Using Two- and Three-Dimensional Human iPSC Culture Systems to Model Psychiatric Disorders

Kimberly M. Christian, Hongjun Song, and Guo-li Ming

1 Introduction

Psychiatric disorders are among the most challenging human diseases to understand at a mechanistic level due to the heterogeneity of symptoms within established diagnostic categories, the general absence of focal pathology, and the genetic complexity inherent in these mostly polygenic disorders. Each of these features presents unique challenges to disease modeling for biological discovery, drug development, or improved diagnostics. In addition, live human neural tissue has been largely inaccessible to experimentation, leaving gaps in our knowledge derived from animal models that cannot fully recapitulate the features of the disease, indirect measures of brain function in human patients, and from analyses of postmortem tissue that can be confounded by comorbid conditions and medication history.

Advances in cellular reprogramming of somatic cells have begun to bridge these gaps and hold the promise of being a novel translational tool to investigate the cellular and molecular mechanisms that contribute to pathology in disease-relevant cell

K. M. Christian (✉)
Department of Neuroscience, Perelman School of Medicine, University of Pennsylvania, Philadelphia, PA, USA

Mahoney Institute for Neurosciences, Perelman School of Medicine, University of Pennsylvania, Philadelphia, PA, USA
e-mail: kchristi@pennmedicine.upenn.edu

H. Song
Department of Neuroscience, Perelman School of Medicine, University of Pennsylvania, Philadelphia, PA, USA

Mahoney Institute for Neurosciences, Perelman School of Medicine, University of Pennsylvania, Philadelphia, PA, USA

Department of Developmental and Cell Biology, Perelman School of Medicine, University of Pennsylvania, Philadelphia, PA, USA

Institute for Regenerative Medicine, Perelman School of Medicine, University of Pennsylvania, Philadelphia, PA, USA

Institute for Epigenetics, Perelman School of Medicine, University of Pennsylvania, Philadelphia, PA, USA

E. DiCicco-Bloom, J. H. Millonig (eds.), *Neurodevelopmental Disorders*,
Advances in Neurobiology 25, https://doi.org/10.1007/978-3-030-45493-7_9

types. A landmark study in 2007 demonstrated that fibroblasts obtained from human adult donors could be reprogrammed into induced pluripotent stem cell (iPSC) lines [1], which can subsequently be differentiated into nearly any cell type in body. Human derived iPSCs (hiPSCs) are similar to human embryonic stem cells (hESCs) that derive from early blastocysts, both of which are capable of generating all the tissue and cell types in the body. Long considered the gold standard in stem cell-based research, there are several well-validated hESC lines that continue to be used in research but these are subject to more regulatory control. Although hiPSC technology has the potential to revolutionize our study of all human disease, there are distinct advantages for psychiatric and neurological disorders that include the ability to generate hiPSC lines that have the same genetic profile as the patient and the ability to generate a renewable source of specific populations of human neurons. In this chapter, we will discuss some of the key opportunities and challenges in hiPSC-based modeling of psychiatric disorders and highlight representative studies using either two- or three-dimensional (2D, 3D) cell cultures that illustrate the potential of each of these approaches to address some of the most critical, outstanding questions in the field. Although we are focusing on only a few studies to frame the discussion of the key issues, it is important to note that there has been a dramatic increase in hiPSC research in recent years and there are many valuable studies that we are unable to include in this chapter due to space limitations.

2 Genetics and Cohort Selection

Since the initial discovery showing the feasibility of generating hiPSCs, we have made great progress in optimizing reprogramming and differentiation protocols but both 2D and 3D iPSC-based studies are still labor-intensive and costly. For this reason, most studies often include a small number of subjects and therefore the choice of donors is critical. Typically, investigators have focused on clinically well-defined patients who are either idiopathic, for whom there is no known genetic risk factor, or those who harbor a causal or risk-associated genetic variant.

G.-l. Ming
Department of Neuroscience, Perelman School of Medicine, University of Pennsylvania, Philadelphia, PA, USA

Mahoney Institute for Neurosciences, Perelman School of Medicine, University of Pennsylvania, Philadelphia, PA, USA

Department of Developmental and Cell Biology, Perelman School of Medicine, University of Pennsylvania, Philadelphia, PA, USA

Institute for Regenerative Medicine, Perelman School of Medicine, University of Pennsylvania, Philadelphia, PA, USA

Department of Psychiatry, Perelman School of Medicine, University of Pennsylvania, Philadelphia, PA, USA

2.1 Monogenic Disorders

There are a few monogenic autism spectrum disorders (ASD) with known causal variants, which has been the focus of some of the first hiPSC-based studies of psychiatric disorders. For example, Rett syndrome, which is a result of mutations in the X-linked gene, *MeCP2*, is one such disorder and an early study revealed specific synaptic deficits in glutamatergic neurons derived from patient iPSCs, including reduced numbers of synapses, spines, and decreases in spontaneous synaptic currents [2]. This study was able to provide proof-of-concept support for the use of hiPSCs to model cellular pathology in 2D cultures using human neurons with disease-relevant mutations and to show that X-inactivation can occur upon neuronal differentiation following reprogramming. Furthermore, this study underscores the utility of hiPSC-based models to identify clear cellular phenotypes suitable for drug screening. The most prevalent monogenic cause of ASD is fragile X syndrome (FXS), which is the result of an expanded CGG trinucleotide repeat in the 5′ untranslated region of fragile X mental retardation gene 1 (*FMR1*). If the expansion is greater than 200 repeats, it leads to epigenetic silencing via CpG hypermethylation and a loss of the fragile X mental retardation protein (FMRP) expression. In one of the first studies to generate iPSC lines from FXS patients, instability of the expansion repeat length was observed after reprogramming, which correlated with methylation and FMRP expression levels [3]. Because the repeat expansion instability occurred even among hiPSC lines from the same subject, this study illustrates the importance of validating any expected genotype and monitoring any mosaicism in the gene of interest both in the fibroblast population before reprogramming and after reprogramming. As with the Rett syndrome study, any X-linked gene should also be monitored for X-inactivation, which can be altered during reprogramming but should be stable in clonal iPSC lines [4, 5]. Upon differentiation of the FXS hiPSC lines, morphological differences have been observed in FXS neurons that exhibit fewer neuronal processes and less complex branching, a phenotype consistent with the role of FMRP in mRNA transport from the nucleus to the dendrites and activity-dependent synaptic development [3, 6]. Additional studies have reported differentiation deficits, as well as impairments in synaptic function and presynaptic vesicle release [7–9]. A recent study used FXS hiPSC-derived neurons, as well as hESC-derived neurons in which the FMR1 gene had been knocked out, to investigate the role of FMRP in homeostatic plasticity mediated by retinoic acid signaling [10]. Importantly, the authors demonstrated that this form of plasticity is present in both hESC- or hiPSC-derived control human neurons, but compromised in FXS neurons. They did not, however, observe any of the overt phenotypes reported in other studies of deficits in differentiation and development and global synaptic dysfunction. Among the many methodological differences that could explain the discrepancy in results, the studies on homeostatic plasticity were predominantly performed in Ngn2-induced glutamatergic neurons that were co-cultured with rodent glial cells and/or rodent neurons. This approach suggests a more nuanced and cell-autonomous deficit in FXS neurons and perhaps that more severe phenotypes can be partly rescued by exposure to non-mutant glial or neural populations, even from other species.

2.2 Polygenic Disorders

In contrast to these monogenic disorders, most psychiatric disorders are polygenic. For disorders such as schizophrenia and bipolar disorder, there are hundreds of risk-associated genes that have been identified. In terms of the strength of association, genetic risk ranges from rare and highly penetrant variants to cumulative risk scores based on multiple common variants [11]. Structurally, risk variants can take many different forms, from discrete single nucleotide polymorphisms (SNPs) to large-scale copy number variations (CNVs) that include both duplications and deletions spanning from a few to hundreds of genes [12]. Adding further complexity, many of these risk variants are often associated with more than one disorder. For example, the 15q11.2–13.1 CNV has been associated with schizophrenia, ASD, and bipolar disorder [13]. Furthermore, some CNVs are associated with diametric risk for different psychiatric disorders based on gene dosage effects. For example, 22q11.2 microdeletions are associated with increased risk for schizophrenia, whereas duplications are associated with ASD [14], which is similar to the diametric risk associations identified for duplications and deletions in 15q11.2 [15].

