Title

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Journal

Stem Cell Reports, 17(2)

ISSN

2213-6711

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Publication Date

2022-02-01

DOI

10.1016/j.stemcr.2021.12.015

Peer reviewed

Stem Cell Reports
Review
ISSCR
OPEN ACCESS

Reaching into the toolbox: Stem cell models to study neuropsychiatric disorders

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https://doi.org/10.1016/j.stemcr.2021.12.015

SUMMARY

Recent advances in genetics, molecular biology, and stem cell biology have accelerated our understanding of neuropsychiatric disorders, like autism spectrum disorder (ASD), major depressive disorder (MDD), bipolar disorder (BD), and schizophrenia (SZ). This progress highlights the incredible complexity of both the human brain and mental illnesses from the biochemical to the cellular level. Contributing to the complexity of neuropsychiatric disorders are their polygenic nature, cellular and brain region interconnectivity, and dysregulation of human-specific neurodevelopmental processes. Here, we discuss available tools, including CRISPR-Cas9, and the applications of these tools to develop cell-based two-dimensional (2D) models and 3D brain organoid models that better represent and unravel the intricacies of neuropsychiatric disorder pathophysiology.

INTRODUCTION

Neuropsychiatric disorders are a major challenge for healthcare systems worldwide due to their significant impact on the quality of life for affected individuals and their families. The World Health Organization (WHO) estimates that 450 million people suffer from mental illnesses worldwide (WHO, 2003). It is estimated that one in four families has at least one member currently suffering from a mental or behavioral health disorder, such as major depressive disorder (MDD), schizophrenia (SZ), or bipolar disorder (BD) (WHO, 2003). Additionally, health system expenditure is projected to cost $6 trillion globally over the next 15 years as mental illness becomes the leading cause of disability (Baingana et al., 2015).

Although there is an urgent need for more research into neuropsychiatric disorders and mental health, the complexity of the central nervous system (CNS) presents a challenge. The diversity of cell types and cellular interactions contribute to the complexity of the CNS (Logan et al., 2019). Genetic and psychosocial factors also play a role in the onset and progression of neuropsychiatric disorders, further complicating the understanding of their pathogenesis.

Despite these challenges, human induced pluripotent stem cells (hiPSCs) have emerged as a valuable tool to improve our understanding of neuropsychiatric conditions. The ability of hiPSCs to recapitulate transcriptomic changes of brain development during differentiation to neural cell types while retaining patient-specific genetic backgrounds has allowed for the *in vitro* study of neuropsychiatric disorder pathophysiology (Logan et al., 2019). hiPSCs can provide insight into the cellular mechanisms underlying neuropsychiatric disorders, granting access to the development of *in vitro* drug screening platforms and patient-tailored therapies (Soldner and Jaenisch, 2018).

The unique properties of pluripotency and self-renewal that PSCs possess provide for complex model systems that can recapitulate the pathogenesis of neuropsychiatric disorders (Logan et al., 2019). Two-dimensional (2D) cocultures, 3D brain organoids, and clustered regularly interspaced short palindromic repeats (CRISPR-Cas9)-edited model systems are useful tools to unravel the complexities of neuropsychiatric disorders (Figure 1). 2D cell cultures are commonly employed stem-cell-based models to study cell autonomous phenotypes and non-cell-autonomous interactions. These cultures are amenable to large-scale experiments and allow for high-throughput drug screenings but lack the tissue complexity required to elucidate many aspects of neuropsychiatric disorder pathophysiology (Soldner and Jaenisch, 2018).

Complex model systems that are more representative of the human brain have emerged from PSC-based technologies. Brain organoids can represent some characteristics of human-specific 3D brain architecture, cellular organization, and spatial-temporal brain development (Camp et al., 2015; Lancaster et al., 2013). Overall, brain organoids provide access to some aspects of neurodevelopment, exhibit evidence of mature cellular networks, can represent brain region interconnectivity, and enable the modeling of drug responses in patient-derived cells, all of which are essential dynamics for investigating neuropsychiatric disorder pathogenesis (Bagley et al., 2017; Lancaster et al., 2013; Quadrato et al., 2017; Trujillo et al., 2019).

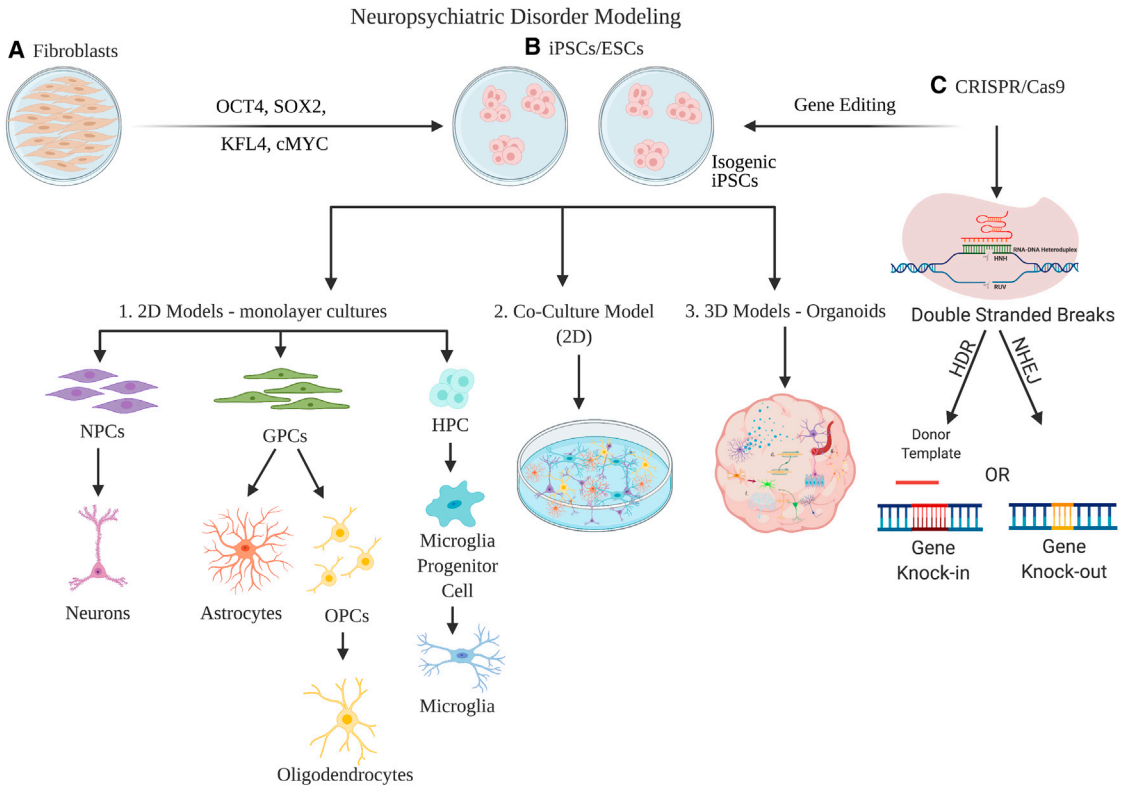


Figure 1. Models for studying neuropsychiatric disorders

(A) Somatic cell (fibroblast) gives rise to iPSCs through a cocktail of transcriptional factors.

(B) iPSCs and ESCs lead to a variety of cell models. 2D models are monolayer cultures of different brain cell types, such as NPCs, GPCs, and hematopoietic progenitor cells (1). Coculture models examine the interaction of different brain cell types, such as neurons, astrocytes, and microglia, in the same culture (2). 3D models (organoids) are more complex structures with multiple cell types and cytoarchitecture that better resembles the human brain (3).

(C) CRISPR-Cas9 is a bioengineering methodology that allows for the introduction of genetic changes in hiPSCs, such as the correction of disorder-causing gene mutations and the introduction of specific mutations into non-affected WT hiPSCs.

Once human stem cells are acquired, genome editing enables the introduction of genetic changes, like the introduction of targeted mutations in wild-type (WT) stem cells (Srikanth et al., 2015; Wang et al., 2015). Genome engineering tools that allow for the programming of a site-specific nuclease, like zinc-finger nucleases (ZFNs), transcription activator-like effector nucleases (TALENs), and CRISPR-Cas9 have improved gene-editing efficiencies in human stem cells (Cong et al., 2013; Hockemeyer et al., 2011; Lombardo et al., 2007; Mali et al., 2013). CRISPR-Cas9 has gained much attention in recent years due to its flexibility and effectiveness in genome engineering and its potential to elucidate the genetic basis of complex disorders.

This review provides an overview of stem cell-based disease models in neuropsychiatry and considers the advantages and challenges surrounding the contributions of these models to our understanding of disorder phenotypes and their mechanisms of action (Table 1).

2D CELL MODELS

Background

hPSCs have been employed for over a decade to model a variety of disorders, including neurodevelopmental and neuropsychiatric disorders, that are exceptionally challenging to study in animals given their subjective manifestations and complex genetic backgrounds (Nestler and Hyman, 2010). hiPSCs generated from patient cells are a source of genetic information that could be contributing to a disease state. Differentiating patient hiPSCs to neural cell types and observing the ensuing phenotypes can illuminate cellular manifestations of neuropsychiatric disorders. Investigating early processes of neurodevelopment through hiPSC specification provides insight into neuropsychiatric disorders rooted in brain development, including Rett syndrome (RTS), autism spectrum disorder (ASD), SZ, and BD (Brennand et al., 2011; Marchetto et al., 2017; Mariani et al., 2015; Stern et al., 2018).

