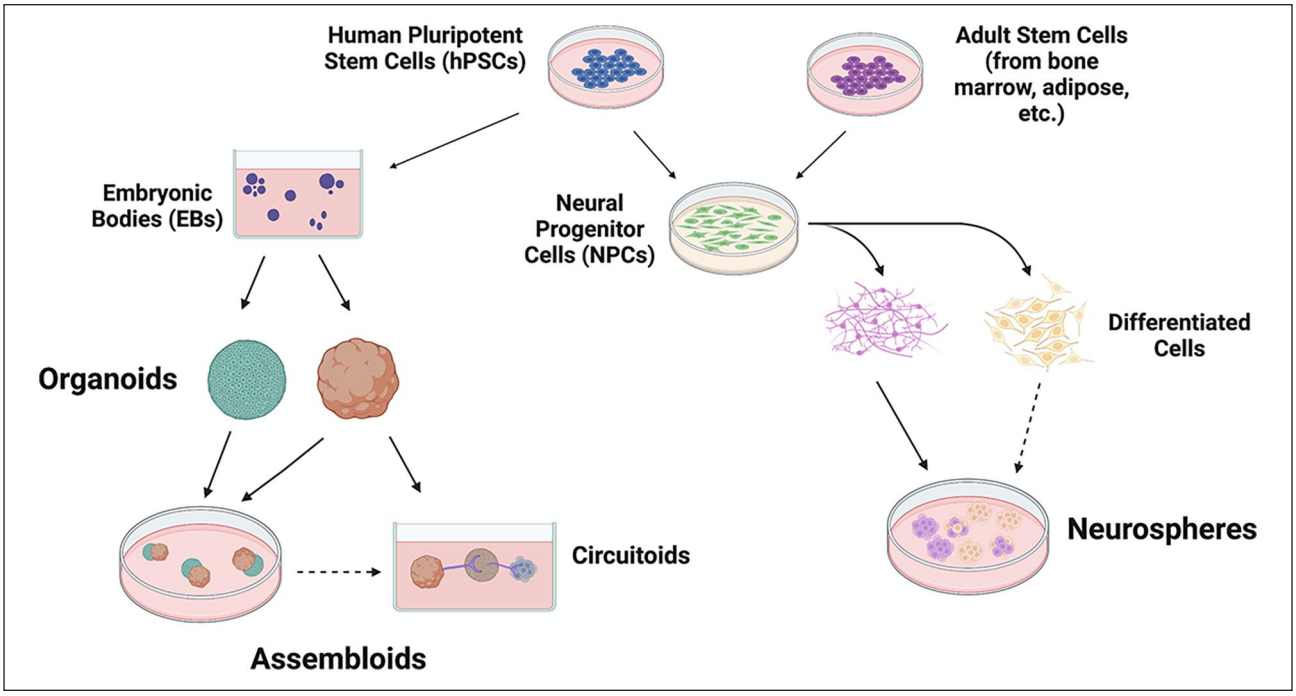

Figure 1. Human stem cells can be used to generate mini brain and spinal cord models. Human pluripotent stem cells (hPSCs), as well as a variety of adult stem cells derived from multiple sources in the body can be differentiated into neural progenitor cells (NPCs). NPCs can then be differentiated into several types of neural lineage cells and aggregated to form neurospheres. HiPSCs can also be aggregated into embryoid bodies, which can be patterned into region-specific CNS organoids, which can then be assembled to create CNS assembloids. Circuitoids are a subset of assembloids which recapitulate neural circuit systems such as the motor pathway and visual system.

and developed from many types of human neural progenitors and other types of stem cells including ciliary epithelium cells, dental pulp stem cells (DPSCs), human umbilical cord blood (hUCB), and bone marrow derived mesenchymal stem cells (Figure 2). In general, these 3D multicellular spheroids, although mechanically simplified, manifest better structural representation and functional design than do 2D cultures, including dynamic cell-cell and cell-matrix interactions that represent some features of the CNS microenvironment.[11,12] Due to their substantial advantages over 2D neuronal cultures, 3D neurospheres pioneered the way for the use of *in vitro* human mini brains and spinal cords for CNS disease modeling.[13–15]

Unlike in long-established 2D cultures, the absence of an attachment surface or scaffold allows mono-dispersed cells to undergo self-assembly and aggregate to form cell specific spheroids.[12] This self-assembly process somewhat mimics developmental cellular processes like embryogenesis, morphogenesis, and organogenesis.[12,16] Spheroid cultures usually form a gradient of oxygen metabolites, soluble signals, and other essential nutrients necessary for an optimal in vivo like microenvironment.[8] Neurospheres can be generated from neural progenitor cells isolated from either adult or fetal CNS.[17,18] They are generally created by culturing originating cells in low adhesion plates.[8] Low adhesion plates have an ultra-low attachment surface coating and a well-defined geometry (U-shaped bottom or V-shaped bottom) in each well to minimize cell adhesion and promote self-aggregation.[19–21]

One of the many advantages of using neurospheres as an *in vitro* human CNS model is the ease with which internal and external cues can be manipulated, proving a better means for modeling different elements of the CNS. Notably, neurospheres may be co-cultured with 2D cells or some acellular matrices, which essentially provide contact-mediated directed differentiation cues. Such co-cultures are able to not only instruct neurospheres to reach specific phenotypes, but promote their reparative properties for therapeutic purposes.[22] This dynamic interaction was exemplified in a model in which neurospheres were developed from adipose stem cell derived neural progenitor cells (ASC-NPCs) followed by co-culture with acellular dermal matrix. Such co-culture was demonstrated as a better therapeutic strategy to repair peripheral nerve injuries when compared to using neurospheres alone.[23] Furthermore, neurospheres have been tested in several rodent transplantation studies targeting tissue regeneration in disease including Alzheimer's disease, macular degeneration, and spinal cord injury (Table 1). Interestingly, transplanted neurospheres were better at restoring native

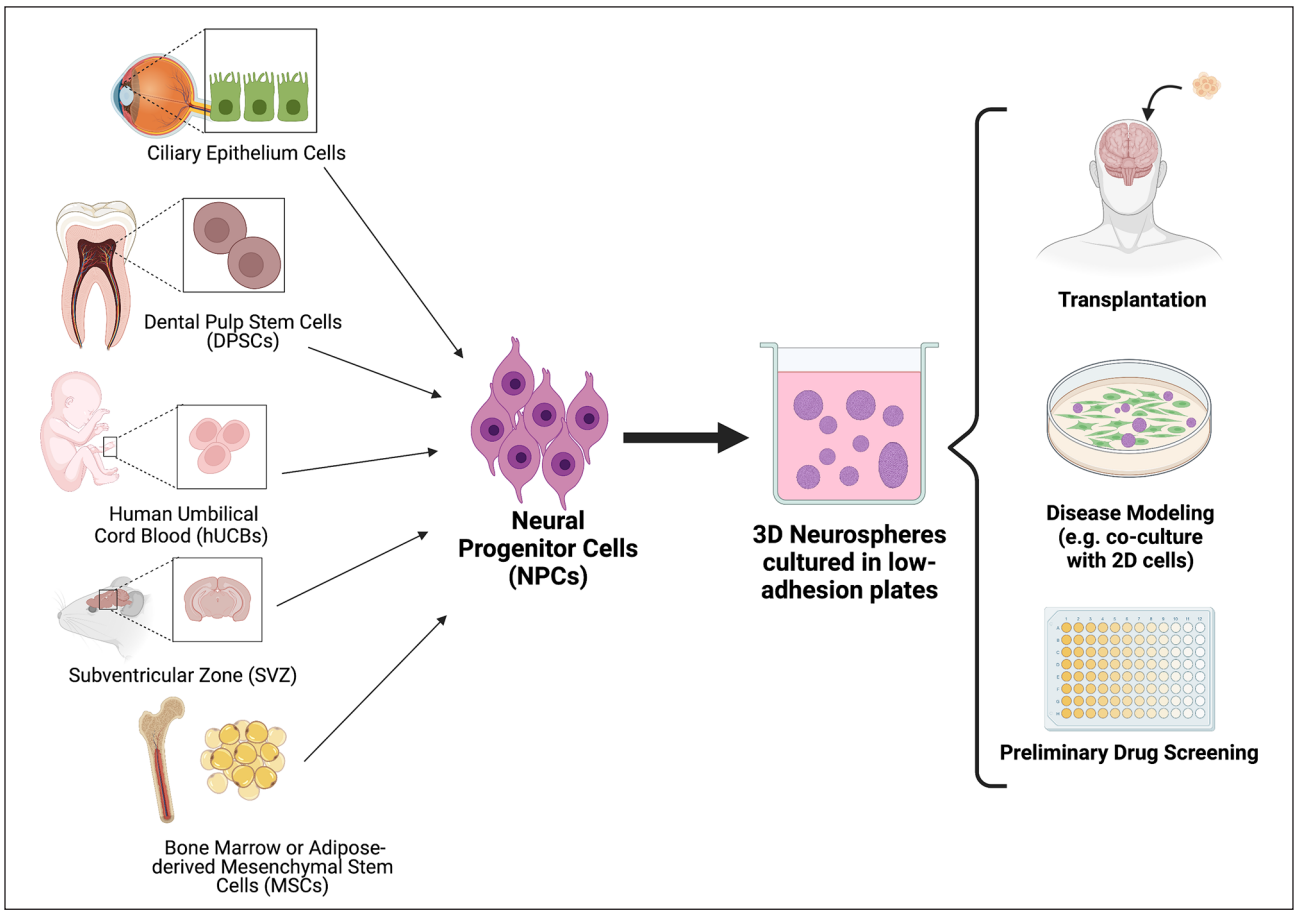

Figure 2. Neurospheres pave the way for studying the CNS in 3D. Neurospheres are generated by culturing neural progenitor cells (NPC) derived from various stem cell sources in low adhesion plates allowing the cells to self-aggregate. This 3D model is currently used for disease modeling via co-culturing with other 2D cells, drug and toxicity screening, and may one day be used for transplantation into humans.

cell populations compared to the transplantation of undifferentiated cells and helped improve certain behavioral phenotypes in cognitively impaired animals.[24,25] Indeed, neurosphere cultures allow us to examine fetal or adult CNS cells outside of their normal environment while maintaining its integrity as a model system for developmental processes and therapeutic intervention. To date, several neurosphere phenotypes have been developed by different laboratories (Table 1). Despite its usefulness, there are significant drawbacks associated with neurosphere technology that limit it from being a frontrunner in modeling complex CNS environments. These limitations include variations in development, maintenance, and size, lack of cellular complexity in specific cell types, uncontrolled proliferation and differentiation of cells, disordered cellular aggregation, and imprecise and unstandardized high-throughput compatible assays for drug screenings.[8] While simple and technically friendly, neurospheres have paved the way for the development of more complex 3D mini brain models, and continue to find utility in the study of the CNS and associated diseases.

