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Review

Human brain organogenesis: Toward a cellular understanding of development and disease

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SUMMARY

The construction of the human nervous system is a distinctly complex although highly regulated process. Human tissue inaccessibility has impeded a molecular understanding of the developmental specializations from which our unique cognitive capacities arise. A confluence of recent technological advances in genomics and stem cell-based tissue modeling is laying the foundation for a new understanding of human neural development and dysfunction in neuropsychiatric disease. Here, we review recent progress on uncovering the cellular and molecular principles of human brain organogenesis *in vivo* as well as using organoids and assembloids *in vitro* to model features of human evolution and disease.

INTRODUCTION

The formation of the human brain—from a microscopic tube of neuroepithelial cells to a complex structure of ~170 billion cells of diverse types and numerous synaptic connections—is a feat of coordinated cellular and molecular development. This dynamic process, known as organogenesis, is initiated by genetic programs that unfold in conjunction with environmental modulators over many years in humans. Understanding the molecular processes that govern organogenesis requires the ability to systematically reconstruct and deconstruct tissue architecture. Consequently, most of the principles of mammalian neurodevelopment have been extrapolated from non-primate model organisms which allow for invasive investigations. Although these studies have yielded fundamental insights, the human-enriched genomic and developmental features that result in advanced cognition remain largely elusive (Sousa et al., 2017a). For instance, what underlies the remarkable expansion of the human cerebral cortex and why does it undergo such a protracted developmental time course over long gestational and childhood periods? Moreover, genetic risk for many neuropsychiatric disorders converges on developmental programs (Sullivan and Geschwind, 2019), yet the molecular and circuit mechanisms of disease pathogenesis remain mostly unknown. A significant challenge in tackling these questions has been the inaccessibility of human tissue, particularly at critical late gestational time periods and within disease-specific genomic contexts, which has impeded rigorous molecular studies of normal and disease-associated human development.

Recent technological advancements in tissue modeling and genomics are transforming our ability to study human brain organogenesis. As is often the case, important discoveries occur on the heels of technological innovation. For example, the classic observations by His, Cajal, and others were made possible by the advent of novel histological methods (Bentivoglio and Mazzarello, 1999). These exquisite, early descriptions of human cellular morphology provided some of the first documentation of human-enriched cellular features. Later, the application of microscopy to developing neural tissue enabled detailed descriptions of primate neocortical progenitors (Rakic, 1971) and human synapse formation (Huttenlocher, 1979). The identification of enlarged progenitor populations in the developing human cortex was made possible by improvements in culture methods that allowed for *ex vivo* analysis of living cells from fetal tissue (Hansen et al., 2010). Additionally, the ability to reprogram somatic cells into induced pluripotent stem (iPS) cells (Takahashi and Yamanaka, 2006) and to differentiate them into neurons (Zhang et al., 2001) and glial cells (Krencik and Zhang, 2011) is providing unprecedented access to human models from patient-derived cells. Coupled with improvements in three-dimensional (3D) self-organization-based approaches (Sasai, 2013; Pașca, 2018) and rapid genetic engineering approaches (Heidenreich and Zhang, 2016), individual genetic loci can now be readily modeled in diverse human brain cell types. Similarly, advancements in single-cell molecular profiling have led to a new era of quantitative description (e.g., detailed maps of molecular features at cellular resolution) (Stuart and Satija, 2019).

This review will focus on recent work that harnesses these emerging technologies to uncover principles of human brain assembly and how its dysfunction results in neuropsychiatric disease. We first describe cellular and molecular insights identified from *in vivo* specimens with particular emphasis on developmental features that distinguish humans from other species. We then highlight advances in building 3D human brain cultures, such as organoids and assembloids, which provide improved experimental systems to molecularly dissect key developmental stages of brain organogenesis *in vitro*. Lastly, we discuss how these same developmental pathways are altered in