Table 1. Summary of the key elements and challenges for each stem-cell-based neuropsychiatric disorder modeling tool

Stem-cell-based tools	Key elements	Challenges
2D models (iPSCs & coculture)	**iPSCs**: • recapitulate early aspects of human neurodevelopment • preserve patient-specific genetic backgrounds • differentiation to cell types of the three germ layers	**iPSCs**: • line-to-line variations • accumulating genetic mutations during the *in vitro* culturing of hiPSCs
	coculture: • explore non-cell-autonomous contributions to disease pathologies • examine contact-dependent and -independent cell interactions	**coculture**: • reproducing cell-plating densities • implicating cell types in observed changes • supporting growth and survival of all cells in coculture media
3D model (brain organoids)	• accesses human-specific neurodevelopmental processes • coincides with spatial-temporal brain development • recapitulates some aspects of brain architecture • evidence of mature cellular networks	• achieving neuronal separation with accurate cytoarchitecture and spatial identities • representing postnatal developmental processes and aging signatures • obtaining advanced cellular network maturation • obtaining the 6 distinct layers and gyrification of human cortex
CRISPR-Cas9-based model	• understanding gene functions in neuropsychiatric disease models • correcting disorder-causing gene mutations • introducing mutations into WT stem cells	• low HDR efficiency to insert foreign DNA at genetic loci • off-target effects • large CRISPR-Cas9 construct sizes negatively impact transduction efficiencies

Neuropsychiatric disorders involve multiple cell types of the CNS, and *in vitro* cocultures are essential for untangling the influences that these cells have on neuropsychiatric disorder pathophysiology. Cocultures of multiple cell types can be established using primary cells and embryonic stem cell (ESC)- or iPSC-derived cells. They allow for the control of multiple variables, such as cell-type ratios and degrees of contact (Meyer and Kaspar, 2017). Neural cocultures can be formed with multiple subtypes of neuronal or glial cells. Although much has been learned from studies employing PSC-derived neurons to explore neuropsychiatric disorder phenotypes, the significant contribution of glial cells, including astrocytes, oligodendrocytes, and microglia, in both healthy and diseased brain states has only recently been explored (Barres, 2008; Vadodaria et al., 2021).

Glial cells are involved in diverse processes at the heart of many neuropsychiatric disorders, from synapse formation, maturation, and pruning to neuroinflammation and blood-brain barrier (BBB) formation (Barres, 2008; Eroglu and Barres, 2010). Dysregulation of synaptic pruning, which is carried out by astrocytes and complement-component-guided microglia, is considered a major contributor to the onset of SZ pathophysiology (Sellgren et al., 2019). However, glial-mediated dysregulation of synaptic pruning is only one of several ways glial cells can contribute to neuropsychiatric disorder pathophysiology. Given the importance of glia in synaptogenesis and neuronal maturation via the secretion of various factors, understanding glial dysfunction will illuminate neuropsychiatric disorder phenotypes associated with neuronal aberrations and could open new avenues for therapy (Molofsky et al., 2012).

While overarching cellular deficits, such as mitochondrial defects, could be present in multiple cell types, unique cell-type responses to the resulting stresses are possible. The exceptionally high metabolic demands of the human brain could explain why general metabolic defects present in many cell types result in neuropsychiatric disorder pathology without causing systemic disease affecting multiple

organs or tissues (Devaraju and Zakharenko, 2017). For example, the activation of the integrated stress response resulting from a particular mitochondrial defect could occur in some cell types and not others, based on their contrasting metabolic states (Mick et al., 2020). Responses to metabolic dysregulation can also vary among neural cell types. Cytochrome *c* oxidase dysfunction will likely cause the upregulation of glycolysis in astrocytes, while evidence suggests that neurons are unable to launch this response, possibly contributing to their sensitivity to energy depletion (Almeida et al., 2004). Coculture of numerous combinations of cell types with various iPSC-derived neural and glial (non-neuronal) cells can elucidate both autonomous and non-autonomous cellular responses to neuropsychiatric disorder pathology. Cocultures more accurately represent the complexity of neuropsychiatric disorder pathogenesis in a human-specific model system.

Key elements of 2D models

Researchers have applied what is known about tissue and cell specification during *in vivo* development to alter the expression of fate-determining genes and signaling pathways in hPSCs, directing their differentiation to cell types implicated in neuropsychiatric disorders. This can be accomplished with small molecules or transcription factors, resulting in cells that are functional and robust (Soldner and Jaenisch, 2018). Unlike the large transcription factor cascades responsible for influencing the transition and specification of cells during development, advances in cell reprogramming have identified a subset of transcriptional regulators capable of reprogramming a fully differentiated cell to a distantly related cell type (Stiles and Jernigan, 2010). For example, the expression of Pax6 alone in astrocytes isolated from the postnatal cerebral cortex of mice is sufficient to convert them into β-tubulin-III, NeuN-positive neurons (Heins et al., 2002).

Subtypes of neurons are also attainable through the direct reprogramming of somatic cells by employing proneural transcription factors in addition to lineage-specifying factors. Excitatory cortical neurons have been generated via the introduction of *BRN2*, *MYT1L*, and *FEZF2* (Miskinyte et al., 2017), while serotonergic neurons were obtained by overexpressing *NKX2.2*, *FEV*, *GATA2*, and *LMX1B* in addition to *ASCL1* and *NGN2* (Vadodaria et al., 2016). Induced glutamatergic, dopaminergic, and GABAergic neurons have also been obtained by employing similar methods (Ho et al., 2015). Several neural cell types associated with various neuropsychiatric disorders are achievable through direct reprogramming of patient cells; nevertheless, additional research is needed to represent the diversity of cell types involved in neuropsychiatric disorder pathology.

Once a target cell type is obtained, either by hPSC-directed differentiation, through direct reprogramming, or from primary tissue samples, the cells can be cocultured to explore non-cell-autonomous interactions and specific molecular pathways in the neuropsychiatric disorder of interest (Figure 2). Multi-layer cocultures of increasing complexity can be established, such as tri-cultures of neurons, astrocytes, and microglia, which have been employed to study the neuroinflammatory response (Goshi et al., 2020). These methods are simple but cannot distinguish contact-dependent and -independent interactions between cell types that have different implications when considering mechanisms of communication and signal transduction. Conditioned media transferred from one cell type to another allows for the study of contact-independent effects; however, achieving physiologically relevant levels of cytokine secretion is challenging, and the simulated interaction is unidirectional. Transwell membranes and sandwich cocultures allow two cell types to be separated following a period of coculture. Both transwell and sandwich cocultures expose two cell types to the same media, dissolved compounds, and released cytokines while allowing the cell types to be evaluated independently for changes in cytokine secretion or cell survival (Meyer and Kaspar, 2017). Moreover, cell-cell interactions and cellular responses to pharmaceutical compounds can be investigated in a reproducible, high-throughput manner using microfluidic chamber assays to measure axonal signaling, migration, and myelination while controlling the degree of contact between cell types (Meyer and Kaspar, 2017). Cocultures allow for the study of neuropsychiatric-disorder-related cell-cell interactions among various cell types and cellular responses to pharmaceutical compounds (Russo et al., 2018; Sellgren et al., 2019; Vadodaria et al., 2021).

Modeling neuropsychiatric disorders using 2D cell culture systems

hPSCs applied in 2D cell culture systems have taught us much about a wide variety of neuropsychiatric disorders, such as ASD, BD, MDD, RTS, and SZ (Table 2). Increased neural progenitor cell (NPC) proliferation caused by dysregulation of the β-catenin/BRN2 transcriptional cascade or *FOXG1* expression was detected in NPCs and neurons derived from idiopathic macrocephalic ASD patients. Abnormal neurogenesis and dysregulation of GABAergic/glutamatergic neuron differentiation have also been observed in idiopathic ASD patient iPSC-derived neurons, leading to functional defects in neuronal networks (Marchetto et al., 2017; Mariani et al., 2015). Importantly, treatment with IGF-1, a compound that is currently in clinical trials for ASD, partially rescued the defects observed in neuronal networks, opening potential avenues for clinical intervention (Marchetto et al., 2017). Subsequent coculture studies

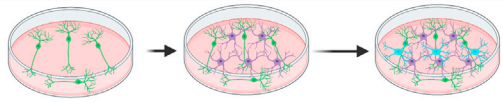
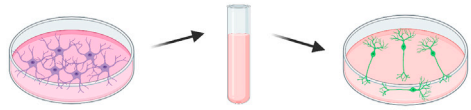
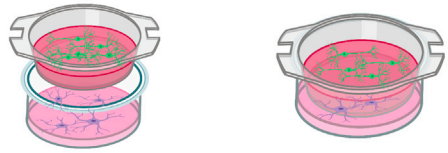
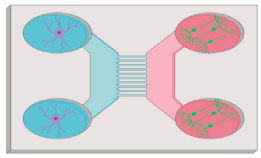
Type	Diagram	Advantages	Disadvantages
Classical co-culture		Easy to set up, easy to manipulate cell concentrations	Cannot differentiate between contact-dependent and independent interactions, media needs to sustain all cell types
Multi-layer co-culture		Easy to set up, affordable, more cell types than classic, so closer to *in vivo*	
Sandwich co-culture		Can separate cell types for RNA/protein characterization after co-culture	Sandwich: more complicated set-up
Conditioned Media Transfer		Easy to set up, well established, shows effects of soluble factors, unidirectional	Can only detect contact-independent interactions
Transwell Insert		Easy to set up, can separate cell types for RNA/protein characterization	Expensive, usually not re-usable
Micro-fluidics chamber		Can manipulate degree of contact of cell types, less cells and reagent needed, possible real time analysis	Complicated to set up, expensive

Figure 2. Advantages and disadvantages of different coculture methods

The classical coculture setup shows astrocytes (purple) plated on top of previously plated neurons (green). The multi-layer coculture plated microglia (blue) on top of astrocytes and neurons. A sandwich coculture shows neurons plated in a well and astrocytes plated on a glass slide, which is placed on top of the neurons cell-side down, separated from the bottom of the well by paraffin. A conditioned media transfer works by transferring conditioned media from one cell type to another. To use a transwell, one cell type would be plated on the plate and the other on the transwell. The transwell would then be inserted on top of the plate, allowing the media and secreted factors to be shared without direct contact occurring between the cell types. A microfluidic device allows for the sharing of media and factors via micro-channels between cell types plated in separate chambers.

probed the effect of ASD astrocytes on control neurons (Russo et al., 2018). ASD-derived astrocytes secreted increased levels of the proinflammatory cytokine interleukin-6 (IL-6) affecting downstream synaptogenesis, and blocking the IL-6 release rescued the synaptogenesis defects.