Ultimately, one of the overarching goals of hiPSC-based research is to determine the degree of mechanistic convergence and divergence in the etiopathology within a single disorder and across clinically related disorders. This is particularly true for polygenic disorders in which patients will likely have distinct underlying genetic risk profiles. For schizophrenia, two of the earliest studies to perform comprehensive phenotyping of neurons differentiated from patient-specific hiPSC lines took different approaches to cohort selection and together were able to provide some information on mechanistic overlap. In one study, the investigators generated hiPSC lines from idiopathic schizophrenia patients [16]. Neurons generated from patient-specific hiPSC lines, as compared to lines generated from age- and sex-matched controls, exhibited several phenotypes including impaired migration, transcriptomic dysregulation, and synaptic deficits [16]. This study was among the first to show that consistent cellular phenotypes could be observed in neurons derived from a cohort of patients who did not share any known genetic or specific environmental risk factor.

Using a different strategy for cohort selection, another study focused on a rare mutation in *DISC1*, a gene that has been implicated in risk for multiple psychiatric disorders including schizophrenia and major depression [17, 18]. This mutation was identified in an American family, Pedigree H, in which some family members harbored a four base pair deletion in *DISC1*. hiPSC lines were generated from family members both with and without the mutation, which were analyzed together with another control line generated from an individual outside of the family to control for genetic background. Several robust cellular phenotypes were observed in forebrain glutamatergic neurons harboring the mutation including widespread transcriptional dysregulation and impaired synapse development and function [19]. Strikingly, several of the cellular phenotypes appeared to be similar to what was observed in the

study of idiopathic SZ patients, suggesting a mechanistic convergence of the influence of different risk factors [20].

Importantly, by focusing on a discrete mutation in*DISC1* that is highly amenable to gene editing, investigators were able to generate pairs of isogenic cell lines by either correcting the mutation in patient lines or introducing the mutation in control lines. Investigation of these isogenic lines revealed that many of the cellular phenotypes could be rescued by repairing the mutation or induced by deleting the four base pairs in *DISC1* [19]. Although this study demonstrated the power of gene editing to reveal a causal relationship between a mutation and molecular/cellular phenotypes, it is important to note that this type of study cannot establish a causal relationship between a mutation and a specific disease, and that a direct mapping of cellular phenotype to clinical presentation of symptoms is unlikely. Even monogenic disorders are still considered heterogeneous at the level of gene expression and clinical symptomatology, illustrating the principle that even when a causal mutation and consistent cellular phenotypes have been identified, there can still be variability in how the disease manifests among individuals, likely involving other environmental, genetic, and epigenetic factors.

Ideally, to determine the causal role of any sequence variant in dysregulated cellular processes, it would be best to generate isogenic lines through targeted gene editing to either repair disease hiPSC lines or introduce the mutation in control lines and to show that the genotype is both required and necessary for the given phenotype. Focal- or mono-genetic variants with high penetrance are much easier to model with advanced gene editing strategies such as the recently developed CRISPR/CAS technologies. For disease-associated CNVs or high-risk polygenic scores affecting multiple genes, while it is relatively easy to generate hiPSC lines directly from patients that express the same genetic changes, it is nearly impossible to correct or introduce the variation in its entirety using current techniques, particularly for large CNVs that span hundreds of genes and diseases with polygenetic factors. On the other hand, manipulating a single gene within the CNV or a SNP is much more feasible, which may pinpoint the causal gene or genes for any observed cellular phenotype. There has been some success in identifying a single gene that may preferentially contribute to certain cellular phenotypes relevant to developmental disorders. For example, hiPSCs generated from the 15q11.2 microdeletion CNV carriers encompassing only four genes were differentiated toward a glutamatergic cortical lineage and deficits were observed in the earliest stages of development in the neural progenitors, which expressed aberrant adherens junctions and defects in apical polarity [21]. The authors identified*CYFIP1* as the gene within the 15q11.2 CNV that was responsible for the phenotype. This type of structural deficit in cortical progenitors could lead to dysregulated formation of the cerebral cortex and potentially contribute to the increased variability observed in some brain regions of schizophrenia patients [22] as well as localized irregularities in cortical structure observed in ASD patients [23].

2.3 Pharmacotherapy Response

In contrast to cohort selection based on genotype, an entirely different approach based on drug responsiveness was exemplified by a study in which the investigators generated iPSC lines from two groups of bipolar patients—those who were responsive to lithium treatment and those who were not [24]. Strikingly, they observed a hyperexcitability phenotype in neurons that could be selectively rescued with lithium, but only in the hiPSC lines derived from lithium-responsive patients. This finding is a remarkable illustration of how hiPSC models can capture some of the heterogeneity at the patient level in terms of treatment efficacy. A similar study used pharmacological responsiveness to selective serotonin reuptake inhibitors (SSRIs) to stratify patients with major depressive disorder, and found a serotonin-induced hyperactivity phenotype only in forebrain neurons derived from hiPSCs generated from patients who were resistant to SSRI treatment [25]. This "top-down" approach to patient stratification based on drug efficacy and treatment response has proven to be a fruitful strategy that has suggested the presence of basal differences in neuromodulatory pathways in a subset of patients, or selective rescue of phenotypes by a drug in cells derived from drug-responsive patients. Both of these open the door for hiPSC-based diagnostic screening and the potential to move toward a personalized medicine approach to better predict effective treatments for individuals.

In sum, these studies provide key examples of the importance of subject selection when designing an iPSC study of psychiatric disorders, regardless of whether 2D and/or 3D culture conditions will be used. For studies based on genetic variants, these should be verified before and after reprogramming and the possibility of isogenic controls should be considered when possible to allow for causal studies linking gene function to cellular phenotypes.

3 2D Models and Directed Differentiation

Following cohort selection, another important decision to be made when designing an hiPSC study is the cell type to be investigated. One of the key advantages of using 2D models is the ability to generate relatively homogenous populations comprised of a desired cell type (Fig. 1a). The earliest studies were not focused on generating highly specific cell types, but rather used less restrictive differentiation protocols to achieve populations enriched for a particular neurotransmitter subtype such as glutamatergic neurons. Typically, differentiation relies on introducing the same signaling molecules that are present during early development to pattern cells toward lineages of different germ layers and then specific tissues and regions [26]. Targeted differentiation protocols have dramatically improved since the introduction of hiPSC technology; however, achieving 100% purity for any specific cell type is still a challenge for the whole stem cell field. The success in generating a "pure" population of a particular cell type also depends on how the