CNS organoids: Self-organizing multicellular hierarchies mimicking distinct CNS regions

Human CNS organoids, including brain and spinal organoids, have drawn substantial attention in the field of neuroscience by becoming more relevant CNS models in relation to anatomical and physiological features of human brains and spinal cords. Unlike neurospheres, CNS organoids are mostly developed from hPSCs and usually represent multicellular structures that are hierarchically organized with sophisticated interstitial compartments. Various cellular phenotypes with orderly arrangement can be identified in these CNS organoids, including neural precursors, different types of neurons, astrocytes, and oligodendrocytes. To date, brain and spinal organoids have become popular modalities for studying region-specific structures, 3D-microenvironments, and entire organs for both the brain and spinal cord, and own an insuperable advantage over animal models by way of their human origin.[31,40,41,77] The first contemporary human cerebral organoid model was established from human embryonic

Table 1. Representative human 3D CNS structures including neurospheres, CNS organoids, and CNS assembloids in disease modeling and therapeutic intervention.

	Representative CNS region	Major cell type(s)	Disease/process modeled	Cell transplantation	Reference(s)
Neurospheres	None	Neural stem or progenitor cells isolated from fetal brain tissue	No	Yes, for an Alzheimer's disease mouse model	Poltavtseva et al.[25]
	None	Dental pulp stem cells (DPSCs) from the dental pulp of wisdom teeth	No	Yes, for regenerative medicine and tissue engineering	Kawase-Koga et al.[26]
	None	Fetal ciliary epithelium cells; human umbilical cord blood derived lineage negative stem cells	No	Yes, for age-related macular degeneration, diabetic retinopathy, glaucoma in mice	Bammidi et al.[24], Coles et al.[27], Bammidi et al.[28]
	None	CNS stem cells	No	Yes, for spinal cord injury in mice	Hooshmand et al.[29], Cummings et al.[30]
Organoids	Cerebrum	Various layer identities of the cortical plate	Neuropsychiatric disorders; neurodevelopment-related diseases; neurotropic infectious diseases	Yes, for stroke and traumatic brain injury	Giandomenico et al.[31], Wang et al.[32], Pain et al.[33], Gulimiheranmu et al.[34], Renner et al.[35], Camp et al.[36], Chan et al.[37], Yang and Shcheglovitov[38], Wang et al.[39]
	Spinal Cord	Spinal motor neurons; interneurons	Spinal Muscular Atrophy	No	Vieira de Sá et al.[40], Khong et al.[41], Hor et al.[42], Hor and S-y[43], Faustino Martins et al.[44]
	Dorsal forebrain; Ventral forebrain	Glutamatergic neurons; GABAergic neurons	Prader-Willi syndrome; Autism spectrum disorders; Rett Syndrome	No	Pașca[45], Bagley et al.[46], De Santis et al.[47], Gomes et al.[48], Bagley et al.[49]
	Brainstem	Midbrain/hindbrain progenitors; noradrenergic and cholinergic neurons; dopaminergic neurons; neural crest lineage cells	-	No	Eura et al.[50]
	Midbrain	Dopaminergic neurons; astrocytes	Parkinson's disease	No	Zagare et al.[51], Jarazo et al.[52], Kim et al.[53], Galet et al.[54]
	Hindbrain	Serotonergic neurons	-	No	Valiulahi et al.[55]
	Hypothalamus	Arcuate nucleus cells	Prader-Willi syndrome	No	Qian et al.[56], Huang et al.[57], Ozaki et al.[58]
	Thalamus	Thalamic neurons; neural progenitor cells; astrocytes	Schizophrenia; depression; autism spectrum disorder; epilepsy	No	Xiang et al.[59], Xiang et al.[60]
	Cerebellum	Cerebellar neurons	Medulloblastoma	No	Nayler et al.[61], Silva et al.[62], Ballabio et al.[63]
	Choroid plexus	Myoepithelial cells	Cerebrospinal fluid secretion	No	Pellegrini et al.[64]
	Hippocampus	Granule neurons; pyramidal neurons	Alzheimer's disease	No	Sakaguchi et al.[65]
	Striatum	Pallial and subpallial progenitors; cortical and striatal neurons; macroglia; mural cells	Autism spectrum disorders	No	Miura et al.[66], Wang et al.[67]

(Continued)

Table 1. (Continued)

	Representative CNS region	Major cell type(s)	Disease/process modeled	Cell transplantation	Reference(s)
Assembloids	Cerebrum (no region identity)	Human glioblastoma organoid-derived cell lines; primary human patient-derived glioblastoma cell lines	Mesenchymal subtype human glioblastoma	Yes, transplanted into the hippocampus of immunodeficient NOD/SCID/ IL2RGKO mice	Ogawa et al.[68]
	Cerebrum (no region identity)	Patient-derived glioma stem cells	Glioblastoma	No	Linkous et al.[69]
	Cerebrum (no region identity)	Human mesodermal progenitor cells	Parkinson's disease; Alzheimer's disease	Yes, tested vascular network functionality by transplanting into the chorion allantois membrane of a chicken embryo	Wörsdörfer et al.[70]
	Cerebral cortex	Pericyte-like cells; cortical neurons; astrocytes	SARS-CoV-2	No	Wang et al.[71]
	Dorsal forebrain; ventral forebrain	GABAergic interneurons; MGE- and LGE/CGE-derived cortical interneurons	Schizophrenia	No	Bagley et al.[46]
	Dorsal forebrain; ventral forebrain	Dorsal and ventral organoid-derived oligodendroglia	Neurodevelopmental disorders associated with myelin defects; CNS injury	No	Kim et al.[72]
	Dorsal forebrain; ventral forebrain	Human GABAergic interneurons	Schizophrenia; autism spectrum disorders; depression; seizure	No	Yuan et al.[73]
	Ventral forebrain; subpallium	Cortical glutamatergic neurons; cortical GABAergic neurons	Timothy syndrome	No	Birey et al.[74]
	Medial ganglionic eminence; cerebral cortex	Human cortical interneurons; radial glial cells; Cajal-Retzius cells; astrocytes	Autism spectrum disorders; Rett syndrome	No	Xiang et al.[75]
	Cerebral cortex; thalamus	Thalamic neurons; neural progenitor cells; astrocytes	Schizophrenia; depression; autism spectrum disorder; epilepsy	No	Xiang et al.[59]
	Striatum; cerebral cortex	Medium spiny neurons; cortical neurons	Phelan-McDermid syndrome; Huntington's disease; Tourette syndrome	No	Miura et al.[66]
	Cerebral cortex; hindbrain/spinal cord; skeletal muscle	Corticofugal neurons; spinal-derived motor neurons; skeletal myoblasts	Multiple sclerosis; spinal cord injury	Yes, studied muscle contraction by co-culturing hSpS into mouse forelimb and hindlimb buds	Andersen et al.[76]

stem cells (hESCs) in 2013 and used for analyzing human brain development, specifically aimed at exploring microcephaly, a condition that cannot be precisely recapitulated in mouse models.[78] Inevitably, this model provided researchers a foundational platform for studying neurological diseases with human specific genetic roots, as well as allowed for the

Table 2. Summary of strengths and weaknesses of various 3D CNS modeling strategies including neurospheres, CNS organoids, CNS assembloids, and Organ-on-a-chip.

	Strengths	Weaknesses
Neurospheres	• Simple procedure • Greater variety and accessibility of originating cell sources • Improved physiological relevance over 2D cultures	• Limited and uncontrolled differentiation capacity • Lack of cellular and structural complexity
Organoids	• Complex cellular composition • Region-specific identities • Hierarchical organization • High adaptability	• Very limited populations of certain CNS cell components • Poor long-term survivability • High variability • Low reproducibility • Undefined maturity
Assembloids	• Improved cellular complexity over organoids • Emergence of region-region circuitry and integrated tissue/organ systems • Expanded customizability	• Complicated and unstandardized procedures • Poor long-term survivability • High variability • Low reproducibility • Undefined maturity
Organ-on-a-chip	• Low-cost and accessible materials • Advanced incorporation of relevant biophysical signaling • Effortless culture maintenance and improved standardization • Wider range of applications	• Laborious and complicated culture preparation • Restricted capacity for multidimensional analyses • Low transplantation potential

closer study of the unique development of the human CNS. Brain organoids are also a promising tool in healthcare's shift toward personalized medicine. Through the reprogramming of patient peripheral blood mononuclear cells or skin fibroblasts into hiPSCs, clinicians can create patient-specific organoid models, in turn allowing them to better tailor treatment regimens to patient needs.[79]

The generation of CNS organoids involves either direct or indirect neural induction, which starts with the aggregation of hPSCs into embryoid bodies (EBs). These EBs are then grown in suspension culture and patterned and matured in the presence of neuronal induction factors and small molecules that drive spatial organization and specific cell fate differentiation (Figure 3).[41,50,64,80–83] As these organoids differentiate and mature, region-specific cell types and tissues begin to emerge including forebrain, midbrain, hippocampus, hypothalamus, spinal cord, etc. (Table 1).[56,84] For instance, dorsal forebrain organoids contain significant amounts of excitatory glutamatergic neurons while ventral forebrain organoids contain a high proportion of inhibitory GABAergic neurons.[84] The potential interaction and connectivity between cell types within brain organoids makes them more advanced models than neurospheres, especially when studying the communication between different cell types in a specific brain region. Thus, brain organoids with specific region identities allow for significant external control with limited variation and reinforce the integrated development of a variety of CNS cell types in a way that cannot be achieved with more basic spheroid models, such as neurospheres.

Spinal cord organoids, a subset of CNS organoids, are usually induced by morphogens and growth factors that caudalize the CNS development. Retinoic acid is introduced to drive differentiation toward spinal motor neurons, while sonic hedgehog (SHH) is used to mimic ventral signals and drive spatial organization of the spinal cord. Growth differentiation factor 11 (GDF-11) is used to increase rostro-caudal patterning of the spinal cord as well as drive differentiation towards motor neurons and interneurons, which may provide either excitatory or inhibitory signals to sensory neurons and other glia.[40,41,77] Indeed, the extreme complexity of the human spinal cord structure may intimidate the development of human spinal organoids, as the spinal cord not only demonstrates an intricate separation of gray and white matter, but also contains a wide variety of interneurons and motor neurons heterogeneously distributed along its length. Despite some advances in their development, the generation of 3D spinal cord tissue from hPSCs may ultimately require assembling region-specific brain organoids, as well as peripheral neural ganglia in more complex constructs.