Recent work demonstrates that iPSC-derived astrocytes from patients with BD also show increased IL-6 expression and secretion. In addition, conditioned medium from BD astrocytes reduced neuronal activity, and this effect was partially blocked by an IL-6-inactivating antibody, suggesting that BD astrocytes are functionally less supportive of neuronal excitability and that this effect is partially mediated by IL-6. The authors also showed that IL-6 levels were elevated in blood from a distinct cohort of BD patients, highlighting the potential role of astrocyte-mediated inflammatory signaling in BD neuropathology (Vadodaria et al., 2021).

Collectively, these findings suggest that glial cell dysfunction could contribute to neuropsychiatric disorder pathophysiology and that *in vitro* models could play an important role in understanding the mechanisms involved in the dysfunction and testing of new therapeutic compounds.

Due to space restrictions, we describe further examples of 2D iPSC-based models to investigate neuropsychiatric disorders in Table 2.

Challenges

While 2D PSC models have advanced our understanding of neuropsychiatric disorder pathophysiology, they are not

Table 2. Selected publications featuring the use of 2D and brain organoid models for investigated neuropsychiatric disorders

Disease	Model system	Source/mutant gene(s)	Cellular phenotype	Rescue	Rescue phenotype	Reference
Autism spectrum disorder	iPSC-derived telencephalic organoids	patient cohort	synaptic overgrowth, increased synaptic connectivity,[a] increased neuronal maturation,[a] increase in inhibitory synapse number, increased GABAergic markers, upregulated *FOXG1* expression	lentiviral infection with short hairpin RNA (shRNA) targeting *FOXG1*	decreased levels of GABAergic neuronal differentiation	Mariani et al., 2015
	iPSC-derived NPCs and neurons	CRISPR-Cas9-edited control cohort/*CHD8*	differentially expressed neural development genes, WNT/β-catenin signaling genes, skeletal system development genes, ASD/SZ risk genes,[a] and *TCF4* upregulation via RNA sequencing[a]	NA	NA	Wang et al., 2015
	iPSC-derived NPCs and neurons	patient cohort/*CTNNB1*, *FRZB*, *FZD6*, *PTEN*, *WNT10B*	NPCs showed increased proliferation and reduced WNT/β-catenin pathway transcriptional expression; neurons exhibited decreased density of excitatory synapses and neuronal activity	LiCl treated NPCs; IGF1 treated neurons	NPCs showed decreased proliferation; neurons showed a rescue of network defects	Marchetto et al., 2017
	iPSC-derived cerebral organoids	CRISPR-Cas9-edited control cohort/*CHD8*	upregulated Wnt/β-catenin signaling and axonal guidance genes, differentially expressed ASD/SZ risk genes,[a] upregulated *TCF4* expression via RNA sequencing[a]	NA	NA	Wang et al., 2017b
	iPSC-derived neurons and astrocytes	patient cohort	patient neurons showed decreased synapse numbers and protein levels, reduced glutamate levels, aberrant electrophysiology;[a] patient astrocytes showed an increase in ROS levels; neurons cocultured with patient astrocytes showed altered neuronal morphology	treatment of anti-IL6 in coculture model	increase in synaptic puncta	Russo et al., 2018
	iPSC-derived cortical neurons and cerebral organoids	patient cohort	**cortical neurons:** complex neurite branching patterns,[a] accelerated development, abnormal temporal maturation[a] **cerebral organoids:** increased CP thickness	NA	NA	Schafer et al., 2019

(*Continued on next page*)

Table 2. *Continued*

Disease	Model system	Source/mutant gene(s)	Cellular phenotype	Rescue	Rescue phenotype	Reference
	iPSC-derived excitatory neurons	CRISPR-Cas9-edited control cohort and patient cohorts/*SHANK2*	patient and CRISPR-Cas9 KO mutant lines exhibited an increase in Synapsin 1 puncta, synaptic connections,[a] dendrite length, and branch complexity[a]	R841X CRISPR-Cas9-corrected excitatory neurons	decreased Synapsin 1 puncta, synaptic connections, dendrite length, branch complexity	Zaslavsky et al., 2019
	PSC-derived PFC-like tissue	CRISPR-Cas9-edited control cohort/*ANKRD11*, *ASH1L*, *ASXL3*, *CHD8*, *CUL3*, *DEAF1*, *DYRK1A*, *GRIN2B*, *KDM5B*, *KMT2A*, *KMT2C*, *RELN*, *SUV420H1*	**class 1 (*ANKRD11*, *ASH1L*, *ASXL3*, *CUL3*, *DEAF1*, *KDM5B*, *KMT2C*, *RELN*):** increased number of NSCs, decreased number of neurons, all lines (except for *ASH1L*) showed decreased neurogenesis after CHIR99021 treatment **class 2 (*CHD8*, *DYRK1A*, *GRIN2B*, *KMT2A*, *SUV420H1*):** decreased number of NSCs, increased number of neurons	NA	NA	Cederquist et al., 2020
	iPSC-derived NPCs	CRISPRi-edited control cohort/*ADNP*, *ASH1L*, *ARID4B*, *CHD2*, *CHD8*, *DYRK1A*, *PTEN*	CRISPRi targeting *ASH1L* and *CHD2* resulted in decreased NPC proliferation; CRISPRi targeting *CHD8* resulted in increased NPC proliferation	NA	NA	Lalli et al., 2020
Bipolar disorder	iPSC-derived hippocampal dentate gyrus (DG) granule-like neurons	patient cohorts (LiCl responder and non-responder)	increased mitochondrial functionality, hyperactive action potential firing,[b] decreased mitochondria size	treatment of neurons with LiCl	LiCl responder neurons exhibited decreased hyperactive action potential firing[b]	Mertens et al., 2015
	iPSC-derived hippocampal DG granule-like neurons	patient cohorts (LiCl responder and non-responder cohorts)	Neuronal hyperexcitability,[b] different electrophysiological properties between LiCl responder and non-responder cohorts	treatment of neurons with LiCl	LiCl responder neurons exhibited decreased number of action potentials and reduced hyperexcitability[b]	Stern et al., 2018
	iPSC-derived cerebral organoids	patient cohorts	downregulated cell adhesion, neurodevelopment, and synaptic biology genes; upregulated immune signaling genes; aberrant ER biology and electrophysiological response	NA	NA	Kathuria et al., 2020

(*Continued on next page*)

Table 2. *Continued*

Disease	Model system	Source/mutant gene(s)	Cellular phenotype	Rescue	Rescue phenotype	Reference
	iPSC-derived hippocampal DG granule-like neurons and CA3 pyramidal neurons	patient cohorts (LiCl responder and non-responder cohorts)	**DG granule-like neurons:** hyperexcitability in LiCl responder and non-responder lines,[b] aberrant sodium, and potassium currents **CA3 pyramidal neurons:** hyperexcitability in LiCl responder neurons,[b] aberrant sodium, and potassium currents	treatment of neurons with α-dendrotoxin, tetraethylammonium chloride, or 4-aminopyridine; treatment of CA3 neurons with LiCl	**treatment with α-dendrotoxin, tetraethylammonium chloride, or 4-aminopyridine:** reduced neuronal excitability in LiCl responder and non-responder lines **treatment with LiCl:** reduced excitability[b] of LiCl responder neurons	Stern et al., 2020
	iPSC-derived hippocampal DG-like neurons	patient cohorts (LiCl responder and non-responder cohorts)	**LiCl non-responder lines:** differentially expressed Wnt pathway genes, decreased *LEF1* expression, decreased Wnt/β-catenin signaling, increased hyperexcitability in *LEF1* shRNA treated neurons **LiCl responder and non-responder lines:** neuronal hyperexcitability[b]	treatment of neurons with valproic acid	increased Wnt/β-catenin signaling activity, decreased hyperexcitability	Santos et al., 2021
	iPSC-derived astrocytes and neurons	patient cohorts and control cohorts	patient astrocytes displayed unique inflammatory transcriptional signature following IL-1β treatment; control neurons cocultured with patient astrocytes treated with IL-1β show decreased neuronal excitability and media showed increased IL-6	treatment of control neurons with supernatant collected from patient astrocytes treated with IL-6-blocking antibody	recovered neuronal activity after exposure to media from astrocytes treated with IL-6-blocking antibody	Vadodaria et al., 2021
Major depressive disorder	iPSC-derived serotonergic neurons	patient cohorts (selective serotonin reuptake inhibitor [SSRI] responder and non-responder)	SSRI non-responder neurons exhibited increased neurite lengths, increased neuronal branching points, decreased expression of *PCDHA6* and *PCDHA8*	treatment of serotonergic neurons with short interfering RNA (siRNA) targeting *PCDHA6/PCDHA8*	increased neurite length in serotonergic neurons	Vadodaria et al., 2019a
	iPSC-derived forebrain neurons	patient cohorts (SSRI responder and non-responder)	SSRI non-responder neurons exhibited hyperactivity and increased calcium spikes after serotonin treatment, increased RNA and protein expression of serotonin receptors 5-HT2A and 5-HT7	treatment of neurons with Lurasidone (5-HT2A and 5-HT7 antagonist)	SSRI non-responder neurons decreased calcium spiking activity similar to control neurons	Vadodaria et al., 2019b