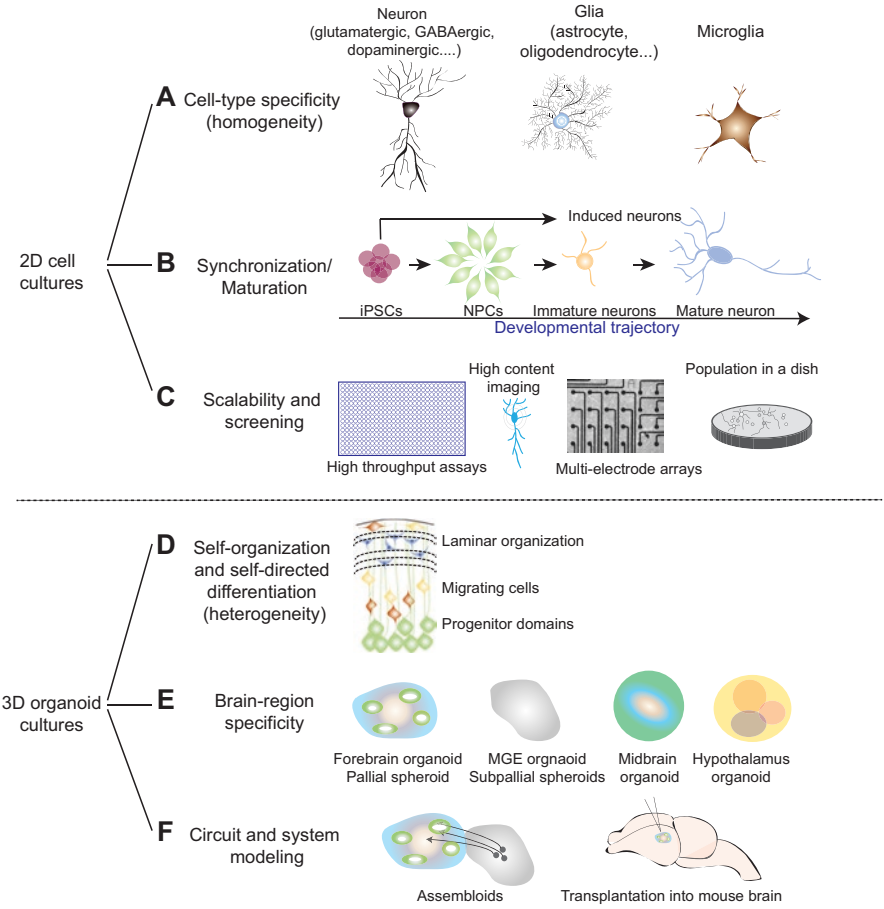

Fig. 1 2D and 3D cultures offer different advantages for modeling biological processes relevant to psychiatric disorders. 2D cell cultures allow for targeted differentiation of highly enriched populations of specific cell types (**a**) with efficient protocols and reproducible results; (**b**) cells can be timelocked during the differentiation process to perform assays in homogenous populations at specific developmental stages; (**c**) 2D cultures are amenable to scaling up to produce large quantities of cells and for high-throughput phenotypic assays; 3D organoid cultures allow for the self-directed fate specification of multiple cell types (**d**); (**e**) recapitulation of neural structures of specific brain regions; and (**f**) rudimentary modeling of neural circuits and axonal targeting

cell type is defined. For example, there are protocols that produce highly enriched populations of forebrain glutamatergic cortical neurons up to ~90% [19]. But this glutamatergic population is comprised of different subsets of cells expressing markers of individual cortical layers, which may have functionally different properties relevant to the disorder under investigation. Similarly, there are efficient protocols for generating GABAergic neurons, but protocols for generating a specific subtype of GABAergic cell, e.g. parvalbumin- or somatostatin-expressing

neurons, are less well developed [27]. Advances in single cell biology are also beginning to reveal that there may be even more granularity in the potential classification of cells that are identified and cross-validated by transcriptomic, morphological, and/or electrophysiological analyses [28, 29]. Further complicating the picture is the fact that there seems to be a dynamic component to neurotransmitter expression and a switching phenomenon that can occur in the developing and adult brain [30]. Despite these caveats, monolayer cell cultures are the most efficient method to produce large quantities of relatively pure populations of target neural cell types for phenotypic analysis.

3.1 Cell-Type-Specific Phenotypes

Using a 2D approach, there have been several key findings in the field of hiPSC-based modeling revealing robust cell-type-specific phenotypes associated with particular disorders that have both provided data to support existing hypotheses and generated new mechanistic hypotheses based on unexpected biological insights. For schizophrenia, early studies such as those described above largely focused on excitatory glutamatergic neurons and observed several overlapping phenotypes related to synaptic plasticity and development. In addition, consistent transcriptional dysregulation was observed in cortical excitatory neurons from multiple patient hiPSC lines with a mutation in *DISC1*, consistent with its role as a hub of a large protein interactome with hundreds of binding partners, including transcription factors [19, 31]. Interestingly, a recent study identified cell-type-specific DISC1 interactomes with enrichment for distinct biological processes in neural progenitor cells and astrocytes differentiated from control iPSCs [32]. Cell-type-specific phenotypes had also been probed and compared between excitatory and inhibitory neurons from idiopathic schizophrenia patient iPSCs. For instance, the protocadherin pathway was identified as a locus of dysregulated gene expression only in GABAergic interneurons, but not in glutamatergic neurons [33]. Dysregulation of this pathway resulted in deficits in dendritic arborization and synapse development of interneurons, a result that was further validated with grafted human interneurons in the mouse cortex as well as in postmortem brain tissue [33].

Targeting brain regions and cell types implicated in cognitive impairments common to psychiatric disorders has led investigators to also focus on the hippocampus. The hippocampus is a critical site for many forms of learning and memory and is comprised of three canonical subregions, CA1, CA3, and dentate gyrus, which form a trisynaptic loop, as well as afferent connections from the entorhinal cortex and efferent projections to other cortical and subcortical regions. Subregion-specific differentiation protocols have been developed for both dentate gyrus [20] and CA3 neurons [34], allowing for the identification of cell-type-specific phenotypes, as well as co-culture systems that can begin to model one of the synaptic connections within the trisynaptic circuit. Selective deficits in spontaneous and evoked activity were observed in the idiopathic

schizophrenia patient-derived CA3 cells [34], which also impacted synaptic activity in DG-CA3 co-cultures. In addition to finding a cell-type-specific deficit in this schizophrenia cohort, this study also demonstrates the possibility that some aspects of neural circuitry can be modeled in 2D cultures. Together, these studies reveal a context-dependent complexity of molecular and cellular phenotypes and we should be cautious when interpreting data from cell cultures with mixed neural cell populations.

3.2 Differentiation as a Phenotype

It is also important to recognize that aberrant differentiation itself may be a phenotype that could contribute to disease-relevant pathology. Brain imaging studies, for example, have reported a progressive reduction in both gray and white matter in schizophrenia patients [35, 36], suggesting that there may be increased neurodegeneration but also a deficit in the oligodendrocyte population contributing to the white matter volume. When comparing imaging results and hiPSC lines generated from six schizophrenia patients and age-matched healthy subjects, reduced white matter volume in vivo was correlated with less efficient differentiation of oligodendrocyte progenitors and overall fewer oligodendrocytes generated from the schizophrenia patient hiPSC lines [37]. This study demonstrates the value of targeted differentiation of a specific cell type to test a hypothesis based on patient observations. And this type of focused and hypothesis-driven investigation of a specific cell type is more tractable in homogeneous 2D cultures.

Cell-type-specific differentiation deficits have also been observed in hiPSC models for Timothy syndrome, a developmental disorder affecting heart, digit formation, and other physiological systems, as well as the nervous system, and is caused by mutations in *CACNA1c*. *CACNA1c* is a gene encoding the voltage-dependent cardiac L-type calcium channel and has been implicated as a risk factor for several psychiatric disorders, including schizophrenia and bipolar disorder. There is also a high co-morbidity of Timothy syndrome with ASD [38]. After differentiating Timothy syndrome hiPSCs toward a neural lineage, a differentiation phenotype was observed in the numbers of cortical neurons derived from the Timothy syndrome hiPSCs that expressed deep vs upper-layer cortical markers [39]. Specifically, there was an increase in cells expressing upper-layer markers, at the expense of cells expressing SATB2, a lower-layer marker that also identifies callosal projection neurons. This observation suggests that there may be a shift in the ratio of neurons projecting to subcortical vs callosal structures related to Timothy syndrome. Although this study was performed in 2D cell cultures, it may still provide clues as to potential structural changes in 3D brain architecture and indeed is consistent with several imaging studies of ASD patients showing reduced volume of corpus callosum but increased volume of frontal lobe cortical thickness [40].