Human CNS organoids have been applied to: (1) disease modeling and drug screening for familial and sporadic neurodevelopmental and neurodegenerative disorders, as well as CNS injuries, (2) elucidating human CNS development for neural differentiation and migration, (3) evaluating neurotoxicity induced by a wide variety of chemicals and pathogens, and (4) testing tissue replacement therapies.[78,85–87] Engineered brain organoid**s** have been validated with applications in modeling Alzheimer's disease, frontotemporal dementia, microcephaly, autism spectrum disorders, traumatic brain injury, and epilepsy, as well as SARS-CoV-2

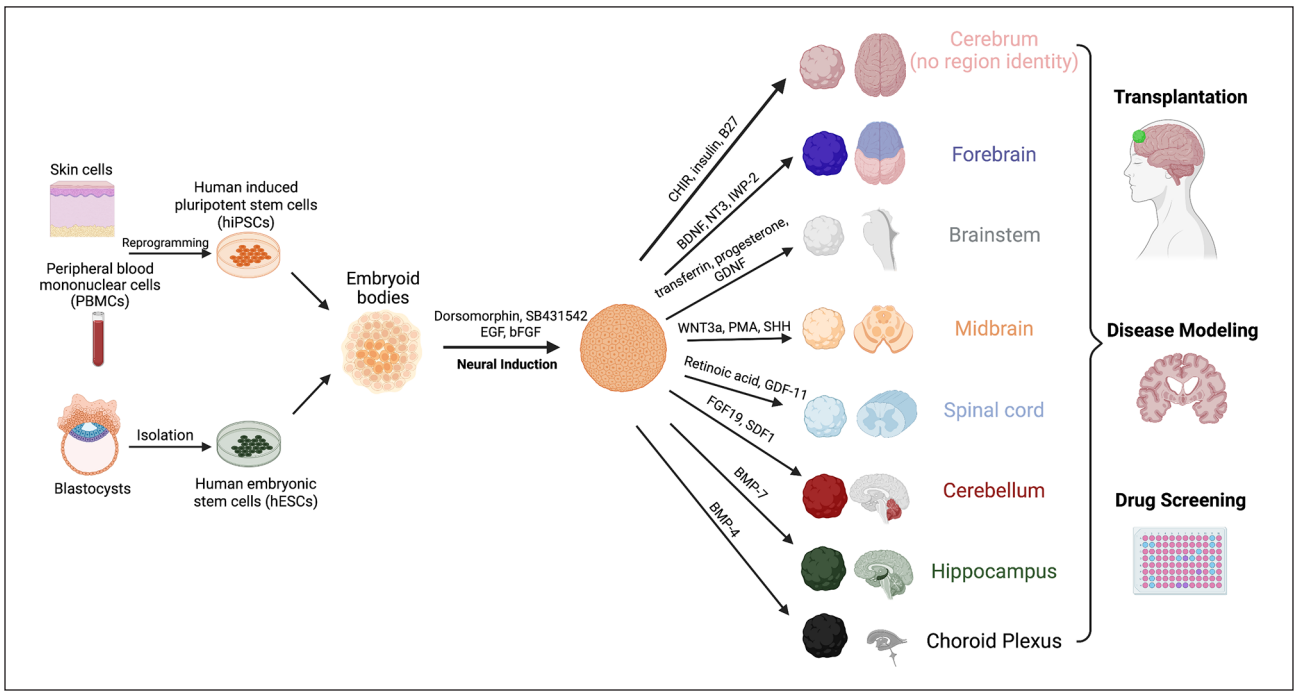

Figure 3. CNS Organoids can be patterned to recapitulate various regions of the brain and spinal cord. hiPSCs and hESCs of different origins can be aggregated to form embryoid bodies, which through the addition of various factors and inhibitors, can be pattern into neuroectodermal-committed organoids. Using unique patterning factors, organoids can be further differentiated toward different regions of the brain or spinal cord. These organoids are useful for modeling neurological and neurodegenerative diseases and can be applied in drug screening studies. They also show potential for use in human transplantation after brain or spinal cord injury.

induced brain damage.[88–94] Brain tumor organoids were used as models to test novel immunotherapies and to screen small compounds in treating some infamous brain cancers, such as glioblastoma.[95–97] Note that several premature spinal organoid models were also developed to model motor neuron diseases such as spinal muscular atrophy and amyotrophic lateral sclerosis.[98] Interestingly, human spinal cord organoids containing dorsal spinal cord interneurons and sensory neurons have been placed on multiple-electrode array chips to monitor electrophysiological activity in response to nociceptive modulators as a means to further understand nociceptive circuitry in the context of pain therapy.[99] Moreover, several transplantation studies of cerebral organoids into rodent models have also been tested as preclinical applications, for example as a means for restoring learning and memory function in traumatic brain injury and ischemic stroke (Table 1).[3,32] The results of these studies showed improvements in neurogenesis stemming from both the native rodent and the transplanted organoids, as well as enhanced axonal regeneration, neuron survival, and synaptic reconstruction.[32] CNS organoid technology provides a great potential to improving *in vitro* CNS disease modeling and offers a new platform for translating bench-side research to therapeutic screenings. Such studies were conducted in both academia and industry settings. Human iPSC derived CNS organoids, combined with machine learning and multi-dimensional phenotypic analyses, were adopted in drug screening studies for schizophrenia, autistic spectrum disorders and epilepsy. A promising molecular profile including novel pathways that are relevant to the disease mechanisms was generated to instruct possible therapeutic interventions.[100] A study to screen therapeutic targets for Zika virus-induced microcephaly was carried out by the collaborative effort from Harvard University and Novartis. Using human cerebral organoids as models, a CRISPR-based strategy was applied to deplete various putative receptors for uncovering entry elements that are essential to Zika viral infection.[101] There is particular value in using human organoids, instead of rodent animals, for these studies since a subpopulation of intermediate progenitor cells that are highly relevant to Zika infection in developing human brains is missing in rodent brains.[102]

Despite advances in CNS organoid modeling strategies, there are still several limitations and technical challenges that hinder CNS organoids from accurately modeling the entire complexity of the CNS. A lack of essential cell types, including microglia and sufficient myelinating oligodendrocytes, limit the study of many CNS diseases such as autoimmune encephalitis, which is implicated by neuroinflammation-trigged neuronal death in the brain.[103] Additionally, a lack of vasculature components not only diminishes the structural complexity of CNS organoids, but restricts their culture duration, expansion, and maturation, partially due to the insidious development of cellular necrotic cores within the organoids. Compensatory strategies that use bioreactor devices and

orbital shakers have shown some relieving benefits but have not done enough to completely overcome these hurdles. To date, even under the most optimized culture conditions, the size and scale of CNS organoids remains only a fraction of that seen in the human brain. One of the biggest concerns in applying human CNS organoids to model neurodegenerative diseases is their limited maturity. While strategies such as prolonged cultivation (>8 months) to accelerate the maturation and aging process in human cortical organoids have been validated through the analysis of key developmental milestones, including modifications in histone deacetylase complex and NMDA receptor signaling, such approaches are not without other significant challenges and complications, and are particularly sensitive to the aforementioned issues of necrosis.[104] Vascularizing organoids is an intriguing method to both accelerate the maturation of neuroglial components and improve long-term cell survival. One study has shown that the overexpression of ETS variant 2 (ETV2) in hESCs resulted in their differentiation to the endothelial phenotype and formed vascular-like structures in cultures. Coupling ETV2-hESCs with untransduced hESCs in a 20:80 ratio led to the formation of vascularized organoids, as cells organized into a physiologically relevant manner and created the beginnings of the blood brain barrier (BBB).[105] While still subject to optimization, these approaches to improving organoid maturation hold extensive value in allowing organoids to be used for modeling aging and neurodegenerative diseases.

Excitingly, recent advances in human CNS organoid technology have led to breakthrough findings in the field. For the first time, brain organoids with choroid plexus-like structures were developed with active human cerebrospinal fluid production.[64] Neuromuscular organoids developed in 2020 have successfully generated self-organizing populations of neurons and skeletal muscle and have demonstrated muscle contraction and neural activity, allowing for the capture of key features of myasthenia gravis and potentially providing the foundation for the study of other neuromuscular diseases.[106] Human brain organoids may potentially be utilized for the study of consciousness, as pioneering investigations have shown that network oscillation recordings done in dorsal forebrain organoids resemble electroencephalography patterns seen in preterm babies.[107,108] Ultimately, CNS organoids have demonstrated their potential as a highly flexible system to be used in a wide array of study designs and applications and will continue to prove their tremendous value for disease modeling and cell therapies.

CNS Assembloids: Assembled and integrated organoids with improved cellular complexity and network connections

Although the field has made great strides with the use of neurosphere and CNS organoid technologies, a more complex model which captures intricate cell-cell interactions and circuitry connections is needed for in-depth study of disease. The rise of assembloid technology has made such requests possible. Assembloids can be described as assembled organoids or other 3D cellular structures with distinct regional identities and multiple cell types, which can be derived from different lineage origins. CNS assembloids usually involve the integration of various hPSC-derived cellular components that add a level of complexity to human mini brain models. They merge different CNS-relevant cell types, fuse region-specific CNS organoids, or create models that blend both CNS cells and organoids, most notably through the addition of non-neuroectodermal cells, such as microglia or endothelial cells.[109] In turn, these models provide insight into the overall spatial communication and network connectivity that occurs within the human brain and the rest of the nervous system.[45] In addition, analysis of the anatomical and functional components of assembloids in both normal and disease models can give a comprehensive understanding of scenarios in which CNS development goes awry as well as inspire novel treatment approaches.[76,110]

Assembloid technology has been used to create models of bodily systems that involve close interaction with the CNS. Such strategies were initially applied to generate the human visual system *in vitro*. Human visual system development involves the coordination of a series of spatial and temporal organizational events, including the elongation of axons from retinal ganglion cells (RGCs) to their postsynaptic targets in the brain.[111] In the human body, most RGCs directly project their axons into the thalamus in the brain. Thus, to study this interface within the visual system, hPSC-derived retinal organoids were engineered and organized into an assembloid model with cortical and thalamic organoids derived from the same hPSCs.[112] In a manner similar to behavior of RGC axons observed during visual system development, RGCs within retinal organoids responded to environmental cues by growing longer and extending deeper into neighboring thalamic organoids. More interestingly, astrocytes originating from the thalamic organoids were identified to retrogradely migrate into tdTomato-labeled retinal organoids in a manner that reflected the *in vivo* cell migration during the development of the human visual system.[112] Additionally, GFP positive axons from the thalamic organoids were seen to project into the cortical organoids to complete the retinal-thalamic-cortical assembloid structure.[112] This early proof-of-concept study showed enormous promise in applying human CNS assembloids to systematic studies of cell-cell and region-region interactions during human brain development. In addition to their applications in studying human brain development, human CNS assembloids have also been used to investigate neurological diseases caused by defective neural circuits. A complex assembloid model was developed to probe Phelan-McDermid syndrome, a disorder related to cortico-striatal deficits and characterized by global developmental delay

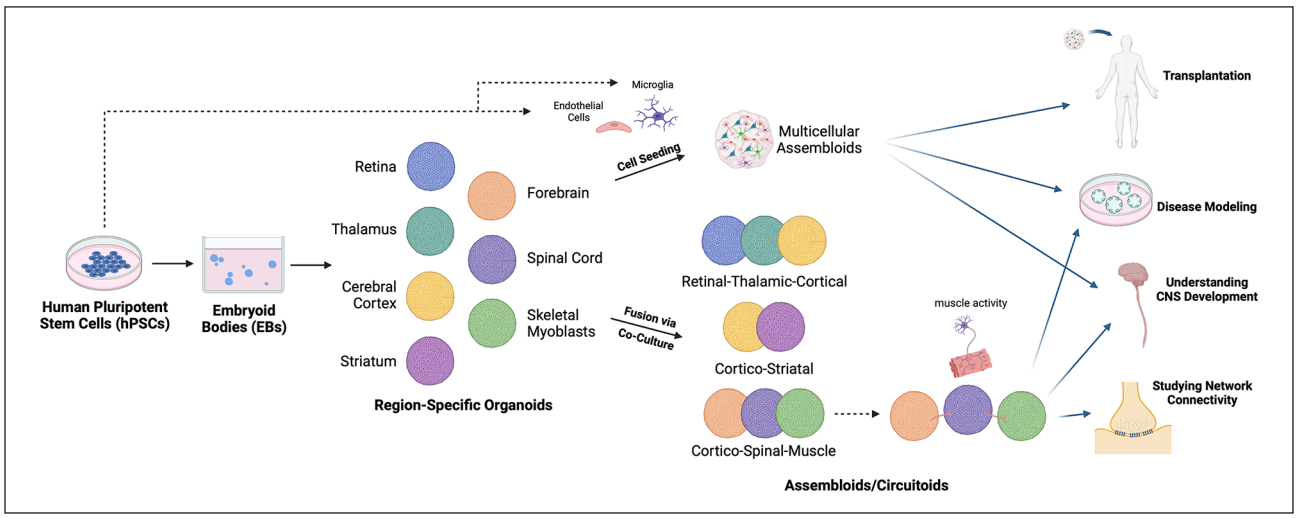

Figure 4. CNS Assembloids allow for the study of region-region circuitry. hiPSCs can be aggregated into embryoid bodies, which can then be differentiated into CNS organoids representative of different brain and spinal cord regions. Organoids can then be co-cultured with other hiPSC-derived cell types to form multicellular assembloids. Organoids with various region identities can also be fused through co-culture to produce assembloids modeling region-region connections. Assembloids can be utilized to study region-region circuitry when modeled as circuitoids. These structures can be used for transplantation studies, disease modeling, understanding CNS development, and studying network connectivity.