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Table 2. *Continued*

Disease	Model system	Source/mutant gene(s)	Cellular phenotype	Rescue	Rescue phenotype	Reference
Rett syndrome	iPSC-derived neurons	patient cohort/*MECP2*	decreased MECP2 protein, glutamatergic synapse number, spine density, soma size,[c] aberrant intracellular calcium signaling, defects in neuronal electrophysiology	ectopic expression of MECP2 and treatment with IGF1 or gentamicin	increase in MECP2 protein levels and glutamatergic synapse number	Marchetto et al., 2010
	iPSC-derived astrocytes cocultured with mouse hippocampal neurons, hESC-derived forebrain neurons, and iPSC-derived interneurons	patient cohort/*MECP2*	neurons cocultured with mutant astrocytes showed decreased soma sizes,[c] neurite lengths, and number of neuronal terminal ends	treatment of model systems with IGF1 or GPE	IGF1 and GPE increased neuronal soma sizes, GPE increased neurite lengths in WT and mutant interneurons, IGF1 decreased neurite length and number of terminal ends in WT and mutant interneurons	Williams et al., 2014
Schizophrenia	iPSC-derived neurons	patient cohorts	neurons exhibited decreased connectivity,[d] decreased pre-synaptic and postsynaptic puncta,[d] increased NRG1 expression via RNA sequencing	treatment with Loxapine (antipsychotic)	neurons showed an increase in connectivity and expression of glutamate receptors	Brennand et al., 2011
	iPSC-derived cerebral organoids	patient cohorts	aberrant cortical development, increased dispersion of NPCs,[d] absence of TBR1 expression in upper cortical layers,[d] decreased reelin protein, decreased expression of FGFR1,[d] and changes in orientation and morphology of calretinin interneurons	NA	NA	Stachowiak et al., 2017
	iPSC-derived NPCs, excitatory neurons	CRISPR-Cas9-edited control cohort/*FURIN*; CRISPRa/i-edited control cohort/*SNAP91*, *TSNARE1*	***FURIN* rs4702:** decreased *FURIN* mRNA and neurite length in excitatory neurons, increased *FURIN* mRNA and neural migration in NPCs ***SNAP91/TSNARE1*:** decreased SYP puncta via CRISPRi,[d] increased puncta for SNAP91 and decreased puncta for TSNARE1 via CRISPRa,[d] decreased puncta size for CRISPRa/i in excitatory neurons	***FURIN* rs4702:** treatment of excitatory neurons with miR338 inhibitor	***FURIN* rs4702:** control and CRISPR-Cas9-edited excitatory neuron *FURIN* RNA expression made more similar	Schrode et al., 2019

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Table 2. *Continued*

Disease	Model system	Source/mutant gene(s)	Cellular phenotype	Rescue	Rescue phenotype	Reference
	iPSC-derived neurons cocultured with monocyte-derived microglia-like cells	patient cohorts	neurons cocultured with microglia-like cells exhibited decreased densities of synaptic spines; microglia-like cells exhibited an increase in synaptic nerve terminal uptake	treatment of model system with minocycline	decreased engulfment of synaptic nerve terminals by microglia-like cells and increased spine density in neurons	Sellgren et al., 2019
	iPSC-derived cerebral organoids	patient cohort	**cerebral organoids exposed to tumor necrosis factor (TNF):** dispersed NPC populations,[d] decreased neuronal networks,[d] cell density, TBR1 cells,[d] neuronal myelination and nuclear FGFR1,[d] increased numbers of cortical GABAergic interneurons and calretinin	NA	NA	Benson et al., 2020
Timothy syndrome	hPSC-derived forebrain spheroid organoids	patient cohorts/*CACNA1C*	increased residual calcium in neurons, increased frequency of saltatory conduction in interneurons	treatment with nimodipine (L-type calcium channel blocker) or roscovitine (cyclin-dependent kinase inhibitor)	rescue of aberrant saltatory conduction activity	Birey et al., 2017
Broad neuropsychiatric disorders	iPSC-derived NPCs and neurons	CRISPR-Cas9-edited control cohort/*DISC1*	decreased DISC1 protein expression in neurons,[e] upregulated Wnt signaling,[e] decreased *FOXG1* and *TBR2* RNA levels in NPCs	treatment of NPCs with XAV939 (Wnt antagonist)	decreased baseline Wnt signaling	Srikanth et al., 2015
	iPSC-derived cerebral organoids	patient cohorts	absence of ventricle-like structures, dispersed cell morphology,[e] increased number of rosettes, decreased size of rosettes, decreased proliferation[e]	treatment of cerebral organoids with XAV939	formation of well-defined ventricle-like structures, rescue of proliferation phenotype	Srikanth et al., 2018
	iPSC-derived cerebral organoids, NPCs, and cortical neurons	patient cohort (discordant monozygotic twins: psychotic twin and healthy twin)	**psychotic twin cerebral organoids:** decreased numbers of NPCs, increased numbers of postmitotic cells, decreased glutamatergic and dopaminergic neuron ratios to postmitotic cells, differentially expressed genes related to Wnt signaling[e] **psychotic twin NPCs:** decreased proliferation,[e]	treatment of cerebral organoids, NPCs, and cortical neurons with LiCl	**cerebral organoids:** rescued progenitor cell population and GABAergic neuron proportion **NPCs:** increased proliferation, decreased expression of GABAergic neuron specification genes **cortical neurons:** decreased inhibitory	Sawada et al., 2020

(*Continued on next page*)

Table 2. *Continued*

Disease	Model system	Source/mutant gene(s)	Cellular phenotype	Rescue	Rescue phenotype	Reference
			increased expression of GABAergic neuron specification genes via RNA sequencing **psychotic twin cortical neurons:** accelerated maturation,[e] decrease in excitatory synapses,[e] increase in inhibitory synaptic puncta,[e] increase in proportion of GABAergic neurons to postmitotic cells		synapses and number of GABAergic neurons	

[a]Reproducible phenotypes observed in more than one study within ASD.
[b]Reproducible phenotypes observed in more than one study within BD.
[c]Reproducible phenotypes observed in more than one study within RTS.
[d]Reproducible phenotypes observed in more than one study within SZ.
[e]Reproducible phenotypes observed in more than one study within all neuropsychiatric disorders investigated.

without their challenges. For example, cocultures have an increased complexity that better represents the human brain when compared with monocultures, but they still lack cell-type diversity, tissue architecture, dynamic growth expansion, and maturation. Additionally, cocultures depend on reproducible cell plating densities and consistent culturing parameters, which can result in differences from one experimental batch to another if not strictly controlled (Logan et al., 2019). Understanding which cell type is responsible for which effect can also be challenging as coculture conditions increase in complexity (Meyer and Kaspar, 2017). Finally, it is essential that coculture media are carefully formulated to support the survival and growth of all cell types within a culture system. Cocultures are an important technique to model the crucial non-cell-autonomous components of neuropsychiatric disorders. Implementing various coculture techniques to elucidate neurological disorders can advance our understanding of the processes governing these conditions and provide access to effective treatment options through personalized medicine.

BRAIN ORGANOIDS

Background

Monolayer cell cultures lack the intricacy and functionality of *in vivo* neural circuits with multiple cell types and long-distance interactions. A system is needed that allows the genetic complexity of neuropsychiatric disorders to "play out," revealing the functional processes contributing to disease phenotypes, much like a compatible computer program is required to run a specific line of code (Ripke et al., 2014). Human brain organoids offer an *in vitro* model system of unprecedented complexity to study human-specific neurodevelopment and neural maturation (Lancaster et al., 2013). This allows researchers to compare phenotypes resulting in altered neurodevelopmental processes in a targeted neuropsychiatric disease with those of healthy individuals (Schafer et al., 2019). Brain organoids are 3D, PSC-derived, self-organizing models that recapitulate some aspects of neurodevelopment, including neurogenesis, gliogenesis, synaptogenesis, cell migration, and cell differentiation.

Brain organoids can be generated by following "unguided" (Lancaster et al., 2013) or "guided" approaches (Kadoshima et al., 2013; Paşca et al., 2015; Qian et al., 2016). During unguided approaches, cerebral organoids are established in the absence of inductive cues. They develop a variety of regional identities organized as discrete domains capable of influencing one another. This protocol relies on the default formation of neuroepithelium in the absence of inductive cues and facilitates neuroepithelial

expansion by establishing the necessary environment for intrinsic signaling to influence development. Following embryoid body (EB) generation from singularized PSCs or entire PSC colonies and neuroectoderm establishment, EBs are embedded in Matrigel, providing a scaffold for more complex tissue growth. In 6–10 days, NPC populations manifest, and in 20–30 days, cells with defined regional identities form (Lancaster et al., 2013). This method recapitulates fundamental mechanisms of mammalian neurodevelopment as well as characteristics of human-specific brain development; however, random tissue heterogeneity is a limitation of this methodology (Lancaster et al., 2013).