3.3 Optimization of Differentiation Protocols in 2D

Efforts are ongoing to develop better differentiation protocols to generate highly enriched populations of neural cell types relevant to psychiatric and neurological disorders. Moving beyond the glutamatergic cortical neurons that were the focus of many of the early studies using patient hiPSCs, investigators are now targeting other neurotransmitter cell types and various brain regions that may contribute to specific symptomatology in patients. Targeted differentiation of dopaminergic neurons has been the focus of many groups due to the selective degeneration of this cell population in Parkinson's disease [41–43]. But it is well established that dopaminergic signaling is also altered in psychiatric disorders such as schizophrenia and addiction, and many antipsychotic drugs used in clinic target the dopamine system [44]. Thus, improved protocols to generate this type of neuron may shed light on the cellular phenotype and mechanisms underlying psychiatric disorders, as well as additional treatments. Likewise, there are focused attempts to optimize differentiation protocols to obtain relatively pure populations of GABA-expressing cortical neurons that arise from the medial ganglionic eminence in the ventral forebrain, a neural cell type affected in many disorders [45, 46]. In general, differentiation from hiPSC cultures follows the same relative timeline during human fetal brain development and GABAergic cells can take many weeks or months to differentiate in culture. Moreover, the efficiency is still relatively low for some of the latest appearing interneuron subtypes, such as parvalbumin-expressing neurons, a subtype that has been associated with excitatory–inhibitory balance and several psychiatric disorders including schizophrenia, bipolar disorder, and ASD [47, 48]. Other cell types that are currently being targeted for directed differentiation include hypothalamic neuropeptidergic neurons [49–51], cholinergic neurons [52], and various glial cell types [53]. With advanced differentiation protocols, hiPSC-based disease cellular models will provide a powerful system to characterize cell autonomous effects as well as cell-type-specific molecular and cellular changes that might contribute to disease pathology, to identify cell-type-specific biomarkers, and to specify the targets of drug action. Moreover, it is also possible to model simple neural networks when multiple neural cell types and glial cells are co-cultured.

4 Scalability, Screening, and Homogeneity

Another distinct advantage of iPSC-based 2D cell culture systems is the ability to scale up and generate large populations of neurons of a specific cellular age that can be grown in a higher-throughput format (Fig. 1c). This is advantageous for both diagnostic screening to identify phenotypes among several iPSC lines as well as for hypothesis-driven or unbiased screening of large compound libraries to rescue established phenotypes for drug discovery.

With respect to standardizing the culture conditions and cells, a major advantage of 2D cell cultures for large-scale screening is the fact that it is easier to synchronize cells during proliferation, differentiation, and postmitotic maturation. Antimitotic agents can be applied to block cell division and eliminate progenitor cells before reseeding to establish a postmitotic population for further differentiation. This ability to initiate a timestamped differentiation process and expose all cells in a monolayer to culture conditions equally allows for targeted investigations of specific stages of neuronal development at the population level (Fig. 1b). For example, one critical stage of neuronal development involves a change in the ratio of KCC2 (chloride exporter) to NKCC1 (chloride importer), and thus intracellular chloride levels, which mediates a polarity switch in GABAergic signaling from hyperpolarizing to depolarizing. In a Rett syndrome study, neurons differentiated from hiPSCs with an *MECP2* mutation exhibited a deficit in the expression of KCC2 [54]. As a result of the sustained low levels of KCC2 expression in these *MECP2* mutant neurons, GABA remained hyperpolarizing in these cells, reflecting aberrant neuronal maturation and a potential locus for widespread dysfunction caused by an excess of depolarizing GABA during an early critical period of development. Observation of this particular phenotype is greatly facilitated in 2D cultures in which the entire population of cells should be maturing at approximately the same time, making it easier to identify aberrations in developmentally and temporally regulated processes.

Once a clear and reproducible cellular phenotype has been discovered, unbiased screens to rescue the phenotype can be performed and effective hits could provide clues to the underlying biology and mechanisms. As a proof of principle for high-throughput drug screening on cellular phenotypes, a recent study using high-content imaging identified 108 compounds out of a library of over 4000 that modulate neurite growth [55]. Alternatively, screens can be performed to identify compounds that can modulate the expression of the target gene or a specific pathway linked to diseases. For example, among prominent risk genes associated with ASD, *SHANK3* has received attention because it encodes a scaffold protein that is highly expressed at the postsynaptic density and is also one of the genes affected by 22q13.3, or Phelan-McDermid deletion syndrome. One study employed a two-step approach to first identify compounds that increased SHANK3 expression in neurons differentiated from embryonic stem cells, which were then functionally validated in neurons derived from patient-specific hiPSCs with a SHANK3 haploinsufficiency [56]. This study highlights the potential utility of disease-relevant cellular models for developing specific disease-modifying treatments. A high-throughput screen has also been carried out to identify compounds that would increase FMRP expression in FXS patient cells, potentially by reversing the epigenetic silencing of the *FMR1* gene [57]. Using a fluorescence resonance energy transfer assay, several compounds, out of a library of approximately 5000, were identified that led to modest increases in FMR1 mRNA. While the increase was below the threshold of clinical relevance, the study demonstrated how unbiased screens may be useful to identify novel modulators of target pathways.

5 3D Models and Neural Architecture

Although the 2D cellular models described above have led to exciting breakthroughs and novel biological insight, there are clear limitations of this approach in modeling the cellular heterogeneity, neural architecture, and interconnected networks that are likely to be critical for understanding the mechanistic bases of psychiatric disorders. Several years after the initial study showing that adult somatic cells could be reprogrammed to pluripotency, laying the foundation for hiPSC-based disease modeling, a pioneering study published new methodology for generating complex 3D structures from self-organizing hiPSCs grown in a spinning bioreactor [58]. This initial report showed that this specialized culture condition led to the formation of 3D organoids that acquired structural properties reminiscent of different brain regions and related tissues. Using a limited differentiation protocol, these early organoids were allowed to follow intrinsic and stochastic patterning signals to generate disparate cell types and heterogeneous structures within a single organoid. Self-organization is a hallmark property of organoids and confirms the importance of cell–cell interactions in modulating the intrinsic capacities of cells to adopt particular fates and migration patterns (Fig. 1d). This exciting new technology development showed the promise of generating diverse cell types of neural tissue but it was difficult to predict which structural arrangements would emerge in each experiment. Whereas 2D cell culture approaches typically emphasize homogeneity allowing for targeted investigations of a single cell type, 3D organoid approaches promote cellular diversity and heterogeneity, as a function of the self-directed fate specification of progenitors and their progeny within a developing system. This reliance on self-organization introduces variability not only across hiPSC lines but also among organoids from the same line grown under the same conditions. Mechanistic studies, however, benefit from reproducibility and organoids present a unique challenge to ensure unbiased selection of sample regions for quantification and sufficient consistency to identify robust phenotypes and perform drug screens.

Since the initial reports showing highly heterogenous compound tissue organoids, many research groups have made concerted efforts to guide organoid differentiation toward specific brain regions of interest for use as models to investigate specific brain disorders [59, 60] (Fig. 1e). Among the first brain-region-specific organoids to be extensively characterized was a model of the developing neocortex [61]. At the time, it proved to be an optimal model to investigate microcephaly, which was the focus of global health crisis related to the reemergent Zika virus. Several other brain-region-specific models have since emerged including models of the cerebellum [62], hippocampus [63], midbrain [60, 64, 65], motor nerves with associated axon fascicles [66] and even assembloids, fusion of two brain-region-specific organoids [67, 68].

In addition to brain region specificity, organoids are also better suited to model some of the gross structural abnormalities associated with various psychiatric disorders, such as changes in brain volume observed in imaging studies of patients. For example, some of the reciprocal chromosomal rearrangements in CNVs in which

both microdeletions and duplications have been associated with increased risk for psychiatric disorders have also been associated with reciprocal differences in brain volume. For CNVs such as 22q11.2, 15q11.2, and 16p11.2, an inverse correlation between gene dosage and brain volume in select brain regions has been observed [14, 69, 70]. Taking advantage of the organoid model, a recent study tested a specific hypothesis regarding brain volume abnormalities focused on the 16p13.11 microduplication CNV [71]. In a small group of patients with the 16p13.11 CNV, magnetic resonance imaging revealed a decrease in cortical volume. Strikingly, correlated to the imaging studies, smaller overall volumes were observed in cerebral organoids generated from those patients' hiPSCs, compared to those from unaffected family control hiPSCs. In addition, aberrant orientation of radial glial progenitors and deficits in neural progenitor cell proliferation were observed. After identifying dysregulation of the NFκB p65 pathway through transcriptomic analysis, pharmacological and genetic rescue targeting this pathway was able to ameliorate the proliferation phenotype. Therefore, well-designed experiments using the right approach to investigate specific phenotypes observed in patients, in this case using organoids to evaluate structural deficits, can lead to new biological insight and targets for drug discovery even from a small cohort of patients.