and severe intellectual impairment.[66,113] To accomplish this, lateral ganglionic eminence (LGE) and cortical organoids were generated from hPSCs. Subsequently, LGE organoids were derived into striatal organoids by introduction of activin A, IWP-2, and SR11237 molecules. These striatal organoids were then combined with cortical organoids to form cortico-striatal assembloids. Neurons arising in the cortical organoids formed synapses with those in striatal organoids, a finding consistent with the behavior of cortico-striatal circuits studied *in vivo*.[66,114] However, cortico-striatal assembloids derived from patients with Phelan-McDermid syndrome showed deficits in neural circuit formation, as well as improper calcium signaling.[66,114] To date, several assembloid models have been developed for investigating CNS diseases (Table 1). These models give researchers the unique ability to explore interneuron migration and axon projection, two main phenotypic defects seen in both neurodevelopmental and neuropsychiatric disorders.[59,66,76,110,111,114]

Recent efforts have been made to study long-range axonal connectivity by "upgrading" assembloids into more advanced structures, often named circuitoids, which are generated through the integration of modular assembloids. One breakthrough in circuitoid technology was the generation of cortico-spinal-muscle circuitry for analysis of the motor pathway.[76] Hindbrain/cervical spinal cord organoids (hSpOs) were initially fused with human forebrain cortical organoids (hFCOs) to create cortico-spinal assembloids. Voltage clamp recordings of hFCO-hSpO assembloid slices showed the establishment of synaptic connections between the two regions. Using an extracellular matrix-coated silicone well, hFCO-hSpO assembloids were coupled with human skeletal myoblasts (hSkMs) to create hFCO-hSpO-hSkM circuitoids. These circuitoids in turn established interconnectivity resulting in the modulation of muscle activity (Figure 4).[76] Previously, such studies could only be performed on motor circuit components that were generated separately in a 3D fashion or through co-culturing with 2D cells.[41,44,115–118] This approach depicted a significant advance beyond prior models by allowing 3D assembly of the motor circuit. Looking forward, this system and others like it might be used to develop an *in vitro* model of spinal cord injury and to evaluate approaches to promote regeneration. Furthermore, assembloids have been used in transplantation based studies to address glioblastoma, motor deficits, Alzheimer's disease, and Parkinson's disease (Table 1).[68,70,119] The results of these studies highlighted that assembloids could integrate with host physiology to modulate muscle contractions, accept native vasculature, and recapitulate tumor-like structures.[68,70,119]

Although currently developed assembloids come close to imitating the human CNS, most models still do not accurately represent its complexity. Presently, most engineered assembloids combine only a few region-specific organoids with very restricted cell types.[84,120] Ideally, a complete assembloid would be a single coherent and interconnected structure that requires the differentiation and integration of organoids with more complete region identities. The incorporation of all relevant cell types, including myelin-forming oligodendrocytes, microglia, ependymal cells, etc., could allow researchers to better understand the cell-cell interactions that are implicated in CNS development and disease.[5,121,122] In models where multiple cell lineages are involved, methods to promote visualization of

cell movement and functionality are essential. Thus, the development of cell type-specific reporters for the live-imaging of these cells has become increasingly important.[5,122] These limitations leave room for improvement in expanding the assembloid model to capture the entire scope of the human brain. Fortunately, as seen by recent advances in the field, scientists are making every effort to optimize assembloid technology. In order to mimic the precise organization of the CNS, some have succeeded in incorporating distinct signaling centers within their assembloid models.[123–125] These signaling centers polarize the assembloids by engineering the cells to secrete (SHH) protein for establishing an SHH gradient.[123] Such methods introduce a sense of spatial organization to the overall system by taking the assembloid components that are initially combined at random and coordinating them by providing a positional identity. In order to keep each of these components alive and growing, some groups have also begun improving the scalability of the assembloid model. Although several researchers have successfully maintained assembloid cultures for several months after assembly, limitations in long-term cell viability make it difficult to conduct studies at more mature stages.[5] The automatization of cell culture maintenance is starting to overcome this problem by allowing for the exploration of CNS diseases that occur later in life or those that involve long-term degeneration.[126] Ultimately, assembloid technology has provided an *in vitro* or *ex vivo* alternative for the use of animal models in human CNS disease modeling and has already showed significant improvement from initial 3D cell culture methods.

Organ-on-a-chip: Utilizing microfluidics for CNS modeling

Key engineering-based modalities, namely the use of microfluidic chips and organ-on-a-chip (OoC) systems, have the potential to bring 3D mini brain cellular structures to life. In the context of building the "next generation human mini brain and spinal cord platform," microfluidic chips are typically polydimethylsiloxane-based (PDMS) systems designed for the purpose of tuning critical physiological parameters including fluid flow, cell-cell mechanical interaction, and cell to cell biochemical signaling.[127] PDMS chips are low in material cost and highly accessible, enabling a wide range of experiments to be performed and the creation of more physiologically-relevant models.[128]

One of the main drawbacks with static culturing methods is their inability to provide sufficient air and nutrient exchange for the robust metabolism of growing 3D cellular structures. While orbital shakers and spinning bioreactors are common solutions for introducing fluid flow in cultured 3D CNS structures, they do not provide a controlled method for regulating the equilibrium between fresh and spent medium, a factor that may influence the real time health of the mini brain. Microfluidic chambers, specifically the use of micropumps, provide the ability to control flow, allowing fresh medium to be pulsed into the 3D microenvironment in a manner much more resemblant to the human vascular system. While this micropump technology has demonstrated its ability to influence and couple with cardiac microtissue, OoC systems on the other hand have been utilized to introduce vasculature into CNS models.[129] One study utilizing OoC systems looked at the effects of brain microvascular endothelial cells (BMEC) on neurogenesis, specifically by leveraging small medium volumes and dual-channel constructs to create vascularized CNS constructs.[130] Another study used chips housing porous polycarbonate membranes to separate two channels filled with endothelial cells and astrocytes, and effectively recreated the permeability and sheer stress properties seen at the BBB.[131] Integrating micropump technology with vascular network forming endothelial cells may one day enable nutrients to be pulsed through vascularized 3D mini brains and spinal cords, an approach that may finally address the issue of progressive necrosis in long-term cultured 3D CNS structures (Figure 5). Other studies have taken different OoC approaches to improve the nutrient accessibility and reproducibility of *in vitro* 3D culture. One proof of concept study used tubular OoC devices to generate organoids on hollow mesh scaffolds and found improved nutrient diffusion, decreased sample heterogeneity and increased neural progenitor populations.[132] Another OoC study used an open-chamber design to optimize the placement and seeding of a cerebral organoid in the center of a chamber, enabling easy access for downstream analyses and more controllability through single organoid studies.[133] The high customizability of OoC systems offers solutions to improve organoid health and long-term survivability, two of the major limitations implicated in 3D CNS culture.

Microfluidic chips also provide unique opportunities for understanding how mechanical forces influence human brain development and activity when combined with mini brain technologies. By controlling the geometry and confines of the culture environment, one can design experiments analyzing the mechanical forces influencing cortical folding during neurodevelopment, which have been implicated in several neurological disorders.[134] One study used an OoC system coupled with a hydrogel filling to culture human brain organoids and study compressive forces on nuclei and how they influence cortical wrinkling.[135] Furthermore, the adaptability of microfluidic technology allows for microsensors to be embedded into OoC systems, providing revolutionary access to the culture environment. Several studies have demonstrated the integration of micro-electrode arrays into OoC systems as a means for measuring the real-time electrical activity of neuronal samples.[136,137] Systems integrating sensors measuring mechanical forces, nucleotide variations, and small