On the other hand, guided approaches have the power to generate more reproducible, homogeneous structures of brain-region-specific organoids (Qian et al., 2018). PSCs are enzymatically detached, converted into EBs, and transferred to neural induction media. Initially, neural induction is achieved by first inhibiting both the bone morphogenetic protein (BMP) and tumor growth factor (TGF)-β signaling pathways, known as dual-SMAD inhibition (Chambers et al., 2009), and then exposing organoids to factors that support and influence the identity of NPC populations to promote further specification (Kadoshima et al., 2013; Paşca et al., 2015). At later time points, factors can be applied to facilitate NPC differentiation and neuronal maturation (Qian et al., 2018).

Key elements of organoids

There has been a growing understanding that the proper orchestration of key events occurring during human brain development is essential for the long-term health of the human brain and that dysregulation of these events could set the stage for the pathophysiology of neuropsychiatric disorders (Arango et al., 2014; Brennand et al., 2011). Neurogenesis, synaptogenesis, atypical trajectories of brain maturation, and cortical folding are key developmental processes implicated in the onset of neuropsychiatric diseases including ASD, BD, and SZ (Arango et al., 2014; Birnbaum et al., 2015; Sarrazin et al., 2018). These processes are executed by a concerted effort of numerous cell types, with glial cells and neurons playing central roles, and can be disrupted by neuroinflammation, hyperoxia, stress, and abnormal spatial-temporal regulation of transcriptomics (Paolicelli et al., 2011; Sarrazin et al., 2018; Sellgren et al., 2019). While 2D, PSC-derived neural rosettes demonstrate basic radial features of neural tube epithelium conserved in vertebrate neural development, such as radial glial (RG) organization and timed cell lineage progression and specification, they lack the heterogeneity and continuity achieved in 3D organoids, which are capable of developing additional, more defined progenitor zones and representing various brain region identities (Lancaster et al., 2013). These more elaborate features of brain development are observed in mammals with large, complex brain structures including sheep, cats, and humans (De Juan Romero and Borrell, 2015). Brain organoid model systems derived from hPSCs offer access to more advanced features of human-specific brain developmental processes implicated in neuropsychiatric disorders that have not been achieved by 2D cell culture methods, such as outer subventricular-like zones (oSVZs), cavities more closely resembling ventricles than their 2D rosette counterparts, and organization similar to that found in the intermediate zone (IZ) and preplate (Lancaster et al., 2013; Qian et al., 2016).

Brain organoids are valuable models for elucidating the mechanisms governing the dysregulation of developmental processes implicated in neuropsychiatric disorder etiology. Brain organoids recapitulate early processes of human brain development and sites of neurogenesis, including the apical-basal orientation of neuroepithelium in ventricular-like zones (VZs). Ventricular RG progenitors (vRGs) undergoing cell division comprise VZs in brain organoids and differentiate into surrounding intermediate progenitors (IPs) and multiple neuronal subtypes (Di Lullo and Kriegstein, 2017; Kadoshima et al., 2013; Lancaster et al., 2013). Unique features of development and dysfunction in the human neocortex have been widely studied by employing forebrain patterned organoid generation protocols (Di Lullo and Kriegstein, 2017; Kadoshima et al., 2013; Paşca et al., 2015; Qian et al., 2018). These organoids exhibit expanded, proliferative SVZs and oSVZs containing outer RG cells (oRGs). The oRGs undergo human-specific oRG mitotic behavior and provide scaffolding for neuronal migration that develops deep and upper layer cortical neuron organization in a way that is not represented in the developing cortex of rodents (Di Lullo and Kriegstein, 2017; Lancaster et al., 2017; Paşca et al., 2015; Qian et al., 2018).

Protocols have been established for the generation of forebrain organoids that develop cortical plate (CP)-like structures enriched with $CTIP2^+$ cells and more distinct oSVZs than those observed in cerebral organoids (Qian et al., 2016). The SVZs in these organoids contain $SOX2^+$ NPCs and $TBR2^+$ intermediate progenitor cells (IPCs), while upper and deep cortical layer markers, like SATB2 and CUX1, are apparent by approximately day 80 of development (Qian et al., 2016). TBR2 is likely critical for coordinating neurogenesis and is generally expressed by IPs migrating to the SVZ where they symmetrically divide into neurons contributing to the exceptional diversity of neuronal subtypes comprising the cortex (Lv et al., 2019). Although these forebrain organoids do not recapitulate all six distinct cortical layers present *in vivo*, investigating the mechanisms behind cortical layer distinction in

organoids could shed light on the SZ- and ASD-associated genes *SATB2* and *CTIP2,* whose major function is the regulation of neuronal differentiation and maturation (Birnbaum et al., 2015; Qian et al., 2016). Comparing single-cell RNA sequencing data of brain organoids to that of the fetal neocortex, researchers have found that cells of developing cerebral-cortex-like structures in human cerebral organoids share commensurate gene expression patterns with those found in the developing human neocortex (Camp et al., 2015). Access to dynamic processes of human neurodevelopment permits their pharmacological and genetic manipulation and improves our understanding of the mechanisms influencing neuropsychiatric disorder onset (Qian et al., 2016).

Not only is it important to understand the priming of neuropsychiatric disorders during prenatal events, but gaining insight into the postnatal period through early adulthood, when some neuropsychiatric disorders present themselves, could help in disease treatment and prevention (Gogtay et al., 2011). We now understand that the human brain undergoes significant remodeling processes postnatally and that it maintains a large amount of plasticity throughout adult life (Paolicelli et al., 2011). While these processes are important for healthy brain maturation and maintenance, they are vulnerable to dysregulation in several major neuropsychiatric disorders. BD, SZ, MDD, and ASD have been linked to postnatal processes including synaptic pruning, complement system regulation, adult neurogenesis, circuit function, and abnormal neurotransmission systems (Di Lullo and Kriegstein, 2017; Feinberg, 1982; Paolicelli et al., 2011; Sarter et al., 2007). A variety of brain regions are implicated in these dysregulated processes, such as the hippocampus, prefrontal cortex (PFC), insular cortex, thalamus, frontal-subcortical circuits, and the basal ganglia, and encompass several cell types including neurons, oligodendrocytes, astrocytes, microglia, and endothelial cells (Mega and Cummings, 1994; Quadrato et al., 2016). These findings suggest that intricate model systems representing the immense complexity of the postnatal brain are required to gain a complete understanding of the pathogenesis of neuropsychiatric disorders (Figure 3). In the following section, we will discuss the progress that has been made in recapitulating this complexity in brain organoid models to successfully represent and elucidate the pathophysiology of neuropsychiatric disorders.

Brain organoids contain neurons that are specific to certain areas of the brain, although these neurons do not demonstrate regional separation as they would *in vivo* (Bhaduri et al., 2020; Qian et al., 2016). Furthermore, neurons in organoids might lack full maturation 82. While several studies have demonstrated functional synapses, spontaneous firing activity, and large-scale neural networks by the neurons of organoids (Paşca et al., 2015; Quadrato et al., 2017; Trujillo et al., 2019), one study has shown that the neurons of some organoids continue to express markers associated with NPC identities in addition to neuronal markers of maturation. While this could be an artifact of lingering protein expression during the transition of cells from NPCs to mature neurons, it has been demonstrated that organoids have a higher overlap of RG cells and mature neuronal markers than that of developing primary cortical tissue and that this marker co-expression does not resolve over time as it does in human brain development, suggesting a failure to reach complete maturation (Bhaduri et al., 2020).

The lack of continued neurogenesis, regional separation of neurons with specific areal identities, and advanced neuronal maturation has been attributed to increased oxidative and metabolic stress within the *in vitro* culture of organoids, hindering the entire representation of developmental and disease-state processes (Bhaduri et al., 2020; Qian et al., 2016). Recombinant leukemia inhibitory factor (LIF) addition during organoid generation has been shown to increase the size of SVZs, enhance the separation of deep and superficial neurons of the cortex, and preserve organoid structure up to 22 weeks (Watanabe et al., 2017). Microgravity-based bioreactors have also been evaluated for their ability to promote 3D mammalian tissue architectures and intracellular processes by reducing fluid shear forces, providing randomized gravitational vectors, and increasing oxygen diffusion (Jessup et al., 1993; Qian et al., 2016). While bioreactors increase oxygen diffusion, engineering brain organoids to develop an endogenous or hPSC-derived, vascular-like perfusable network or implanting organoids into the brains of mice for their subsequent vascularization more effectively reduces apoptosis and cellular stress to facilitate the modeling of brain development beyond the second trimester (Cakir et al., 2019; Mansour et al., 2018; Pham et al., 2018). Recently, researchers demonstrated that 3D human cortical organoids cultured at specific conditions survived for up to 300 days and were comparable transcriptionally and epigenetically to postnatal human brain stages, paralleling *in vivo* development (Gordon et al., 2021) These results suggest that it is possible to capture components from human *in vivo* brain developmental programs, including birth, using the organoid technology.