6 Spatiotemporal Influences on Epigenetics and Epitranscriptomics

In addition to the risk-associated genetic variants that may be associated with the etiopathology of various psychiatric disorders, we are just beginning to understand the degree to which gene expression can be dynamically modulated by environmental factors, epigenetic regulation, gene–gene interactions, and chromosomal architecture, as well as post-transcriptional [72] and post-translational modifications. All of these factors interact to further define the dynamic landscape of protein expression in a brain region and cell-type-specific manner and can influence susceptibility to psychiatric disorders. Brain-region-specific epigenetic profiles have been associated with psychiatric disorders and trait heritability [73], and cell-type-specific chromosomal conformations, for example, have been shown to be associated with schizophrenia risk loci in differentiating progenitor cells [74]. This complexity makes it extremely challenging to identify individual risk profiles and dissect the relative contributions of each component to the emergence of pathology and symptomatology. Brain organoids offer a more holistic approach to modeling the formation of neural systems and provide a platform to interrogate non-cell autonomous influences on the molecular dynamics of developmental processes. In comparison to fetal brain tissue at different gestational ages, brain-region-specific organoids can recapitulate transcriptional [61] and epigenetic signatures of fetal brain development [75], allowing for targeted investigations of early critical windows for long-lasting changes in gene expression.

7 Neural Systems as a Therapeutic Target

It has long been appreciated that the specific patterns of connectivity among neurons are fundamental to brain function. Indeed, several symptomatic domains are thought to involve anatomically distributed neural systems, such as the cortico-striatal-thalamic loops that are implicated in major depressive disorder, obsessive compulsive disorder, and substance abuse disorders [76]. Delineating relevant pathology in terms of neural circuits may be a better approach to understanding the cell and region-specific biological processes that play a causal role in psychiatric disorders, but these systems are more difficult to model in vivo due to potential species-specific differences and are also difficult to address therapeutically. Most pharmaceutical interventions will have off-target effects arising from the impact of drugs on functionally distinct systems that share a common molecular substrate. We need better strategies to target specific brain areas or to define a neural system in a way that distinguishes it from other circuitry. Although still in the early stages of protocol development, 3D cultures hold the promise of being able to dissect circuitry that may facilitate the development of rationally designed circuit-specific therapeutics.

However, we need to first be able to model components of neural circuitry, including accurate targeting of afferent and efferent projections of appropriate cell types. A major step in this direction are the recent studies showing that organoids, or neural spheroids, can be patterned to either a dorsal forebrain (pallium) or ventral forebrain (subpallium) identity and then assembled together to model the environment during fetal brain development when interneurons from the subpallium migrate and integrate into cortical circuits in the pallium [67, 68, 77]. These "assembloids" can approximate early migration events during fetal development and potentially reveal new phenotypes that would not be observable when analyzing region-specific organoids alone (Fig. 1f). Future studies may demonstrate the feasibility of fusing more mature brain regions together to model circuitry in the adult brain.

An alternative strategy to evaluate targeting and integration of hiPSC-derived neural cell types with appropriate presynaptic and postsynaptic partners is to transplant organoids to animal models to assess long-term survival, maturation, and integration. hiPSC-derived cerebral organoids transplanted to a cortical region in the mouse brain can survive for several months and show signs of successful integration into the local circuitry, including extensive axonal growth and evidence of presynaptic targeting of synapses on neurons in the host brain [78]. Importantly, these organoids became vascularized within the host brain, allowing circulating molecular signals to reach to the inner core of the organoid, which may promote long-term survival and maturation.

One challenge in the capacity of organoids to model relevant properties of neural systems is the inclusion of all relevant cell types. Following the same temporal sequence of cell type differentiation in human brain development, glial cells and inhibitory interneurons also emerge later in the developmental trajectory of hiPSC-

derived brain organoids. To achieve a more representative distribution of cell types that exist in the mature brain, organoids must be cultured for longer periods of time, which is still a challenge. Efforts to encourage differentiation of astrocytes and oligodendrocytes in 3D cultures have been promising [79, 80]. As opposed to cell types that may arise from the same progenitor population and emerge at different timepoints, other critical populations may arise from a different embryonic origin and need to be co-cultured together with the organoids. Among these populations, microglia have garnered significant attention because these cells are the resident macrophages of the central nervous system. As resident immune cells, microglia are essential to model features of the neuroimmune axis that are not only important for infectious disease but are increasingly appreciated for their role in mediating long-term effects of stress and other factors that may contribute to psychiatric disorders [81]. Several groups have developed differentiation protocols for hiPSC-derived macrophages and microglia, which can be co-cultured in both 2D and 3D platforms [82] to better model the intact physiological environment.

8 Challenges and Future Directions

A theme that is emerging from the application of new technologies such as single cell biology and machine learning is that as we increase both the size of our datasets and the resolution of our analytical methods, some of the traditional categorical distinctions we have relied on for classification may be more fluid than previously thought. This can impact our understanding and investigation of psychiatric disorders at multiple levels. At the clinical level, there has been a growing movement to reconsider clinical diagnostic categories. In 2009, the NIMH proposed a new framework for the investigation of mental disorders based on Research Domain Criteria (RDoC). Currently, there are six recognized domains of human function that cover cognitive, social, and sensorimotor behaviors as well as affective and motivational states. The idea behind these domains is to provide another mode of interpretation for the constellation of symptoms exhibited by a patient, not to supplant the traditional diagnostic criteria. Although there are ongoing debates among clinicians about how and whether to implement this approach in the clinic [83], the RDoC framework can be informative in the design of iPSC studies during patient and cohort selection, or in trying to stratify and compare results across studies. At the cellular level, there is growing evidence that many of the bedrock criteria traditionally used to determine cell identity may be insufficient to distinguish among functionally discrete populations. Because organoids rely, at least in part, on self-organization to generate multiple constitutive cell types for a specific brain region, this approach can be less subject to bias about the importance of particular cell types. Organoids may be particularly useful for the identification and classification of human neural cell types based on single cell sequencing, morphology, and electrophysiological analyses in a more physiologically relevant context [84].

Defining, and achieving, cellular maturity is another challenge for the field. An important question is the extent to which organoids that recapitulate early brain developmental processes can also represent properties of a fully mature brain. For example, some organoid models have been shown to highly correlate with human fetal brain development at the structural, transcriptional, and epigenetic levels through the second trimester. These models may be able to capture dysregulation of early developmental processes that lead to the aberrant formation of neuronal networks, which could effectively form a neural substrate for increased susceptibility to psychiatric disorders. Indeed, there is an increasing appreciation for the idea that there is a developmental component for the majority of psychiatric disorders, which makes a developmental model highly relevant, even for later onset disorders. To identify effective therapies for adults, though, it is important to ask whether these approaches can also model features of the adult brain. Studies using treatment responsivity to stratify patient selection and identify cellular phenotypes suggest that 2D platforms may be an effective tool to generate populations of cells that can be used in diagnostic and therapeutic screening for adult patients. For organoids, although the global structure and transcriptional profiles mirror early brain development, this stage of development is also a period of heightened synaptogenesis and dendritic remodeling, which remain as core features of synaptic plasticity throughout life. As a model of disease-relevant biological processes, the 3D system can provide mechanistic insight into dysregulated plasticity that can affect neural function in the adult brain.