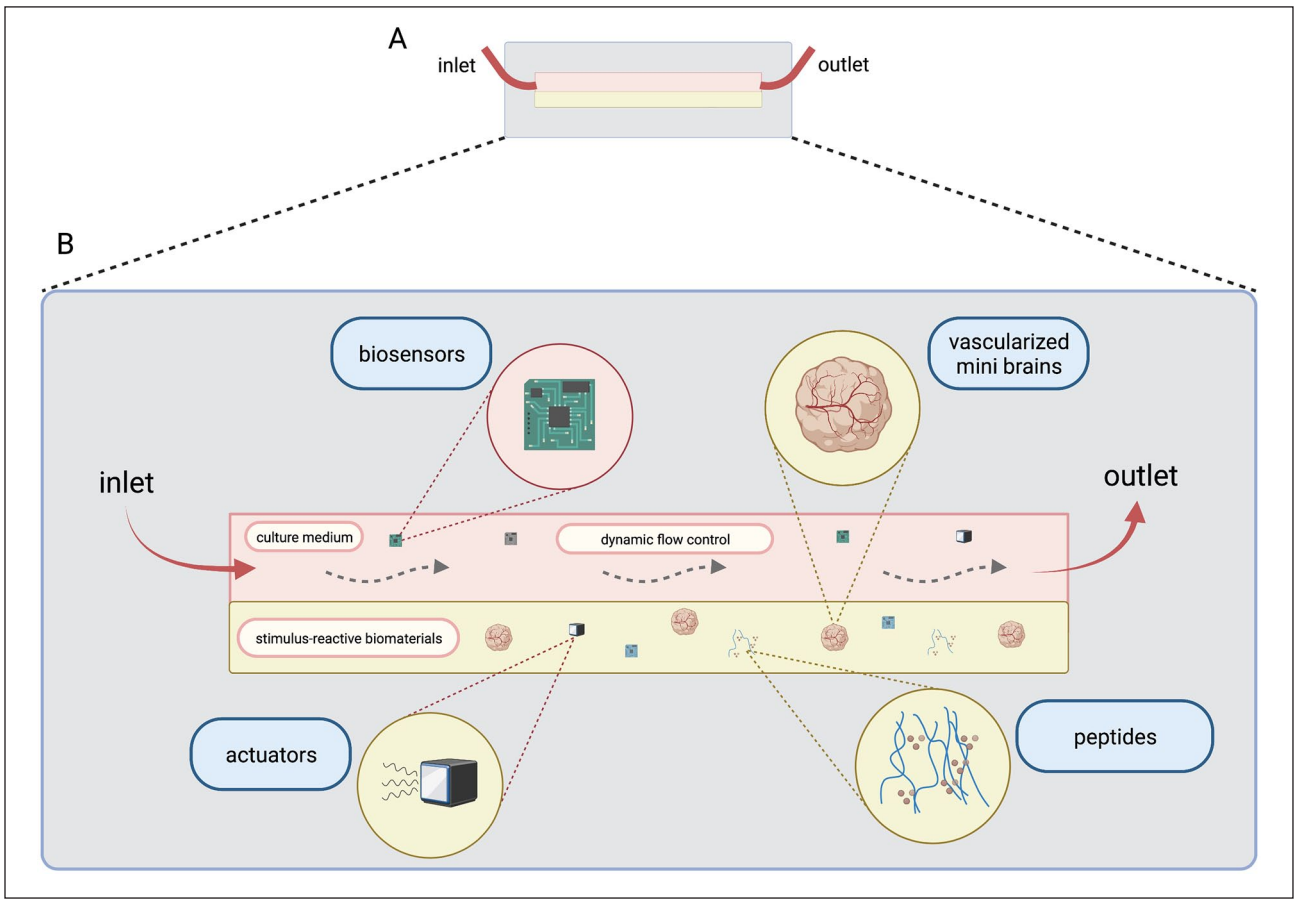

Figure 5. Customizable microfluidic chips provide mini brain models with a variety of physiologically critical stimuli. (A) Side view of microfluidic chip system with inlet and outlet points. (B) Enlarged side view of microfluidic 3D environment with detailed manipulations. The ability to control the flow of culture medium as well as continuously remove spent medium and toxins allows for better sample survivability and maturation. Engineered stimulus-reactive biomaterials enhanced with peptides may be designed for specific biochemical signaling purposes and may be coupled with other microfluidic components to create more physiologically relevant extracellular environments. Under flow conditions, vascularized mini brains may be able to recreate the intricate circulatory system responsible for the nourishment of the brain. A variety of biosensors may be integrated into microfluidic systems to provide real time feedback of culture conditions and can be coupled with actuators that can make real-time changes to critical parameters.

molecule release have also been reported.[138–140] In turn, one can begin to realize the utility for a microchip with dynamic force control, particularly in the context of studying traumatic brain injury. Approaches to modeling traumatic brain injury in human mini brain models often include needle-stick injury, sheer injury via a moving plate, or even high intensity focused ultrasound (HIFU) induced mechanical injury, all of which have their own caveats.[86,141] Microfluidic chip technology may be the solution that laboratories need to expand such study of traumatic brain injury, especially given the multidimensionality of the injury and how it relates to other neurodegenerative disorders such as Alzheimer's disease and frontotemporal dementia. Ultimately, OoC systems and microfluidic technology open the door to new approaches in uncovering the environmental factors implicated in both normal and neuropathological processes. Despite their fixed constructs and currently limited transplantation potential, their full potential in the field of 3D CNS modeling is yet to be realized.

Biomaterials: Key supporting elements to in vitro 3D CNS environment

The advancement of biomaterial technology has proven to be monumental in improving the feasibility of human mini brain and spinal cord models to be used as reliable *in vitro* and *ex vivo* models. Spearheaded by Matrigel, a commercially available mouse-derived mixture rich in ECM proteins, many types of hydrogels provide the ability to tune specific mechanical and biochemical properties of the extracellular matrix, which is important for modulating behaviors like neural process development, synapse formation,

and cell migration.[142] While culturing neurospheres, organoids, and assembloids embedded in Matrigel has been and continues to be a popular approach, there are several drawbacks to Matrigel, particularly its animal origin and relative heterogeneity, that limit it from being deeply translational in the study of 3D CNS structures. Synthetic hydrogels on the other hand, can be consistently reproduced and provide a base for manipulating parameters relevant to the application they are involved in. Dynamic covalent chemistry (DCC), which relies on the reversible formation and breakage of covalent bonds, has emerged as a powerful tool for controlling the crosslinking and subsequent viscoelastic properties of a hydrogel. The potential application of DCC in *in vitro* modeling technology includes precise coordination of cell differentiation, migration, and maturation within the 3D environment.[143,144] One study utilizing DCC to link DNA strands to polyacrylamide hydrogels showed that hydrogel stiffness contributes to neural sprouting.[119] Given that the mechanical properties of the brain change throughout development, hydrogels may be utilized to promote more complex organoid and assembloid spatial organization, as the stiffness and stress-relaxation characteristics of hydrogels housing 3D mini brains can be tuned in real-time to match developmental parameters seen in the developing brain.[145] For example, one study used photo-responsive crosslinkers to temporally and spatially tune the stiffness of a hydrogel, in turn demonstrating the ability to optically control cell migration.[146] Precision control of local hydrogel properties may be useful in directing the migration of microglia or endothelial cells, which can be seeded into assembloid-containing hydrogel scaffolds to replicate the infiltration of those non-neuronal cells into the CNS during embryonic development.[147] Moreover, hyaluronic acid (HA) incorporated hydrogels have been shown to improve the stability of hydrogels in 3D cultures. It should be noted that HA hydrogels are particularly important in neural based applications given the high HA composition in the human brain ECM, as it presents a great promise in their application for advancing mini brain and spinal cord technology.[75,148] One study investigating how migration is affected in certain neurodevelopmental disorders, such as Rett Syndrome, showed that methacrylate modified HA hydrogels improved neural migration in neurosphere cultures.[149] Another study identified the utility of HA in promoting axon regeneration and angiogenesis, as severed axons at the site of spinal cord injury in mice were found to extend into transplanted HA-composed hydrogels.[150] HA hydrogels containing stem-cell derived axon bundles are already being tested for clinical applications to restore damaged pathways in patients with Parkinson's disease.[151] Expanding this type of approach to include semi-mature organoid structures may be able to address a wider range of conditions with larger implicated injury sites. In addition, the covalent integration of peptides and other signaling molecules into these "designer hydrogels" opens a new avenue for manipulating culture conditions and further exploring brain properties (Figure 5). The peptide Arg-Gly-Asp (RGD), for example, is commonly used to increase cell adhesion, a property important for cell migration and injury recovery.[152] One study utilized polyethylene glycol (PEG) hydrogels supplemented with RGD-derivative peptides to produce 3D neural constructs of neural, mesenchymal, and microglial cells, and showed this approach was able to consistently reproduce CNS constructs of similar RNA-seq profiles.[153] Similarly, amphipathic β-sheet forming peptides such as EAK16, RADA16, and IKVAV have been developed to improve mammalian cell adhesion while also remaining incredibly stable over a variety of temperatures, pHs, or enzymatic ranges.[154] One study showed that the laminin-mimetic IKVAV sequence was able to promote bone marrow-derived mesenchymal stem cell differentiation into the neuroectodermal lineage after just one week, a finding particularly important in improving the generation and throughput of neurosphere cultures.[155] These biodegradable scaffolds go beyond improving the *in vitro* culture systems and have already shown potential in treating Alzheimer's disease and Parkinson's disease from a neuroregenerative approach. Given their synthetic origin, peptide-modified scaffolds are great candidates for organoid-transplantation solutions and future work hopes to explore their utility in these approaches.[156] Ultimately, the continued integration of innovative peptides with hydrogels into 3D CNS cultures will enhance both *in vitro* modeling capabilities as well as inspire novel therapeutic solutions for neurodegeneration and CNS injury.

The real potential of biomaterials begins to reveal itself in "smart hydrogels," which can perhaps integrate nanotechnology to increase their real time influence on 3D culture. The integration of biochemical sensors and actuators into these hydrogels may allow them to change their properties in response to culture conditions in real time (Figure 5). Stimulus-reactive hydrogels have been implemented in a wide variety of biomedical applications and have so far been designed to use temperature, pH, compressive, light, electric field, magnetic, ultrasound, antigenic, ionic, and enzymatic stimuli to induce desired behaviors in certain systems.[157] While their utility in 3D CNS culture has yet to be fully realized, they hold significant promise in improving culture conditions by providing researchers with a wider range of levers to pull in the customization of their models. For example, several studies have captured the influence of electric fields on nervous tissue development, particularly in terms of cell differentiation, proliferation, and axon growth.[158,159] In turn, studies utilizing conductive nanomaterials, such as polypyrrole, polyaniline, and polythiophene, have demonstrated their ability to respond to electrical stimulation in the form of direct, biphasic, alternating, and pulse currents to modulate local cell migration, gene expression, maturation, and process extension of neural progenitors and mature neurons.[158,160–162] Precise electrical stimulation for enhancing the development and maturation of a 3D CNS model may decrease the need to

add large cocktails of bioactive compounds and growth factors, which often have off-target effects that confound study results. The design and implementation of smart hydrogels may in turn provide us with a level of control previously unimaginable in engineering complex but orderly 3D mini brains and spinal cords. This level of precision is exactly what the human mini brain and spinal cord field needs to prove translational in the study of human neurodevelopment and many kinds of neurodegenerative disorders.

Conclusion and perspective

In vitro and *ex vivo* human 3D CNS models have great potential in meeting Neuroscience's growing need for more advanced CNS modeling platforms. Neurospheres, CNS Organoids, CNS assembloids, OoC systems, and their combinations are several modeling strategies that allow for great flexibility and ingenuity in mechanistic study for CNS development and disorders, as well as fostering translational solutions to treat neurological disorders. Despite the great promise of contemporary engineered human 3D CNS technology, there are still issues in its variability, maturity, and reproducibility that need to be overcome before its large-scale application in translational biomedical research (see their pros and cons in Table 2). Efforts to reduce the variability of generating these 3D structures, especially for organoids and assembloids, involve defining the type and number of the originating cells, standardizing the reagents and procedures of the development process, and adjusting the timing for the patterning and maturation strategy. In particular, reduced variability has been achieved through using more committed cell sources and optimized time-windows for patterning.[163,164] Ideally, automated systems with liquid and plate handling should also relieve some of the concerns in culture variability by standardizing medium changes, embryoid body formation, and nutrient distribution in culture. Well-defined and standardized biocompatible materials combined with 3D printing technology have shown to improve the reproducibility of using organoids and assembloids as disease models.[165–167] Strikingly, integration of endothelium in these engineered 3D CNS tissues not only escalated the complexity of these structures, but advanced their maturity (our unpublished data).[168,169] Innovative prolonged culture protocols, as well as those to accelerate the cell growth and differentiation also showed promise in promoting maturation.[62,170] Slice cultures derived from organoids provide an air-liquid interface allowing improved oxygen penetration into the core structure, which also facilitates neuronal differentiation, axonal growth and synaptic formation.[171,172] Tubular organoids and other OoC systems have also been able to address some of the shortcomings of 3D *in vitro* culture.[132] Additionally, supporting materials, including bioreactors, microfluidic chips, biomaterials, etc. have proven to be critical in optimizing these methods for laboratory and clinical use. Current research highlights using human mini brains and spinal cords to underline the rapid development of 3D human CNS models in the bioengineering and neuroscience fields and aspires to stimulate further innovations and advances in overcoming some remaining hurdles that limit their applications.

Author contributions

LQ and SK initiated the work and designed the idea. All authors contributed and revised the final version of the manuscript.

Declaration of conflicting interests

The author(s) declared no potential conflicts of interest with respect to the research, authorship, and/or publication of this article.