Lastly, representing human brain regions implicated in neuropsychiatric disorders is important for understanding the brain-region-specific cell types involved and the interconnectivity between different communicating brain subunits. Transcriptional regulators have been applied during brain organoid development to direct the fate of organoids toward specific brain regions, including the midbrain,

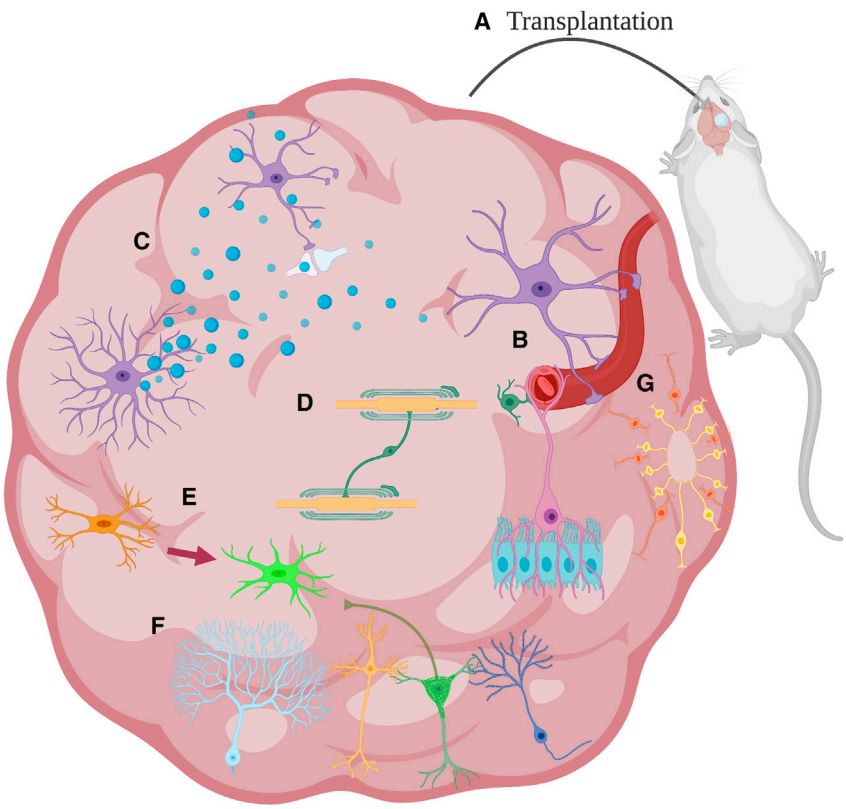

Figure 3. A complete brain organoid model for studying human neuropsychiatric diseases

(A) Vascularization of organoids increases neuronal maturation and specification by reducing oxidative stress and can be accomplished via implanting organoids into mice or by engineering endogenous vascular-like networks.

(B) Increasing the ratios of astrocytes, pericytes, and microglia in organoids will promote a functional BBB system that better supports NSC populations, neuronal circuits, and sustained neurogenesis.

(C) Supporting the enrichment and maturation of astrocytes in organoids will improve neuronal maturation, synapse regulation, and neuronal circuit formation through direct interactions of astrocytes with synapses and astrocyte-secreted factors.

(D) Generating organoids with myelinating oligodendrocytes will improve electrical transmission of neurons in disease models.

(E) Introducing microglia allows researchers to study microglial behavior and inflammation in neuropsychiatric diseases.

(F) Enriching organoids with specific neuronal subtypes and brain region identities by implementing developmental patterning paradigms could elucidate neuropsychiatric diseases implicating certain populations of cells, brain regions, or cell-cell interactions.

(G) VZs composed of vRGs and surrounded by an SVZ made up of oRGs recapitulate human-specific cortex development.

hypothalamus, hippocampus, and cerebellum (Jo et al., 2016; Muguruma et al., 2015; Qian et al., 2018; Sakaguchi et al., 2015). Basal ganglionic organoids have also been established by improving the long-term maintenance of basal RGs in the SVZ via LIF addition (Watanabe et al., 2017). Once these brain-region-specific organoids are generated, they can be fused together to study their interconnectivity and routes of communication. The fusion of distinct cortical brain regions has provided insight into neuropsychiatric disorders (Bagley et al., 2017; Birey et al., 2017), while the fusion of developed thalamic organoids with cortical organoids has taught us about thalamic neuronal maturation, axon targeting, and synaptogenesis (Xiang et al., 2019). Future studies of thalamic organoids could elucidate the role that the thalamus plays as an information relay hub and how its dysregulation influences neuropsychiatric disorders such as MDD, SZ, ASD, and BD (Radenbach et al., 2010; Xiang et al., 2019; Young et al., 2004).

Modeling neuropsychiatric disorders using brain organoids

Brain organoids have been successfully generated from patients' hiPSCs representing a variety of neuropsychiatric disorders, such as ASD, BD, and SZ (Table 2). Recent studies employing telencephalic and forebrain organoids developed from idiopathic ASD patients with macrocephaly identified neurodevelopmental alterations associated with aberrant cell proliferation, cell-cycle timing irregularities, unbalanced inhibitory neuron differentiation likely influenced by an upregulation in FOXG1 expression, exuberant synaptogenesis, temporal deviations in cortex development, and increased neuronal branching (Mariani et al., 2015; Schafer et al., 2019). Direct comparisons to neural stem cell (NSC) monolayer systems and forebrain organoids derived from a subset of the cohort verified no differences in the ability of ASD RGs to differentiate to $TBR1^{+}$ neurons; however, an increased thickness of the CP was observed in ASD iPSC-derived organoids, demonstrating the advantage that 3D organoid models have in representing architectural aberrations of brain development. Together, the 2D monolayer and 3D organoid systems identified transcriptional dysregulation and aberrations in cortical neuron development associated with ASD.

In addition to cortical or forebrain organoids, spheroids and organoids that represent the ventral and dorsal subregions of the forebrain have also been developed.

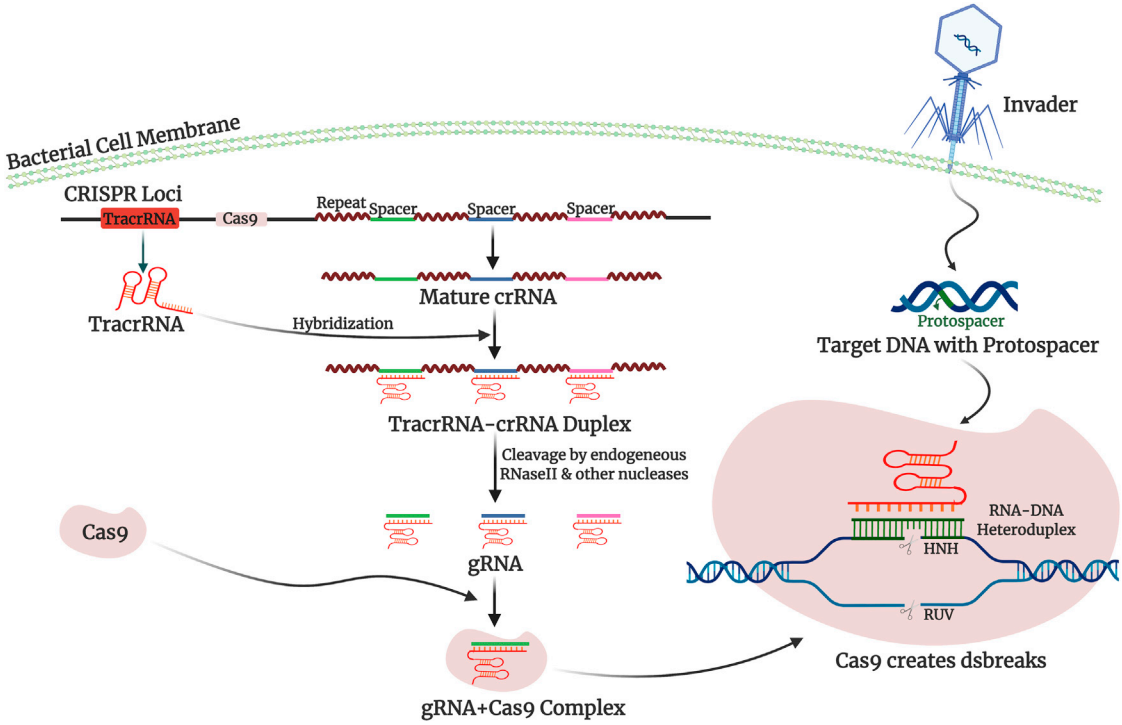

Figure 4. CRISPR-Cas9 mechanism of action in bacteria

CRISPR loci are processed to make mature crRNA and tracrRNA, which are further hybridized to form a gRNA. Spacers are regions of the bacteriophage DNA that are interspersed between the repeat sequences in CRISPR loci, providing adaptive immunity. The gRNA and the Cas9 form a complex, which binds to the invading DNA at the protospacer, forming the RNA-DNA heteroduplex. Cas9 then creates DSBs, ultimately disintegrating the invader's DNA.

Glutamatergic-neuron-containing dorsal forebrain spheroids were fused with GABAergic interneuron-containing ventral forebrain spheroids to observe the subsequent migration, integration, and maturation of interneurons within the cortical system (Bagley et al., 2017; Birey et al., 2017). The perturbation of this mid-to-late gestation developmental process is thought to contribute to neuropsychiatric disorders like Timothy syndrome (characterized by ASD) and epilepsy (Birey et al., 2017). Additional examples of works employing organoid technologies to the study of neuropsychiatric disorders can be found in Table 2.