For both 2D and 3D hiPSC models of psychiatric disorders, it will become increasingly important to model the effects of environmental factors to approximate gene–environment interactions. Perturbagens may reveal risk gene-associated deficits in intracellular pathways in response to stress or inflexibility at the cellular or network level. Environmental factors may include exogenous toxins, drugs of abuse, or aberrant levels of endogenous hormones or neuromodulators. In addition to identifying biological processes that are affected at the cellular level by risk gene variants, there may be additional phenotypes that emerge in the presence of both a risk-associated mutation and exposure to an environmental stressor. And conversely, there may be some hiPSC lines that are not affected by the exogenous stressors, suggesting a resilience that could be investigated mechanistically. Finally, future studies should consider the possibility of sex differences when selecting a patient cohort and analyzing the data. Thus far, most studies have not reported any differences in cellular phenotypes in neural cells derived from male or female hiPSC lines, but sample sizes are typically small and some phenotypes may require a larger cohort size to detect. And given the literature on sexually dimorphic circuitry underlying different disorders and symptomatic domains, which can be further modulated by stress [85, 86], differences may be more likely to emerge at the structural level, which could be easier to detect in 3D cultures.

In sum, 2D and 3D cellular platforms from patient hiPSCs can provide complementary, and potentially confirmative, data to study psychiatric disorders. 2D cultures are advantageous when investigating a known specific cell type, scaling up for higher-throughput phenotypic or drug screens and for generating more mature neu-

rons. 3D organoids, on the other hand, facilitate the investigation of specific brain regions over single cell types and can model features of neural structure, neuronal migration, and axonal targeting and interactions between different brain regions, to some extent. Both platforms are valuable and can be used at different stages of the investigation depending on the current state of knowledge about the biology of the disorder, the particular cohort being studied, and whether specific hypotheses are being tested or a more unbiased approach to discovery is warranted.

References

1. Takahashi, K., Tanabe, K., Ohnuki, M., Narita, M., Ichisaka, T., Tomoda, K., et al. (2007). Induction of pluripotent stem cells from adult human fibroblasts by defined factors. *Cell, 131*(5), 861–872.
2. Marchetto, M. C., Carromeu, C., Acab, A., Yu, D., Yeo, G. W., Mu, Y., et al. (2010). A model for neural development and treatment of Rett syndrome using human induced pluripotent stem cells. *Cell, 143*(4), 527–539.
3. Sheridan, S. D., Theriault, K. M., Reis, S. A., Zhou, F., Madison, J. M., Daheron, L., et al. (2011). Epigenetic characterization of the FMR1 gene and aberrant neurodevelopment in human induced pluripotent stem cell models of fragile X syndrome. *PLoS One, 6*(10), e26203.
4. Barakat, T. S., Ghazvini, M., de Hoon, B., Li, T., Eussen, B., Douben, H., et al. (2015). Stable X chromosome reactivation in female human induced pluripotent stem cells. *Stem Cell Reports, 4*(2), 199–208.
5. Tchieu, J., Kuoy, E., Chin, M. H., Trinh, H., Patterson, M., Sherman, S. P., et al. (2010). Female human iPSCs retain an inactive X chromosome. *Cell Stem Cell, 7*(3), 329–342.
6. Doers, M. E., Musser, M. T., Nichol, R., Berndt, E. R., Baker, M., Gomez, T. M., et al. (2014). iPSC-derived forebrain neurons from FXS individuals show defects in initial neurite outgrowth. *Stem Cells and Development, 23*(15), 1777–1787.
7. Telias, M., Kuznitsov-Yanovsky, L., Segal, M., & Ben-Yosef, D. (2015). Functional deficiencies in fragile X neurons derived from human embryonic stem cells. *The Journal of Neuroscience, 35*(46), 15295–15306.
8. Telias, M., Segal, M., & Ben-Yosef, D. (2013). Neural differentiation of Fragile X human embryonic stem cells reveals abnormal patterns of development despite successful neurogenesis. *Developmental Biology, 374*(1), 32–45.
9. Telias, M., Segal, M., & Ben-Yosef, D. (2016). Immature responses to GABA in fragile X neurons derived from human embryonic stem cells. *Frontiers in Cellular Neuroscience, 10*, 121.
10. Zhang, Z., Marro, S. G., Zhang, Y., Arendt, K. L., Patzke, C., Zhou, B., et al. (2018). The fragile X mutation impairs homeostatic plasticity in human neurons by blocking synaptic retinoic acid signaling. *Science Translational Medicine, 10*, 452.
11. Sullivan, P. F., Agrawal, A., Bulik, C. M., Andreassen, O. A., Borglum, A. D., Breen, G., et al. (2018). Psychiatric genomics: An update and an agenda. *The American Journal of Psychiatry, 175*(1), 15–27.
12. Bergen, S. E., Ploner, A., Howrigan, D., O'Donovan, M. C., Group CNVA, The Schizophrenia Working Group of the Psychiatric Genomics C, et al. (2019). Joint contributions of rare copy number variants and common SNPs to risk for schizophrenia. *The American Journal of Psychiatry, 176*(1), 29–35.
13. Malhotra, D., & Sebat, J. (2012). CNVs: Harbingers of a rare variant revolution in psychiatric genetics. *Cell, 148*(6), 1223–1241.

14. Lin, A., Ching, C. R. K., Vajdi, A., Sun, D., Jonas, R. K., Jalbrzikowski, M., et al. (2017). Mapping 22q11.2 gene dosage effects on brain morphometry. *The Journal of Neuroscience, 37*(26), 6183–6199.
15. Crespi, B. J., & Crofts, H. J. (2012). Association testing of copy number variants in schizophrenia and autism spectrum disorders. *Journal of Neurodevelopmental Disorders, 4*(1), 15.
16. Brennand, K. J., Simone, A., Jou, J., Gelboin-Burkhart, C., Tran, N., Sangar, S., et al. (2011). Modelling schizophrenia using human induced pluripotent stem cells. *Nature, 473*(7346), 221–225.
17. Chiang, C. H., Su, Y., Wen, Z., Yoritomo, N., Ross, C. A., Margolis, R. L., et al. (2011). Integration-free induced pluripotent stem cells derived from schizophrenia patients with a DISC1 mutation. *Molecular Psychiatry, 16*(4), 358–360.
18. Sachs, N. A., Sawa, A., Holmes, S. E., Ross, C. A., DeLisi, L. E., & Margolis, R. L. (2005). A frameshift mutation in disrupted in schizophrenia 1 in an American family with schizophrenia and schizoaffective disorder. *Molecular Psychiatry, 10*(8), 758–764.
19. Wen, Z., Nguyen, H. N., Guo, Z., Lalli, M. A., Wang, X., Su, Y., et al. (2014). Synaptic dysregulation in a human iPS cell model of mental disorders. *Nature, 515*(7527), 414–418.
20. Yu, D. X., Di Giorgio, F. P., Yao, J., Marchetto, M. C., Brennand, K., Wright, R., et al. (2014). Modeling hippocampal neurogenesis using human pluripotent stem cells. *Stem Cell Reports, 2*(3), 295–310.
21. Yoon, K. J., Nguyen, H. N., Ursini, G., Zhang, F., Kim, N. S., Wen, Z., et al. (2014). Modeling a genetic risk for schizophrenia in iPSCs and mice reveals neural stem cell deficits associated with adherens junctions and polarity. *Cell Stem Cell, 15*(1), 79–91.
22. Brugger, S. P., & Howes, O. D. (2017). Heterogeneity and homogeneity of regional brain structure in schizophrenia: A meta-analysis. *JAMA Psychiatry, 74*(11), 1104–1111.
23. Stoner, R., Chow, M. L., Boyle, M. P., Sunkin, S. M., Mouton, P. R., Roy, S., et al. (2014). Patches of disorganization in the neocortex of children with autism. *The New England Journal of Medicine, 370*(13), 1209–1219.
24. Mertens, J., Wang, Q. W., Kim, Y., Yu, D. X., Pham, S., Yang, B., et al. (2015). Differential responses to lithium in hyperexcitable neurons from patients with bipolar disorder. *Nature, 527*(7576), 95–99.
25. Vadodaria, K. C., Ji, Y., Skime, M., Paquola, A., Nelson, T., Hall-Flavin, D., et al. (2019). Serotonin-induced hyperactivity in SSRI-resistant major depressive disorder patient-derived neurons. *Molecular Psychiatry, 24*(6), 795–807.
26. Mertens, J., Marchetto, M. C., Bardy, C., & Gage, F. H. (2016). Evaluating cell reprogramming, differentiation and conversion technologies in neuroscience. *Nature Reviews. Neuroscience, 17*(7), 424–437.
27. Soliman, M. A., Aboharb, F., Zeltner, N., & Studer, L. (2017). Pluripotent stem cells in neuropsychiatric disorders. *Molecular Psychiatry, 22*(9), 1241–1249.
28. Tasic, B., Menon, V., Nguyen, T. N., Kim, T. K., Jarsky, T., Yao, Z., et al. (2016). Adult mouse cortical cell taxonomy revealed by single cell transcriptomics. *Nature Neuroscience, 19*(2), 335–346.
29. Holguera, I., & Desplan, C. (2018). Neuronal specification in space and time. *Science, 362*(6411), 176–180.
30. Spitzer, N. C. (2017). Neurotransmitter switching in the developing and adult brain. *Annual Review of Neuroscience, 40*, 1–19.
31. Porteous, D. J., Millar, J. K., Brandon, N. J., & Sawa, A. (2011). DISC1 at 10: Connecting psychiatric genetics and neuroscience. *Trends in Molecular Medicine, 17*(12), 699–706.
32. Wilkinson, B., Evgrafov, O. V., Zheng, D., Hartel, N., Knowles, J. A., Graham, N. A., et al. (2019). Endogenous cell type-specific disrupted in schizophrenia 1 interactomes reveal protein networks associated with neurodevelopmental disorders. *Biological Psychiatry, 85*(4), 305–316.