Funding

The author(s) disclosed receipt of the following financial support for the research, authorship, and/or publication of this article: The work on using hiPSC derived models in the authors' laboratory was supported by NIH grant R01NS115977 awarded to LQ; the Lisa Dean Moseley Foundation grant awarded to LQ; Spastic Paraplegia Foundation grant awarded to LQ, and the grant from the CURE program via Drexel University College of Medicine to LQ (CURE Grant SAP Number: 4100083087).

ORCID iD

Simeon Kofman https://orcid.org/0000-0003-3599-5258

References

1. Zhao X and Bhattacharyya A. Human models are needed for studying human neurodevelopmental disorders. *Am J Hum Genet* 2018; 103: 829–857.
2. Piehl F. Current and emerging disease-modulatory therapies and treatment targets for multiple sclerosis. *J Intern Med* 2021; 289: 771–791.
3. Bao Z, Fang K, Miao Z, et al. Human cerebral organoid implantation alleviated the neurological deficits of traumatic brain injury in mice. *Oxid Med Cell Longev* 2021; 2021: 6338722.
4. Kelley KW and Pașca SP. Human brain organogenesis: toward a cellular understanding of development and disease. *Cell* 2022; 185: 42–61.
5. Miura Y, Li MY, Revah O, et al. Engineering brain assembloids to interrogate human neural circuits. *Nat Protoc* 2022; 17: 15–35.
6. Corrò C, Novellasdemunt L and Li VSW. A brief history of organoids. *Am J Physiol Cell Physiol* 2020; 319: C151–C165.
7. Azar J, Bahmad HF, Daher D, et al. The use of stem cell-derived organoids in disease modeling: an update. *Int J Mol Sci* 2021; 22: 7667.
8. Fang Y and Eglen RM. Three-dimensional cell cultures in drug discovery and development. *SLAS Discov* 2017; 22: 456–472.
9. Reynolds BA and Weiss S. Generation of neurons and astrocytes from isolated cells of the adult mammalian central nervous system. *Science* 1992; 255: 1707–1710.

10. Pastrana E, Silva-Vargas V and Doetsch F. Eyes wide open: a critical review of sphere-formation as an assay for stem cells. *Cell Stem Cell* 2011; 8: 486–498.
11. Cui X, Hartanto Y and Zhang H. Advances in multicellular spheroids formation. *J R Soc Interface* 2017; 14: 20160877.
12. Achilli TM, Meyer J and Morgan JR. Advances in the formation, use and understanding of multi-cellular spheroids. *Expert Opin Biol Ther* 2012; 12: 1347–1360.
13. da Silva Siqueira L, Majolo F, da Silva APB, et al. Neurospheres: a potential in vitro model for the study of central nervous system disorders. *Mol Biol Rep* 2021; 48: 3649–3663.
14. de Oliveira NB, Irioda AC, Stricker PEF, et al. Natural membrane differentiates human adipose-derived mesenchymal stem cells to neurospheres by mechanotransduction related to YAP and AMOT proteins. *Membranes* 2021; 11: 687.
15. Benitez JA, Finlay D, Castanza A, et al. PTEN deficiency leads to proteasome addiction: a novel vulnerability in glioblastoma. *Neuro Oncol* 2021; 23: 1072–1086.
16. Foty RA, Pfleger CM, Forgacs G, et al. Surface tensions of embryonic tissues predict their mutual envelopment behavior. *Development* 1996; 122: 1611–1620.
17. Jensen JB and Parmar M. Strengths and limitations of the neurosphere culture system. *Mol Neurobiol* 2006; 34: 153–161.
18. Tropepe V, Hitoshi S, Sirard C, et al. Direct neural fate specification from embryonic stem cells: a primitive mammalian neural stem cell stage acquired through a default mechanism. *Neuron* 2001; 30: 65–78.
19. Lee ST, Chu K, Jung KH, et al. Direct generation of neurosphere-like cells from human dermal fibroblasts. *PLoS One* 2011; 6: e21801.
20. Yang E, Liu N, Tang Y, et al. Generation of neurospheres from human adipose-derived stem cells. *Biomed Res Int* 2015; 2015: 743714.
21. Pauly MG, Krajka V, Stengel F, et al. Adherent vs. Free-floating neural induction by dual SMAD inhibition for neurosphere cultures derived from human induced pluripotent stem cells. *Front Cell Dev Biol* 2018; 6: 3.
22. Klein C, Butt SJ, Machold RP, et al. Cerebellum- and forebrain-derived stem cells possess intrinsic regional character. *Development* 2005; 132: 4497–4508.
23. Syu WZ, Hueng DY, Chen WL, et al. Adipose-derived neural stem cells combined with acellular dermal matrix as a neural conduit enhances peripheral nerve repair. *Cell Transplant* 2019; 28: 1220–1230.
24. Bammidi S, Bali P, Kalra J, et al. Transplantation efficacy of human ciliary epithelium cells from fetal eye and lin-ve stem cells from umbilical cord blood in the murine retinal degeneration model of laser injury. *Cell Transplant* 2020; 29: 963689720946031.
25. Poltavtseva RA, Samokhin AN, Bobkova NV, et al. Effect of transplantation of neural stem and progenitor cells on memory in animals with Alzheimer's type neurodegeneration. *Bull Exp Biol Med* 2020; 168: 589–596.
26. Kawase-Koga Y, Fujii Y, Yamakawa D, et al. Identification of neurospheres generated from human dental pulp stem cells in xeno-/serum-free conditions. *Regen Therapy* 2020; 14: 128–135.
27. Coles BL, Angénieux B, Inoue T, et al. Facile isolation and the characterization of human retinal stem cells. *Proc Natl Acad Sci U S A* 2004; 101: 15772–15777.
28. Bammidi S, Modgil S, Kalra J, et al. Human fetal pigmented ciliary epithelium stem cells have regenerative capacity in the murine retinal degeneration model of laser injury. *Curr Neurovasc Res* 2019; 16: 187–193.
29. Hooshmand MJ, Sontag CJ, Uchida N, et al. Analysis of host-mediated repair mechanisms after human CNS-stem cell transplantation for spinal cord injury: correlation of engraftment with recovery. *PLoS One* 2009; 4: e5871.
30. Cummings BJ, Uchida N, Tamaki SJ, et al. Human neural stem cells differentiate and promote locomotor recovery in spinal cord-injured mice. *Proc Natl Acad Sci U S A* 2005; 102: 14069–14074.
31. Giandomenico SL, Sutcliffe M and Lancaster MA. Generation and long-term culture of advanced cerebral organoids for studying later stages of neural development. *Nat Protoc* 2021; 16: 579–602.
32. Wang SN, Wang Z, Xu TY, et al. Cerebral organoids repair ischemic stroke brain injury. *Transl Stroke Res* 2020; 11: 983–1000.
33. Pain B, Baquerre C and Coulpier M. Cerebral organoids and their potential for studies of brain diseases in domestic animals. *Vet Res* 2021; 52: 65.
34. Gulimiheranmu M, Li S and Zhou J. in vitro recapitulation of neuropsychiatric disorders with pluripotent stem cells-derived brain organoids. *Int J Environ Res Public Health* 2021; 18: 12431.
35. Renner M, Lancaster MA, Bian S, et al. Self-organized developmental patterning and differentiation in cerebral organoids. *EMBO J* 2017; 36: 1316–1329.
36. Camp JG, Badsha F, Florio M, et al. Human cerebral organoids recapitulate gene expression programs of fetal neocortex development. *Proc Natl Acad Sci U S A* 2015; 112: 15672–15677.
37. Chan WK, Griffiths R, Price DJ, et al. Cerebral organoids as tools to identify the developmental roots of autism. *Mol Autism* 2020; 11: 58.
38. Yang G and Shcheglovitov A. Probing disrupted neurodevelopment in autism using human stem cell-derived neurons and organoids: an outlook into future diagnostics and drug development. *Dev Dyn* 2020; 249: 6–33.
39. Wang Z, Wang S-N, Xu TY, et al. Cerebral organoids transplantation improves neurological motor function in rat brain injury. *CNS Neurosci Ther* 2020; 26: 682–697.
40. Vieira de Sá R, Cañizares Luna M and Pasterkamp RJ. Advances in central nervous system organoids: A focus on organoid-based models for motor neuron disease. *Tissue Eng Part C Methods* 2021; 27: 213–224.
41. Khong ZJ, Hor JH and Ng SY. Spinal cord organoids add an extra dimension to traditional motor neuron cultures. *Neural Regen Res* 2019; 14: 1515–1516.
42. Hor JH, Soh ESY, Tan LY, et al. Cell cycle inhibitors protect motor neurons in an organoid model of spinal muscular atrophy. *Cell Death Dis* 2018; 9: 1100.
43. Hor J-H and Ng S-Y. Chapter 11 - generating ventral spinal organoids from human induced pluripotent stem cells. In: JR Spence (ed.) *Methods in Cell Biology*. San Diego, CA: Academic Press, 2020, pp.257–277.
44. Faustino Martins JM, Fischer C, Urzi A, et al. Self-organizing 3D human trunk neuromuscular organoids. *Cell Stem Cell* 2020; 26: 172–186.e176.