Challenges

Forebrain organoids partially recapitulate processes occurring during the first trimester of neocortical development; yet, between approximately days 60–100 of organoid development, the apical-basal organization of neuroepithelium begins to break down, and by day 100, there is a disordered mix of NPCs, neurons, and glial cell types often surrounding a necrotic core (Bhaduri et al., 2020; Qian et al., 2016). Neurons surrounding the necrotic core do continue to mature, demonstrating spontaneously active neuronal networks and more robust dendritic spine formation than what is observed in 2D neuronal cultures (Quadrato et al., 2017; Trujillo et al., 2019). Although the first 100 days of neurogenesis from the SZs and SVZs might be sufficient for studying some components of neuropsychiatric disorder etiologies, it has been suggested that neurogenesis continues beyond gestational week 20 in humans, necessitating the continued expansion of proliferative neuroepithelium to recapitulate later developmental processes including representative amounts of excitatory and inhibitory neurons (Malik et al., 2013).

While the addition of LIF, the implementation of bioreactors, and the incorporation of endogenous and exogenous simple vasculature have improved neuronal maturation, identity, and cortical layer separation in organoids, they have not been able to consistently achieve all six distinct cortical layers found *in vivo* (Cakir et al., 2019; Mansour et al., 2018; Pham et al., 2018; Qian et al., 2020; Watanabe et al., 2017). Continued neocortical expansion and NPC proliferation results in the gyrification of the human cortex, a process that distinguishes humans from lissencephalic species (including mice) and whose dysregulation has been associated with neuropsychiatric disorders (Sarrazin et al., 2018). Folding in cerebral organoids has been

accomplished by establishing organoids from CRISPR-Cas9-edited *PTEN* knockout PSCs to increase NPC proliferation and delay neuronal differentiation but not through intrinsic properties of development (Li et al., 2017). This research has demonstrated key mechanisms governing gyrification, although it may not represent the entire orchestration of the process and the potentially dysregulated mechanisms contributing to neuropsychiatric disorder pathogenesis.

Although brain organoid technologies have been advancing quickly over the last 5 years, there is still much room for the optimization of these models. Variability between brain organoids and batches of brain organoids, including cellular heterogeneity, continues to occur despite the implementation of defined transcriptional regulators, embedding materials, and media (Bhaduri et al., 2020). Automated approaches are being employed to improve reproducibility, homogeneity, and scalability while maintaining architectural complexity of midbrain organoids (Renner et al., 2020). Yet, additional optimization is required to apply automated technologies for the consistent generation of brain organoids representing additional brain region identities. Furthermore, brain organoid generation efficiencies can be low if the PSCs employed to generate organoids contain genetic mutations affecting their properties of pluripotency and self-renewal (Qian et al., 2018). To truly understand the intricacies of neuropsychiatric disorders, from the genetic level to the transcriptome and cell physiology to brain architecture and circuitry, it will be necessary to push the boundaries for the establishment of consistent, physiologically relevant human brain organoid model systems.

CRISPR-CAS9 GENE EDITING

Background

Discovered in the adaptive immune system of bacteria, CRISPR-Cas is an exceptional tool for studying both gene function and regulation while unearthing novel drug targets for the development of therapeutics. Recent advances in CRISPR technologies have allowed for the efficient manipulation of DNA in mammalian cells and its implementation in 2D cell culture and 3D brain organoid model systems. This has made CRISPR a pivotal tool for investigating diseases with complex genetic backgrounds including polygenic neuropsychiatric disorders (Cong et al., 2013; Gasiunas et al., 2012; Jinek et al., 2012). In this section, we will review recent advances in CRISPR technology and its application to establish PSC-based disease models for elucidating neuropsychiatric disorders.

Key elements of CRISPR-Cas9 gene editing

The CRISPR-Cas type II system consists of a Cas9 endonuclease and guide RNA (gRNA) composed of CRISPR RNA (crRNA) and transactivating crRNA (tracrRNA) (Cong et al., 2013) (Figure 4). The gRNA recognizes specific sequences of DNA, and Cas9 cleaves the DNA through two nuclease domains, HNH and RuvC, generating double-stranded breaks (DSBs) at the targeted site (Gasiunas et al., 2012) (Figure 4). For the Cas9 complex to bind to the target DNA and create DSBs, Cas9 requires a three-nucleotide sequence (NGG), known as the protospacer adjacent motif (PAM), downstream of the protospacer (Mojica et al., 2009). The PAM region is crucial for the CRISPR-Cas9 system to distinguish between itself and the viral DNA (Marraffini and Sontheimer, 2010), although recent progress in CRISPR-Cas technology has increased the versatility of the system with the identification or engineering of Cas endonucleases with PAM plasticity (Collias and Beisel, 2021). The ability to transplant CRISPR into distantly related organisms and the programmability of the Cas protein makes it a valuable tool in the study of human disease (Cho et al., 2013; Cong et al., 2013; Jinek et al., 2012; Mali et al., 2013; Wang et al., 2015).

DSBs created by CRISPR-Cas9 instigate cellular repair mechanisms. Subsequent DNA repair can take the form of either homology-directed repair (HDR) or non-homologous end-joining (NHEJ) (Cong et al., 2013). The HDR pathway is considered "error-free," as providing a homologous DNA template allows for the integration of the desired DNA sequence at DSB sites via homologous recombination. This differs from "error-prone" NHEJ repair mechanisms that can cause frameshift mutations at target sites by adding nucleotide insertions or deletions (indels) (Hsu et al., 2014). Mutations in the Cas9 endonuclease have been introduced that render either the HNH or RuvC domain inactive, resulting in a Cas9 Nickase that can establish single-stranded breaks in DNA, enabling the introduction of point mutations or staggered cuts. This method reduces the amount of off-target editing and indels by increasing HDR and facilitating the introduction of longer insertions (Ran et al., 2013).

The CRISPR-Cas9 system has undergone substantial advancements by acquiring new functions in genome editing. Technological innovations of this system have delved into epigenetic modulation of chromatin to better understand psychiatric disorders. This strategy exploits a nuclease-dead Cas9 (dCas9) protein that has inactivated Cas9 nuclease domains yet can still locate specific genetic loci and induce the methylation or acetylation of histone proteins, resulting in transcriptional activation or repression (Kang et al., 2019; Sajwan and Mannervik, 2019). Epigenetic modifications might be introduced into cell-based model systems of neuropsychiatry to investigate the influence that age-related genetic changes have on the presentation of disease phenotypes. Additionally, dCas9 can interact with transcriptional activator

domains, such as VP64, and repressor domains, establishing changes in transcriptional expression. This is accomplished either by a CRISPR gene activation system (CRISPRa) or through CRISPR interference (CRISPRi) (Gilbert et al., 2013). Researchers can use these tools to probe the polygenic nature of several neuropsychiatric disorders by altering the expression of associated genes and investigating their influence or representing their dysregulation through genomic indels, corrections, activation, or inactivation (Cong et al., 2013; Kang et al., 2019; Mali et al., 2013; Savell and Day, 2017; Wang et al., 2015). CRISPRi, CRISPRa, CRISPR knockouts (KOs), and different Cas enzymes to target mRNA or DNA in various ways can be employed using pooled sgRNA expression libraries to generate pooled screens for the interrogation of cell-autonomous phenotypes in iPSC-derived neural cells. To investigate non-cell-autonomous phenotypes in iPSC-derived neural cells, arrayed screens can be established wherein sgRNAs target one gene in each well (Tian et al., 2019). Additionally, the influence that single genetic perturbations have on the transcriptome of individual cells can be observed using CRISPR droplet sequencing (CROP-seq) and could prove to be valuable in pinpointing critical genes involved in the etiology of polygenic neuropsychiatric disorders (Datlinger et al., 2017).

Modeling neuropsychiatric disorders using CRISPR-Cas9-based systems

CRISPR-Cas9 technologies have been implemented in several hiPSC-based disorder models to better understand mental illness by investigating the physiological effects of genetic alterations.

To better understand the etiology of ASD, CRISPR-Cas9 technology has been employed to investigate the effects of the ASD candidate gene *CHD8* by establishing a CRISPR-Cas9-induced heterozygous KO of *CHD8* in patient-derived hiPSCs (Wang et al., 2015) and, later, in patient hiPSC-derived cerebral organoids (Wang et al., 2017b). Following reprogramming of control and CRISPR-Cas9-edited fibroblasts to hiPSCs, the resulting hiPSCS were directed to NPCs, and neurons and the transcriptomic profiles of these cells were compared. *CHD8* was found to be a key regulator of several pathways implicated in ASD and SZ pathologies, including pathways associated with the identified psychiatric risk genes *TCF4* and *NRXN1* (Wang et al., 2015). The role of *TCF4* during neurodevelopment has been studied in the rat prefrontal neocortex using CRISPR-Cas9 to inhibit *TCF4* expression *in vivo* (Rannals et al., 2016). This caused a reduction in the action potential spiking frequency of Cas9-edited cells, implicating KCNQ1 and SCN10A voltage-gated channel receptors. *NRXN1* transcribes a presynaptic cell adhesion molecule (CAM), whose interaction with postsynaptic neuroligins allows for effective synaptic connections and neurotransmission (Onay et al., 2016). *NRXN1* KO mutations in human ESC (hESC)-derived neurons did not influence differentiation or synapse formation, as electrophysiological recordings and synaptic density appeared unaffected, despite decreased spontaneous currents (Pak et al., 2015). This decline in spontaneous current is likely due to the *NRXN1* KO mutation hampering the ability of neurons to release neurotransmitters.

Recent research by Cederquist and colleagues has sought to develop a multiplex hPSC platform to study a group of genes implicated in ASD (Cederquist et al., 2020). Using CRISPR-Cas9, 27 isogenic KO lines derived from different patients were cocultured together and differentiated to PFC lineages. Eight lines were found to exhibit a reduction in neurogenesis due to abnormal WNT/β-catenin signaling, whereas five lines showed an enhancement in neurogenesis. Further examples from studies implementing CRISPR in 2D cell culture and 3D brain organoid models for the investigation of complex genetic backgrounds contributing to neuropsychiatric disorders can be found in Table 2.