33. Shao, Z., Noh, H., Bin Kim, W., Ni, P., Nguyen, C., Cote, S. E., et al. (2019). Dysregulated protocadherin-pathway activity as an intrinsic defect in induced pluripotent stem cell-derived cortical interneurons from subjects with schizophrenia. *Nature Neuroscience, 22*(2), 229–242.
34. Sarkar, A., Mei, A., Paquola, A. C. M., Stern, S., Bardy, C., Klug, J. R., et al. (2018). Efficient generation of CA3 neurons from human pluripotent stem cells enables modeling of hippocampal connectivity in vitro. *Cell Stem Cell, 22*(5), 684–697.
35. Cannon, T. D., Chung, Y., He, G., Sun, D., Jacobson, A., van Erp, T. G., et al. (2015). Progressive reduction in cortical thickness as psychosis develops: A multisite longitudinal neuroimaging study of youth at elevated clinical risk. *Biological Psychiatry, 77*(2), 147–157.
36. Cropley, V. L., Klauser, P., Lenroot, R. K., Bruggemann, J., Sundram, S., Bousman, C., et al. (2017). Accelerated gray and white matter deterioration with age in schizophrenia. *The American Journal of Psychiatry, 174*(3), 286–295.
37. McPhie, D. L., Nehme, R., Ravichandran, C., Babb, S. M., Ghosh, S. D., Staskus, A., et al. (2018). Oligodendrocyte differentiation of induced pluripotent stem cells derived from subjects with schizophrenias implicate abnormalities in development. *Translational Psychiatry, 8*(1), 230.
38. Freitas, B. C., Trujillo, C. A., Carromeu, C., Yusupova, M., Herai, R. H., & Muotri, A. R. (2014). Stem cells and modeling of autism spectrum disorders. *Experimental Neurology, 260*, 33–43.
39. Pasca, S. P., Portmann, T., Voineagu, I., Yazawa, M., Shcheglovitov, A., Pasca, A. M., et al. (2011). Using iPSC-derived neurons to uncover cellular phenotypes associated with Timothy syndrome. *Nature Medicine, 17*(12), 1657–1662.
40. Pagnozzi, A. M., Conti, E., Calderoni, S., Fripp, J., & Rose, S. E. (2018). A systematic review of structural MRI biomarkers in autism spectrum disorder: A machine learning perspective. *International Journal of Developmental Neuroscience, 71*, 68–82.
41. Xue, Y., Zhan, X., Sun, S., Karuppagounder, S. S., Xia, S., Dawson, V. L., et al. (2019). Synthetic mRNAs drive highly efficient iPS cell differentiation to dopaminergic neurons. *Stem Cells Translational Medicine, 8*(2), 112–123.
42. Cobb, M. M., Ravisankar, A., Skibinski, G., & Finkbeiner, S. (2018). iPS cells in the study of PD molecular pathogenesis. *Cell and Tissue Research, 373*(1), 61–77.
43. Yan, Y., Yang, D., Zarnowska, E. D., Du, Z., Werbel, B., Valliere, C., et al. (2005). Directed differentiation of dopaminergic neuronal subtypes from human embryonic stem cells. *Stem Cells, 23*(6), 781–790.
44. McCutcheon, R. A., Abi-Dargham, A., & Howes, O. D. (2019). Schizophrenia, dopamine and the striatum: from biology to symptoms. *Trends in Neurosciences, 42*(3), 205–220.
45. Tao, Y., & Zhang, S. C. (2016). Neural subtype specification from human pluripotent stem cells. *Cell Stem Cell, 19*(5), 573–586.
46. Liu, Y., Liu, H., Sauvey, C., Yao, L., Zarnowska, E. D., & Zhang, S. C. (2013). Directed differentiation of forebrain GABA interneurons from human pluripotent stem cells. *Nature Protocols, 8*(9), 1670–1679.
47. Ferguson, B. R., & Gao, W. J. (2018). PV interneurons: Critical regulators of E/I balance for prefrontal cortex-dependent behavior and psychiatric disorders. *Frontiers in Neural Circuits, 12*, 37.
48. Volk, D. W., Sampson, A. R., Zhang, Y., Edelson, J. R., & Lewis, D. A. (2016). Cortical GABA markers identify a molecular subtype of psychotic and bipolar disorders. *Psychological Medicine, 46*(12), 2501–2512.
49. Wang, L., Meece, K., Williams, D. J., Lo, K. A., Zimmer, M., Heinrich, G., et al. (2015). Differentiation of hypothalamic-like neurons from human pluripotent stem cells. *The Journal of Clinical Investigation, 125*(2), 796–808.
50. Wang, L., Egli, D., & Leibel, R. L. (2016). Efficient generation of hypothalamic neurons from human pluripotent stem cells. *Current Protocols in Human Genetics, 90*, 21.