45. Pașca SP. The rise of three-dimensional human brain cultures. *Nature* 2018; 553: 437–445.
46. Bagley JA, Reumann D, Bian S, et al. Fused cerebral organoids model interactions between brain regions. *Nat Methods* 2017; 14: 743–751.
47. De Santis R, Etoc F, Rosado-Olivieri EA, et al. Self-organization of human dorsal-ventral forebrain structures by light induced SHH. *Nat Commun* 2021; 12: 6768.
48. Gomes AR, Fernandes TG, Vaz SH, et al. Modeling Rett syndrome with human patient-specific forebrain organoids. *Front Cell Dev Biol* 2020; 8: 610427.
49. Bagley JA, Reumann D, Bian S, et al. Fused dorsal-ventral cerebral organoids model human cortical interneuron migration. *bioRxiv* 2017; 131250.
50. Eura N, Matsui TK, Luginbühl J, et al. Brainstem organoids from human pluripotent stem cells. *Front Neurosci* 2020; 14: 538. DOI: 10.3389/fnins.2020.00538
51. Zagare A, Gobin M, Monzel AS, et al. A robust protocol for the generation of human midbrain organoids. *STAR Protocols* 2021; 2: 100524.
52. Jarazo J, Barmpa K, Modamio J, et al. Parkinson's disease phenotypes in patient neuronal cultures and brain organoids improved by 2-hydroxypropyl-β-cyclodextrin treatment. *Mov Disord* 2022; 37: 80–94.
53. Kim H, Park HJ, Choi H, et al. Modeling G2019S-LRRK2 sporadic Parkinson's disease in 3D midbrain organoids. *Stem Cell Reports* 2019; 12: 518–531.
54. Galet B, Cheval H and Ravassard P. Patient-derived midbrain organoids to explore the molecular basis of Parkinson's disease. *Front Neurol* 2020; 11: 1005.
55. Valiulahi P, Vidyawan V, Puspita L, et al. Generation of caudal-type serotonin neurons and hindbrain-fate organoids from hPSCs. *Stem Cell Reports* 2021; 16: 1938–1952.
56. Qian X, Jacob F, Song MM, et al. Generation of human brain region-specific organoids using a miniaturized spinning bioreactor. *Nat Protoc* 2018; 13: 565–580.
57. Huang W-K, Wong SZH, Pather SR, et al. Generation of hypothalamic arcuate organoids from human induced pluripotent stem cells. *Cell Stem Cell* 2021; 28: 1657–1670. e1610.
58. Ozaki H, Suga H and Arima H. Hypothalamic-pituitary organoid generation through the recapitulation of organogenesis. *Dev Growth Differ* 2021; 63: 154–165.
59. Xiang Y, Tanaka Y, Cakir B, et al. hESC-Derived thalamic organoids form reciprocal projections when fused with cortical organoids. *Cell Stem Cell* 2019; 24: 487–497.e487.
60. Xiang Y, Cakir B and Park I-H. Generation of regionally specified human brain organoids resembling thalamus development. *STAR protocols* 2020; 1: 100001.
61. Nayler S, Agarwal D, Curion F, et al. Single-cell sequencing of human iPSC-derived cerebellar organoids shows recapitulation of cerebellar development. *bioRxiv* 2020. DOI: 10.1101/2020.07.01.182196
62. Silva TP, Fernandes TG, Nogueira DES, et al. Scalable generation of mature cerebellar organoids from human pluripotent stem cells and characterization by immunostaining. *J Vis Exp* 2020; 160: e61143.
63. Ballabio C, Anderle M, Gianesello M, et al. Modeling medulloblastoma in vivo and with human cerebellar organoids. *Nat Commun* 2020; 11: 583.
64. Pellegrini L, Bonfio C, Chadwick J, et al. Human CNS barrier-forming organoids with cerebrospinal fluid production. *Science* 2020; 369: eaaz5626.
65. Sakaguchi H, Kadoshima T, Soen M, et al. Generation of functional hippocampal neurons from self-organizing human embryonic stem cell-derived dorsomedial telencephalic tissue. *Nat Commun* 2015; 6: 8896.
66. Miura Y, Li MY, Birey F, et al. Generation of human striatal organoids and cortico-striatal assembloids from human pluripotent stem cells. *Nat Biotechnol* 2020; 38: 1421–1430.
67. Wang Y, Chiola S, Yang G, et al. Modeling autism-associated SHANK3 deficiency using human cortico-striatal organoids generated from single neural rosettes. *bioRxiv* 2021. DOI: 10.1101/2021.01.25.428022
68. Ogawa J, Pao GM, Shokhirev MN, et al. Glioblastoma model using human cerebral organoids. *Cell Rep* 2018; 23: 1220–1229.
69. Linkous A, Balamatsias D, Snuderl M, et al. Modeling Patient-derived glioblastoma with cerebral organoids. *Cell Rep* 2019; 26: 3203–3211.e3205.
70. Wörsdörfer P, Dalda N, Kern A, et al. Generation of complex human organoid models including vascular networks by incorporation of mesodermal progenitor cells. *Sci Rep* 2019; 9: 15663.
71. Wang L, Sievert D, Clark AE, et al. A human three-dimensional neural-perivascular 'assembloid' promotes astrocytic development and enables modeling of SARS-CoV-2 neuropathology. *Nat Med* 2021; 27: 1600–1606.
72. Kim H, Xu R, Padmashri R, et al. Pluripotent stem cell-derived cerebral organoids reveal human oligodendrogenesis with dorsal and ventral origins. *Stem Cell Reports* 2019; 12: 890–905.
73. Yuan F, Fang KH, Hong Y, et al. LHX6 is essential for the migration of human pluripotent stem cell-derived GABAergic interneurons. *Protein Cell* 2020; 11: 286–291.
74. Birey F, Andersen J, Makinson CD, et al. Assembly of functionally integrated human forebrain spheroids. *Nature* 2017; 545: 54–59.
75. Xiang Y, Tanaka Y, Patterson B, et al. Fusion of regionally specified hPSC-derived organoids models human brain development and. *Cell Stem Cell* 2017; 21: 383–398.e387.
76. Andersen J, Revah O, Miura Y, et al. Generation of functional human 3D cortico-motor assembloids. *Cell* 2020; 183: 1913–1929.e26.
77. Hor JH and Ng SY. Generating ventral spinal organoids from human induced pluripotent stem cells. *Methods Cell Biol* 2020; 159: 257–277.
78. Lancaster MA, Renner M, Martin CA, et al. Cerebral organoids model human brain development and microcephaly. *Nature* 2013; 501: 373–379.
79. Kim Y, Rim YA, Yi H, et al. The generation of human induced pluripotent stem cells from blood cells: an efficient protocol using serial plating of reprogrammed cells by centrifugation. *Stem Cells Int* 2016; 2016: 1329459.
80. Lancaster MA and Knoblich JA. Generation of cerebral organoids from human pluripotent stem cells. *Nat Protoc* 2014; 9: 2329–2340.
81. Das D, Li J, Cheng L, et al. Human forebrain organoids from induced pluripotent stem cells: A Novel Approach to

model repair of ionizing radiation-induced DNA damage in human neurons. *Radiat Res* 2020; 194: 191–198.
82. Smits LM and Schwamborn JC. Midbrain organoids: a new tool to investigate Parkinson's disease. *Front Cell Dev Biol* 2020; 8: 359.
83. Jacob F, Pather SR, Huang W-K, et al. Human pluripotent stem cell-derived neural cells and brain organoids reveal SARS-CoV-2 neurotropism predominates in choroid plexus epithelium. *Cell Stem Cell* 2020; 27: 937–950. e939.
84. Sloan SA, Andersen J, Paşca AM, et al. Generation and assembly of human brain region-specific three-dimensional cultures. *Nat Protoc* 2018; 13: 2062–2085.
85. Park JC, Jang SY, Lee D, et al. A logical network-based drug-screening platform for Alzheimer's disease representing pathological features of human brain organoids. *Nat Commun* 2021; 12: 280.
86. Lai JD, Berlind JE, Fricklas G, et al. A model of traumatic brain injury using human iPSC-derived cortical brain organoids. *bioRxiv* 2020; 7: 180299.
87. Zheng Y, Zhang F, Xu S, et al. Advances in neural organoid systems and their application in neurotoxicity testing of environmental chemicals. *Genes Environ* 2021; 43: 39.
88. Papaspyropoulos A, Tsolaki M, Foroglou N, et al. Modeling and targeting Alzheimer's disease with organoids. *Front Pharmacol* 2020; 11: 396.
89. Lines G, Casey JM, Preza E, et al. Modelling frontotemporal dementia using patient-derived induced pluripotent stem cells. *Mol Cell Neurosci* 2020; 109: 103553.
90. Wang L, Li Z, Sievert D, et al. Loss of NARS1 impairs progenitor proliferation in cortical brain organoids and leads to microcephaly. *Nat Commun* 2020; 11: 4038.
91. Qian X, Nguyen HN, Song MM, et al. Brain-region-specific organoids using mini-bioreactors for modeling ZIKV Exposure. *Cell* 2016; 165: 1238–1254.
92. Pereira JD, DuBreuil DM, Devlin AC, et al. Human sensorimotor organoids derived from healthy and amyotrophic lateral sclerosis stem cells form neuromuscular junctions. *Nat Commun* 2021; 12: 4744.
93. Nieto-Estévez V and Hsieh J. Human Brain organoid models of developmental epilepsies. *Epilepsy Curr* 2020; 20: 282–290.
94. Willner MJ, Xiao Y, Kim HS, et al. Modeling SARS-CoV-2 infection in individuals with opioid use disorder with brain organoids. *J Tissue Eng* 2021; 12: 2041731420985299.
95. Dong X, Xu SB, Chen X, et al. Human cerebral organoids establish subcortical projections in the mouse brain after transplantation. *Mol Psychiatry* 2021; 26: 2964–2976.
96. Majc B, Novak M, Kopitar-Jerala N, et al. Immunotherapy of glioblastoma: current strategies and challenges in tumor model development. *Cells* 2021; 10: 265.
97. Rybin MJ, Ivan ME, Ayad NG, et al. Organoid models of glioblastoma and their role in drug discovery. *Front Cell Neurosci* 2021; 15: 605255.
98. Lee J-H, Shin H, Shaker MR, et al. Human spinal cord organoids exhibiting neural tube morphogenesis for a quantifiable drug screening system of neural tube defects. *bioRxiv* 2020.
99. Ao Z, Cai H, Wu Z, et al. Human spinal organoid-on-a-chip to model nociceptive circuitry for pain therapeutics discovery. *Anal Chem* 2022; 94: 1365–1372.
100. System1 Biosciences. https://system1.bio/ (accessed 2 June 2022).
101. Salick MR, Wells MF, Eggan K, et al. Modelling Zika virus infection of the developing human brain in vitro using stem cell derived cerebral organoids. *J Vis Exp* 2017; 127: 56404. DOI: 10.3791/56404
102. Marshall JJ and Mason JO. Mouse vs man: organoid models of brain development & disease. *Brain Res* 2019; 1724: 146427.
103. Zabala A, Vazquez-Villoldo N, Rissiek B, et al. P2X4 receptor controls microglia activation and favors remyelination in autoimmune encephalitis. *EMBO Mol Med* 2018; 10: e8743.
104. Gordon A, Yoon SJ, Tran SS, et al. Long-term maturation of human cortical organoids matches key early postnatal transitions. *Nat Neurosci* 2021; 24: 331–342.
105. Cakir B, Xiang Y, Tanaka Y, et al. Engineering of human brain organoids with a functional vascular-like system. *Nat Methods* 2019; 16: 1169–1175.
106. Faustino Martins JM, Fischer C, Urzi A, et al. Self-organizing 3D human trunk neuromuscular organoids. *Cell Stem Cell* 2020; 27: 498.
107. Lavazza A. 'Consciousnessoids': clues and insights from human cerebral organoids for the study of consciousness. *Neurosci Conscious* 2021; 7: niab029.
108. Trujillo CA, Gao R, Negraes PD, et al. Complex oscillatory waves emerging from cortical organoids model early human brain network development. *Cell Stem Cell* 2019; 25: 558–569.e557.
109. Sabogal-Guáqueta AM, Marmolejo-Garza A, de Pádua VP, et al. Microglia alterations in neurodegenerative diseases and their modeling with human induced pluripotent stem cell and other platforms. *Prog Neurobiol* 2020; 190: 101805.
110. Luo J and Li P. Human pluripotent stem cell-derived brain organoids as in vitro models for studying neural disorders and cancer. *Cell Biosci* 2021; 11: 99.
111. Murcia-Belmonte V and Erskine L. Wiring the binocular visual pathways. *Int J Mol Sci* 2019; 20: E3282.
112. Fligor CM, Lavekar SS, Harkin J, et al. Extension of retinofugal projections in an assembled model of human pluripotent stem cell-derived organoids. *Stem Cell Reports* 2021; 16: 2228–2241.
113. Phelan K and McDermid HE. The 22q13.3 deletion syndrome (Phelan-McDermid syndrome). *Mol Syndromol* 2012; 2: 186–201.
114. Shepherd GM. Corticostriatal connectivity and its role in disease. *Nat Rev Neurosci* 2013; 14: 278–291.
115. Ogura T, Sakaguchi H, Miyamoto S, et al. Three-dimensional induction of dorsal, intermediate and ventral spinal cord tissues from human pluripotent stem cells. *Development* 2018; 145: dev162214.
116. Sances S, Bruijn LI, Chandran S, et al. Modeling ALS with motor neurons derived from human induced pluripotent stem cells. *Nat Neurosci* 2016; 19: 542–553.
117. Shi Y, Lin S, Staats KA, et al. Haploinsufficiency leads to neurodegeneration in C9ORF72 ALS/FTD human induced motor neurons. *Nat Med* 2018; 24: 313–325.
118. Steinbeck JA, Jaiswal MK, Calder EL, et al. Functional connectivity under optogenetic control allows modeling of human neuromuscular disease. *Cell Stem Cell* 2016; 18: 134–143.