Challenges

Great strides have been made over the last decade in CRISPR-Cas9-based research, increasing the efficiency and accessibility of DNA editing. However, challenges remain with CRISPR-Cas9-based approaches that should be considered when designing experiments and selecting genome-editing technology. One limitation is the low efficiency of HDR when using the CRISPR-Cas9 system to insert foreign DNA into targeted genetic loci (Shen et al., 2014). Techniques to increase HDR, including the inhibition of NHEJ-related processes, have allowed for more accurate DNA insertions (Chu et al., 2015). Researchers have also turned to Cas9 nickases to increase HDR and to improve the efficiency of Cas9 genetic insertions (Ran et al., 2013). Moreover, off-target effects are common, resulting in the editing of the genetic code at undesired loci (Moon et al., 2019). Careful design of crRNA is essential to reduce off-target effects. Additionally, larger CRISPR construct sizes negatively impact transfection efficiencies (Mali et al., 2013). Overcoming these challenges is crucial to accessing the full potential of CRISPR-Cas9 genome-editing technology and better representing the genetic complexity of neuropsychiatric disorders.

FUTURE DIRECTIONS

The discovery of rapid, efficient, and non-invasive methods to reprogram patient somatic cells to iPSCs

has strengthened our ability to establish relevant model systems that illuminate the mechanisms underlying neuropsychiatric disorders and to pursue drug discovery (Brennand et al., 2011; Marchetto et al., 2017; Xu et al., 2016). Yet, further advances are required to better represent the complexity of neuropsychiatric disorder pathogenesis. In the following sections, we will discuss possibilities for improving model systems of neuropsychiatric disorders and the tools required to accomplish this. We will also consider ways in which these model systems can be employed to elucidate mechanisms governing certain neuropsychiatric disorder phenotypes.

Not only are hPSC-based models crucial for studying human-specific mechanisms underlying neuropsychiatric disorders, but hiPSCs are essential to accelerating drug development and personalized medicine. Previous works testing drug candidates on patient-derived hiPSCs and observing subsequent cellular responses have demonstrated the strength of hiPSC modeling to uncover therapeutic options. Several antipsychotic drugs were evaluated on neuronal cultures derived from the hiPSCs of SZ patients to assess their ability to rescue neuronal connectivity deficits and other phenotypes found in SZ neurons (Brennand et al., 2011). Furthermore, the effects of IGF-1 were tested in hiPSC-derived neurons from RTS and ASD patients, revealing a reversal of neuronal phenotypes (Marchetto et al., 2010, 2017). Further standardization and scaling up of hiPSC model systems could allow for large-scale preclinical trials of pharmaceutical compounds to treat neuropsychiatric disorders. Research employing hiPSCs to predict patients' responses to potential therapeutics suggests that a similar process could occur in hospital settings, relinquishing patients from the taxing undertaking of sampling drug options until they find one that provides relief (McNeish et al., 2015; Vadodaria et al., 2019a, 2019b). Yet, the translatability of results gained from *in vitro* hiPSC drug candidate studies to treatment options for patients is still largely unknown, necessitating animal modeling and clinical trials.

Once implicated cell types are obtained, they can be implemented in coculture systems to further recapitulate the mechanisms underlying the pathogenesis of neuropsychiatric disorders. Cocultures mimicking complex homeostatic brain processes, including models of the human BBB and neuroinflammatory responses, might one day be applied to understand how the dysregulation of these processes contributes to neuropsychiatric condition pathophysiology (Appelt-Menzel et al., 2017). Protocol optimization and standardization in coculture practices are required for the scalable, reproducible modeling of neuropsychiatric disorders.

Brain organoids have granted researchers unprecedented access to early, human-specific neurodevelopmental processes whose dysregulation is hypothesized to be at the origin of several neuropsychiatric disorders. Access to SVZs and OSVZs in human brain organoid models could be a valuable tool for investigating what could be human-specific mTOR pathway mutations implicated in focal cortical dysplasia and ASD, which are suggested to disproportionally affect oRGs found in these structures (Pollen et al., 2019). Furthermore, developing cortex-like structures in organoids might be used to elucidate the human-specific role of the *MEF2C* gene associated with ASD and SZ as identified through genome-wide association studies (GWASs) (Li et al., 2008). *MEF2C* deletion in rodents is known to result in the abnormal migration of neurons in the developing neocortex; however, observing the consequences of its deletion in a human-specific neocortex organoid model might reveal uniquely human consequences (Ripke et al., 2014).

Much of early neurogenesis contributing to the rapid expansion of the human neocortex is represented in brain organoids and could reveal the processes required to innately recreate the complexities of gyrification without introducing genetic mutations (Di Lullo and Kriegstein, 2017; Li et al., 2017). Although forebrain organoids do not demonstrate the distinct separation of all six cortical layers present *in vivo*, investigating the mechanisms behind cortical layer distinction could shed light on the SZ- and ASD-associated genes *SATB2* and *CTIP2*, whose major function is the regulation of neuronal differentiation and maturation (Birnbaum et al., 2015; Qian et al., 2020). Researchers have tested the placement of inducible signaling centers within organoids that produce a gradient of transcriptional regulators, a process more representative of the patterning of brain tissue as it occurs *in vivo*. It might be possible to implement signaling center technologies to refine the spatial organization, maturation, and identities of neurons, including the improved separation of neurons comprising the six cortical layers (Cederquist et al., 2019). The pursuit to better model cortical brain development and organization in organoids could improve our understanding of neuronal migration, positioning, and maturation disruptions in patients with SZ, BD, or ASD (Birnbaum et al., 2015; Qian et al., 2020).

In the last decade, researchers have expanded upon current CRISPR-Cas9 technology, pushing it beyond the boundaries of its original function. CRISPRa and CRISPRi are effective when repressing or activating a gene through transcriptional regulation (Gilbert et al., 2013). Schrode and colleagues employed both CRISPRa and CRISPRi to assess the differential expression of synaptophysin (SYP) in excitatory neurons expressing SNAP91 and TSNARE1,

genes associated with synaptic dysregulation in patients with SZ (Schrode et al., 2019). Additionally, multiplexing CRISPR-Cas9 strategies offer promise in decoding and reversing neuropsychiatric disorder phenotypes by targeting the polygenic nature of these illnesses (McCarty et al., 2020; Savell and Day, 2017). Multiplexed CRISPR-Cas9 approaches have been employed to correct mutations in the dystrophin gene restoring dystrophin expression in cells derived from patients with Duchenne muscular dystrophy (Ousterout et al., 2015). Furthermore, researchers have engineered Cas endonucleases that more accurately perform base editing and have achieved 10- to 100-fold lower Cas9-independent off-target editing events (Doman et al., 2020). The first clinical trials to administer CRISPR-Cas9 directly into the body with the goal of removing a mutation causing Leber's congenital amaurosis 10, the leading cause of childhood blindness, are underway (Ledford, 2020). Prior to this, only one CRISPR-based therapy, CTX001, had entered clinical trials for the treatment of sickle cell disease by editing patients' hematopoietic stem cells *ex vivo* and reintroducing them into the respective patients' bodies (Paganelli, 2019). The application of CRISPR-based technologies in the treatment and prevention of neuropsychiatric disorders is foreseeable, either through the design of more representative model systems or through the development of *in vivo* therapeutics (Wang et al., 2017a). Although it is unlikely that a single tool or technique will cure or explain all neuropsychiatric disorders, a concerted effort of many strategies may, someday, accomplish this goal.

SUMMARY

In the last decade, stem cell research has undergone a vast amount of innovation through the generation of novel methods for disease modeling. Advances in hiPSC research have allowed for the modeling of several neuropsychiatric disorders, paving the way for future research into personalized medicine and more efficient drug discovery processes. Work in brain organoid and coculture models will continue to improve our understanding of complex neuropsychiatric disorders and the cellular interrelationships of specific disorder pathologies. The usage of CRISPR-Cas9 in neuropsychiatric disease modeling has been advantageous for exploring the genetic underpinnings of these complex disorders, providing a more efficient means of genetic engineering to better understand the functionality of genetic loci implicated in disorders. Optimizing and pursuing representative PSC-based model systems in neuropsychiatry will advance the understanding of human neuropsychiatric disorders and promote the discovery of effective, personalized treatment options.

AUTHOR CONTRIBUTIONS

Conceptualization and supervision, J.T.W., S.F., A.S., and M.C.M.; data curation and writing, J.T.W., S.F., A.S., A.P.D.M., V.R., and S.K.B.; reviewing and editing, J.T.W., S.F., A.S., A.P.D.M., V.R., S.K.B., and M.C.M.

CONFLICTS OF INTEREST

The authors declare no competing interests.

ACKNOWLEDGMENTS

We are grateful for the funding provided to MCM by the Larry L. Hillblom Foundation and to SF by 954 the T32 Ruth L. Kirschstein Institutional National Research Service Award through the National 955 Institutes of Health (1T32GM133351-01) and distributed by UCSD's Pathways in Biological 956 Sciences (PiBS) training program. The authors thank J.R. Jones, S.L. Parylak, and S.T. Schafer for critical reading of the manuscript. Figures were created with BioRender.com. Due to word and reference constraints, we were not able to cite every source that may have helped advance and inspire research mentioned in this manuscript.

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