51. Merkle, F. T., Maroof, A., Wataya, T., Sasai, Y., Studer, L., Eggan, K., et al. (2015). Generation of neuropeptidergic hypothalamic neurons from human pluripotent stem cells. *Development, 142*(4), 633–643.
52. Hu, Y., Qu, Z. Y., Cao, S. Y., Li, Q., Ma, L., Krencik, R., et al. (2016). Directed differentiation of basal forebrain cholinergic neurons from human pluripotent stem cells. *Journal of Neuroscience Methods, 266*, 42–49.
53. Zheng, W., Li, Q., Zhao, C., Da, Y., Zhang, H. L., & Chen, Z. (2018). Differentiation of glial cells from hiPSCs: Potential applications in neurological diseases and cell replacement therapy. *Frontiers in Cellular Neuroscience, 12*, 239.
54. Tang, X., Kim, J., Zhou, L., Wengert, E., Zhang, L., Wu, Z., et al. (2016). KCC2 rescues functional deficits in human neurons derived from patients with Rett syndrome. *Proceedings of the National Academy of Sciences of the United States of America, 113*(3), 751–756.
55. Sherman, S. P., & Bang, A. G. (2018). High-throughput screen for compounds that modulate neurite growth of human induced pluripotent stem cell-derived neurons. *Disease Models & Mechanisms, 11*, 2.
56. Darville, H., Poulet, A., Rodet-Amsellem, F., Chatrousse, L., Pernelle, J., Boissart, C., et al. (2016). Human pluripotent stem cell-derived cortical neurons for high throughput medication screening in autism: A proof of concept study in SHANK3 haploinsufficiency syndrome. *eBioMedicine, 9*, 293–305.
57. Kumari, D., Swaroop, M., Southall, N., Huang, W., Zheng, W., & Usdin, K. (2015). High-throughput screening to identify compounds that increase fragile X mental retardation protein expression in neural stem cells differentiated from fragile X syndrome patient-derived induced pluripotent stem cells. *Stem Cells Translational Medicine, 4*(7), 800–808.
58. Lancaster, M. A., Renner, M., Martin, C. A., Wenzel, D., Bicknell, L. S., Hurles, M. E., et al. (2013). Cerebral organoids model human brain development and microcephaly. *Nature, 501*(7467), 373–379.
59. Amin, N. D., & Pasca, S. P. (2018). Building models of brain disorders with three-dimensional organoids. *Neuron, 100*(2), 389–405.
60. Qian, X., Jacob, F., Song, M. M., Nguyen, H. N., Song, H., & Ming, G. L. (2018). Generation of human brain region-specific organoids using a miniaturized spinning bioreactor. *Nature Protocols, 13*(3), 565–580.
61. Qian, X., Nguyen, H. N., Song, M. M., Hadiono, C., Ogden, S. C., Hammack, C., et al. (2016). Brain-region-specific organoids using mini-bioreactors for modeling ZIKV exposure. *Cell, 165*(5), 1238–1254.
62. Muguruma, K., Nishiyama, A., Kawakami, H., Hashimoto, K., & Sasai, Y. (2015). Self-organization of polarized cerebellar tissue in 3D culture of human pluripotent stem cells. *Cell Reports, 10*(4), 537–550.
63. Sakaguchi, H., Kadoshima, T., Soen, M., Narii, N., Ishida, Y., Ohgushi, M., et al. (2015). Generation of functional hippocampal neurons from self-organizing human embryonic stem cell-derived dorsomedial telencephalic tissue. *Nature Communications, 6*, 8896.
64. Jo, J., Xiao, Y., Sun, A. X., Cukuroglu, E., Tran, H. D., Goke, J., et al. (2016). Midbrain-like organoids from human pluripotent stem cells contain functional dopaminergic and neuromelanin-producing neurons. *Cell Stem Cell, 19*(2), 248–257.
65. Monzel, A. S., Smits, L. M., Hemmer, K., Hachi, S., Moreno, E. L., van Wuellen, T., et al. (2017). Derivation of human midbrain-specific organoids from neuroepithelial stem cells. *Stem Cell Reports, 8*(5), 1144–1154.
66. Kawada, J., Kaneda, S., Kirihara, T., Maroof, A., Levi, T., Eggan, K., et al. (2017). Generation of a motor nerve organoid with human stem cell-derived neurons. *Stem Cell Reports, 9*(5), 1441–1449.
67. Sloan, S. A., Andersen, J., Pasca, A. M., Birey, F., & Pasca, S. P. (2018). Generation and assembly of human brain region-specific three-dimensional cultures. *Nature Protocols, 13*(9), 2062–2085.

68. Bagley, J. A., Reumann, D., Bian, S., Levi-Strauss, J., & Knoblich, J. A. (2017). Fused cerebral organoids model interactions between brain regions. *Nature Methods, 14*(7), 743–751.
69. Niarchou, M., Chawner, S., Doherty, J. L., Maillard, A. M., Jacquemont, S., Chung, W. K., et al. (2019). Psychiatric disorders in children with 16p11.2 deletion and duplication. *Translational Psychiatry, 9*(1), 8.
70. Sonderby, I. E., Gustafsson, O., Doan, N. T., Hibar, D. P., Martin-Brevet, S., Abdellaoui, A., et al. (2018). Dose response of the 16p11.2 distal copy number variant on intracranial volume and basal ganglia. *Molecular Psychiatry, 25*(3), 584–602.
71. Johnstone, M., Vasistha, N. A., Barbu, M. C., Dando, O., Burr, K., Christopher, E., et al. (2019). Reversal of proliferation deficits caused by chromosome 16p13.11 microduplication through targeting NFkappaB signaling: an integrated study of patient-derived neuronal precursor cells, cerebral organoids and in vivo brain imaging. *Molecular Psychiatry, 24*(2), 294–311.
72. Kadumuri, R. V., & Janga, S. C. (2018). Epitranscriptomic code and its alterations in human disease. *Trends in Molecular Medicine, 24*(10), 886–903.
73. Rizzardi, L. F., Hickey, P. F., Rodriguez DiBlasi, V., Tryggvadottir, R., Callahan, C. M., Idrizi, A., et al. (2019). Neuronal brain-region-specific DNA methylation and chromatin accessibility are associated with neuropsychiatric trait heritability. *Nature Neuroscience, 22*(2), 307–316.
74. Rajarajan, P., Borrman, T., Liao, W., Schrode, N., Flaherty, E., Casino, C., et al. (2018). Neuron-specific signatures in the chromosomal connectome associated with schizophrenia risk. *Science, 362*, 6420.
75. Luo, C., Lancaster, M. A., Castanon, R., Nery, J. R., Knoblich, J. A., & Ecker, J. R. (2016). Cerebral organoids recapitulate epigenomic signatures of the human fetal brain. *Cell Reports, 17*(12), 3369–3384.
76. Fettes, P., Schulze, L., & Downar, J. (2017). Cortico-striatal-thalamic loop circuits of the orbitofrontal cortex: Promising therapeutic targets in psychiatric illness. *Frontiers in Systems Neuroscience, 11*, 25.
77. Birey, F., Andersen, J., Makinson, C. D., Islam, S., Wei, W., Huber, N., et al. (2017). Assembly of functionally integrated human forebrain spheroids. *Nature, 545*(7652), 54–59.
78. Mansour, A. A., Goncalves, J. T., Bloyd, C. W., Li, H., Fernandes, S., Quang, D., et al. (2018). An in vivo model of functional and vascularized human brain organoids. *Nature Biotechnology, 36*(5), 432–441.
79. Sloan, S. A., Darmanis, S., Huber, N., Khan, T. A., Birey, F., Caneda, C., et al. (2017). Human astrocyte maturation captured in 3D cerebral cortical spheroids derived from pluripotent stem cells. *Neuron, 95*(4), 779–790.
80. Marton, R. M., Miura, Y., Sloan, S. A., Li, Q., Revah, O., Levy, R. J., et al. (2019). Differentiation and maturation of oligodendrocytes in human three-dimensional neural cultures. *Nature Neuroscience, 22*(3), 484–491.
81. Salam, A. P., Borsini, A., & Zunszain, P. A. (2018). Trained innate immunity: A salient factor in the pathogenesis of neuroimmune psychiatric disorders. *Molecular Psychiatry, 23*(2), 170–176.
82. Muffat, J., Li, Y., Yuan, B., Mitalipova, M., Omer, A., Corcoran, S., et al. (2016). Efficient derivation of microglia-like cells from human pluripotent stem cells. *Nature Medicine, 22*(11), 1358–1367.
83. Ross, C. A., & Margolis, R. L. (2018). Research domain criteria: Cutting edge neuroscience or Galen's humors revisited? *Molecular Neuropsychiatry, 4*(3), 158–163.
84. Quadrato, G., Nguyen, T., Macosko, E. Z., Sherwood, J. L., Min Yang, S., Berger, D. R., et al. (2017). Cell diversity and network dynamics in photosensitive human brain organoids. *Nature, 545*(7652), 48–53.
85. Bangasser, D. A., & Valentino, R. J. (2014). Sex differences in stress-related psychiatric disorders: Neurobiological perspectives. *Frontiers in Neuroendocrinology, 35*(3), 303–319.
86. Tiwari, A., & Gonzalez, A. (2018). Biological alterations affecting risk of adult psychopathology following childhood trauma: A review of sex differences. *Clinical Psychology Review, 66*, 69–79.