119. Jiang FX, Yurke B, Schloss RS, et al. The relationship between fibroblast growth and the dynamic stiffnesses of a DNA crosslinked hydrogel. *Biomaterials* 2010; 31: 1199–1212.
120. Vogt N. Assembloids. *Nat Methods* 2021; 18: 27–27.
121. Makrygianni EA and Chrousos GP. From brain organoids to networking assembloids: implications for neuroendocrinology and stress medicine. *Front Physiol* 2021; 12: 621970.
122. Marton RM and Pașca SP. Organoid and assembloid technologies for investigating cellular crosstalk in human brain development and disease. *Trends Cell Biol* 2020; 30: 133–143.
123. Cederquist GY, Asciolla JJ, Tchieu J, et al. Specification of positional identity in forebrain organoids. *Nat Biotechnol* 2019; 37: 436–444.
124. Miura Y and Pașca SP. Polarizing brain organoids. *Nat Biotechnol* 2019; 37: 377–378.
125. Fedorchak NJ, Iyer N and Ashton RS. Bioengineering tissue morphogenesis and function in human neural organoids. *Semin Cell Dev Biol* 2021; 111: 52–59.
126. Lavon N, Zimerman M and Itskovitz-Eldor J. Scalable expansion of pluripotent stem cells. *Adv Biochem Eng Biotechnol* 2018; 163: 23–37.
127. Zheng F, Xiao Y, Liu H, et al. Patient-specific organoid and organ-on-a-chip: 3D cell-culture meets 3D printing and numerical simulation. *Adv Biol* 2021; 5: e2000024.
128. Amirifar L, Shamloo A, Nasiri R, et al. Brain-on-a-chip: Recent advances in design and techniques for microfluidic models of the brain in health and disease. *Biomaterials* 2022; 285: 121531.
129. Rismani Yazdi S, Shadmani A, Bürgel SC, et al. Adding the 'heart' to hanging drop networks for microphysiological multi-tissue experiments. *Lab Chip* 2015; 15: 4138–4147.
130. Sances S, Ho R, Vatine G, et al. Human iPSC-derived endothelial cells and microengineered organ-chip enhance neuronal development. *Stem Cell Reports* 2018; 10: 1222–1236.
131. Booth R and Kim H. Characterization of a microfluidic in vitro model of the blood-brain barrier (μBBB). *Lab Chip* 2012; 12: 1784–1792.
132. Ao Z, Cai H, Wu Z, et al. Tubular human brain organoids to model microglia-mediated neuroinflammation. *Lab Chip* 2021; 21: 2751–2762.
133. Salmon I, Grebenyuk S, Abdel Fattah AR, et al. Engineering neurovascular organoids with 3D printed microfluidic chips. *Lab Chip* 2022; 22: 1615–1629.
134. Garcia KE, Kroenke CD and Bayly PV. Mechanics of cortical folding: stress, growth and stability. *Philos Trans R Soc Lond B Biol Sci* 2018; 373: 20170321.
135. Karzbrun E, Kshirsagar A, Cohen SR, et al. Human brain organoids on a chip reveal the physics of folding. *Nat Phys* 2018; 14: 515–522.
136. Scott A, Weir K, Easton C, et al. A microfluidic microelectrode array for simultaneous electrophysiology, chemical stimulation, and imaging of brain slices. *Lab Chip* 2013; 13: 527–535.
137. Pearce TM, Wilson JA, Oakes SG, et al. Integrated microelectrode array and microfluidics for temperature clamp of sensory neurons in culture. *Lab Chip* 2005; 5: 97–101.
138. Kim G-A, Ginga NJ and Takayama S. Integration of sensors in gastrointestinal organoid culture for biological analysis. *Cell Mol Gastroenterol Hepatol* 2018; 6: 123–131.e121.
139. Hajialyani M, Hosseinzadeh L and Wu JJ. Microfluidics-integrated sensors toward rapid detection of single nucleotide variations. *ACS Omega* 2021; 6: 24297–24303.
140. Ketterer S, Hövermann D, Guebeli RJ, et al. Transcription factor sensor system for parallel quantification of metabolites on-chip. *Anal Chem* 2014; 86: 12152–12158.
141. Jgamadze D, Johnson VE, Wolf JA, et al. Modeling traumatic brain injury with human brain organoids. *Curr Opin Biomed Eng* 2020; 14: 52–58.
142. Zhao Z, Vizetto-Duarte C, Moay ZK, et al. Composite hydrogels in three-dimensional in vitro models. *Front Bioeng Biotechnol* 2020; 8: 611.
143. Chaudhuri O, Gu L, Klumpers D, et al. Hydrogels with tunable stress relaxation regulate stem cell fate and activity. *Nat Mater* 2016; 15: 326–334.
144. Rizwan M, Baker AEG and Shoichet MS. Designing hydrogels for 3D cell culture using dynamic covalent crosslinking. *Adv Healthc Mater* 2021; 10: e2100234.
145. Ryu Y, Iwashita M, Lee W, et al. A shift in tissue stiffness during hippocampal maturation correlates to the pattern of neurogenesis and composition of the extracellular matrix. *Front Aging Neurosci* 2021; 13: 709620.
146. Wu X, Huang W, Wu WH, et al. Reversible hydrogels with tunable mechanical properties for optically controlling cell migration. *Nano Res* 2018; 11: 5556–5565.
147. Ranawat N and Masai I. Mechanisms underlying microglial colonization of developing neural retina in zebrafish. *eLife* 2021; 10: e70550.
148. Figueiredo T, Jing J, Jeacomine I, et al. Injectable self-healing hydrogels based on boronate ester formation between hyaluronic acid partners modified with benzoxaborin derivatives and saccharides. *Biomacromolecules* 2020; 21: 230–239.
149. Zhang Z-N, Freitas BC, Qian H, et al. Layered hydrogels accelerate iPSC-derived neuronal maturation and reveal migration defects caused by MeCP2 dysfunction. *Proc Natl Acad Sci USA* 2016; 113: 3185–3190.
150. Wei YT, He Y, Xu CL, et al. Hyaluronic acid hydrogel modified with nogo-66 receptor antibody and poly-L-lysine to promote axon regrowth after spinal cord injury. *J Biomed Mater Res Part B Appl Biomater* 2010; 95: 110–117.
151. Gordián-Vélez WJ, Chouhan D, España RA, et al. Restoring lost nigrostriatal fibers in Parkinson's disease based on clinically-inspired design criteria. *Brain Res Bull* 2021; 175: 168–185.
152. Cui FZ, Tian WM, Hou SP, et al. Hyaluronic acid hydrogel immobilized with RGD peptides for brain tissue engineering. *J Mater Sci Mater Med* 2006; 17: 1393–1401.
153. Schwartz MP, Hou Z, Propson NE, et al. Human pluripotent stem cell-derived neural constructs for predicting neural toxicity. *Proc Natl Acad Sci U S A* 2015; 112: 12516–12521.
154. Gray VP, Amelung CD, Duti IJ, et al. Biomaterials via peptide assembly: Design, characterization, and application in tissue engineering. *Acta Biomater* 2022; 140: 43–75.
155. Ji W, Álvarez Z, Edelbrock AN, et al. Bioactive nanofibers induce neural transdifferentiation of human bone marrow mesenchymal stem cells. *ACS Appl Mater Interfaces* 2018; 10: 41046–41055.

156. Khan J, Rudrapal M, Bhat EA, et al. Perspective insights to bio-nanomaterials for the treatment of neurological disorders. *Front Bioeng Biotechnol* 2021; 9: 724158.
157. El-Husseiny HM, Mady EA, Hamabe L, et al. Smart/stimuli-responsive hydrogels: cutting-edge platforms for tissue engineering and other biomedical applications. *Mater Today Bio* 2022; 13: 100186.
158. Zhu R, Sun Z, Li C, et al. Electrical stimulation affects neural stem cell fate and function in vitro. *Exp Neurol* 2019; 319: 112963.
159. Xu K, Liu X, Li X, et al. Effect of electrical and electromechanical stimulation on PC12 cell proliferation and axon outgrowth. *Front Bioeng Biotechnol* 2021; 9: 757906.
160. George PM, Bliss TM, Hua T, et al. Electrical preconditioning of stem cells with a conductive polymer scaffold enhances stroke recovery. *Biomaterials* 2017; 142: 31–40.
161. Yang K, Yu SJ, Lee JS, et al. Electroconductive nanoscale topography for enhanced neuronal differentiation and electrophysiological maturation of human neural stem cells. *Nanoscale* 2017; 9: 18737–18752.
162. Jin Y, Seo J, Lee JS, et al. Triboelectric nanogenerator accelerates highly efficient nonviral direct conversion and in vivo reprogramming of fibroblasts to functional neuronal cells. *Adv Mater* 2016; 28: 7365–7374.
163. Ha J, Kang JS, Lee M, et al. Simplified brain organoids for rapid and robust modeling of brain disease. *Front Cell Dev Biol* 2020; 8: 594090.
164. Nickels SL, Modamio J, Mendes-Pinheiro B, et al. Reproducible generation of human midbrain organoids for in vitro modeling of Parkinson's disease. *Stem Cell Res* 2020; 46: 101870.
165. Lancaster MA, Corsini NS, Wolfinger S, et al. Guided self-organization and cortical plate formation in human brain organoids. *Nat Biotechnol* 2017; 35: 659–666.
166. Haremaki T, Metzger JJ, Rito T, et al. Self-organizing neuruloids model developmental aspects of Huntington's disease in the ectodermal compartment. *Nat Biotechnol* 2019; 37: 1198–1208.
167. Knight GT, Lundin BF, Iyer N, et al. Engineering induction of singular neural rosette emergence within hPSC-derived tissues. *eLife* 2018; 7: e37549.
168. Bergmann S, Lawler SE, Qu Y, et al. Blood-brain-barrier organoids for investigating the permeability of CNS therapeutics. *Nat Protoc* 2018; 13: 2827–2843.
169. Nzou G, Wicks RT, Wicks EE, et al. Human cortex spheroid with a functional blood brain barrier for high-throughput neurotoxicity screening and disease modeling. *Sci Rep* 2018; 8: 7413.
170. Durens M, Nestor J, Williams M, et al. High-throughput screening of human induced pluripotent stem cell-derived brain organoids. *J Neurosci Methods* 2020; 335: 108627.
171. Giandomenico SL, Mierau SB, Gibbons GM, et al. Cerebral organoids at the air-liquid interface generate diverse nerve tracts with functional output. *Nat Neurosci* 2019; 22: 669–679.
172. Qian X, Su Y, Adam CD, et al. Sliced human cortical organoids for modeling distinct cortical layer formation. *Cell Stem Cell* 2020; 26: 766–781.e769.