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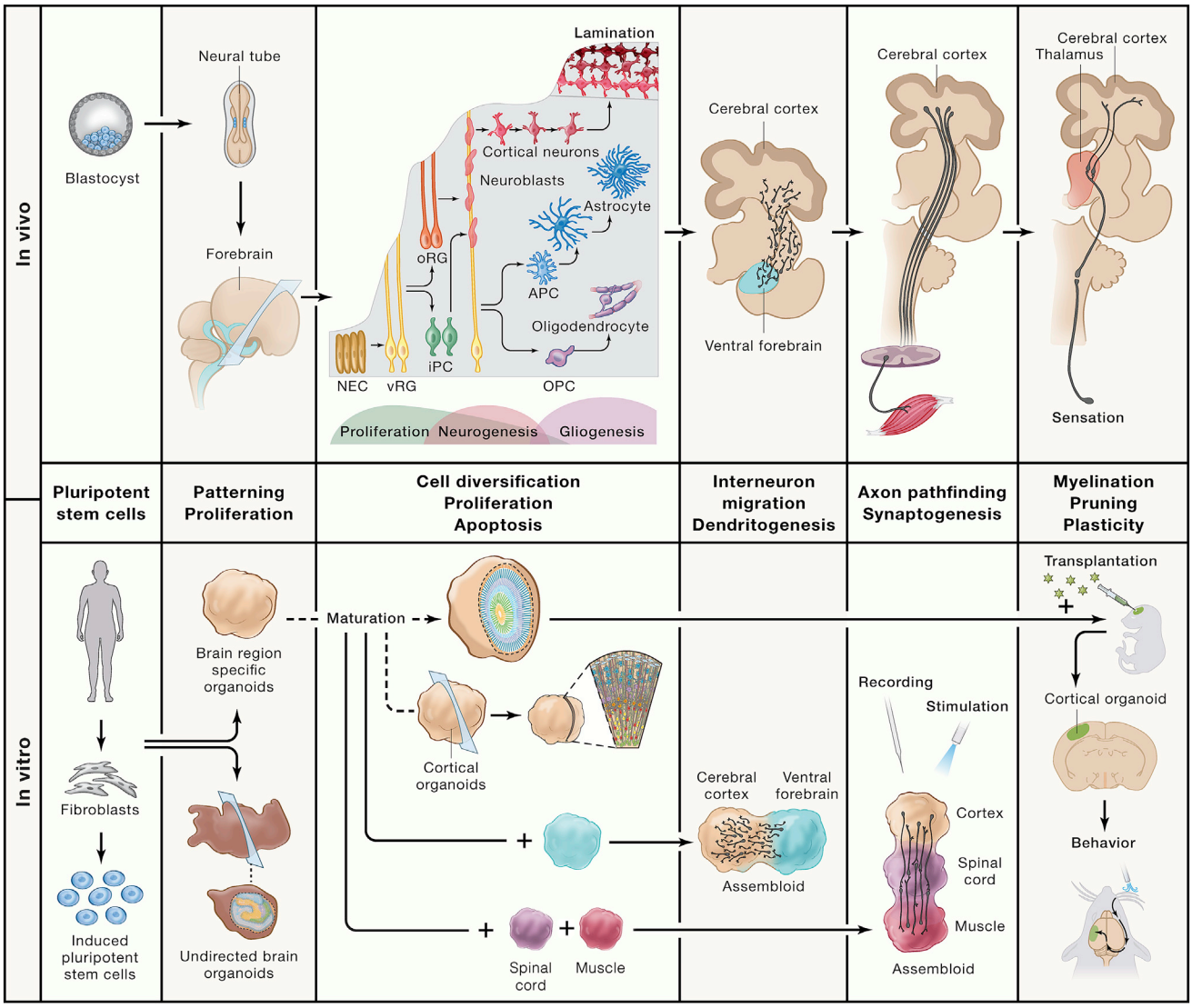

Figure 1. Overview of human brain organogenesis *in vitro* and *in vivo*
Key human brain developmental processes that occur *in vivo* (top) and can be modeled *in vitro* (bottom).

neuropsychiatric disease and consider efforts to uncover pathogenic mechanisms using *in vitro* systems. Moving forward, the combined analysis of both *in vivo*, *ex vivo*, and *in vitro* experimental models will lead to a new and rigorous biological understanding of the human nervous system.

Cellular organization of the developing human brain

The development of the mammalian brain follows conserved developmental processes that govern its cellular organization from pluripotent stem cells to a differentiated and highly complex mature nervous system (Figure 1).

Progenitor cell expansion and specification

Early in development, the anterior neural tube consists of a relatively uniform layer of neuroepithelial cells organized around a ventricular zone (VZ). As organogenesis progresses, these progenitor cells give rise to ventricular radial glia (vRG, also known as apical progenitors), which extend basal processes toward the pial surface that span the entire cortical thickness, while their apical processes maintain VZ contact (Figure 1) (Bystron et al., 2008; Lui et al., 2011). In the early developing cerebral cortex, vRG can self-renew via symmetric divisions or give rise to intermediate progenitor cells (IPCs) or neurons via asymmetric divisions, whereas early neuroepithelial progenitors primarily undergo symmetric cell divisions that expand the progenitor population. As progenitor cells accumulate, the subventricular zone (SVZ) arises and continues to expand through mid-gestation, culminating in the formation of the outer SVZ (oSVZ). The oSVZ is a defining feature of large mammalian cortices and consists of IPCs and progenitors known as outer radial glia (oRG, or basal radial glia progenitors) that lack apical processes but retain contact with the pial surface by extending a long basal process akin to that of vRG (Hansen et al., 2010; Fietz et al., 2010). Mid-way

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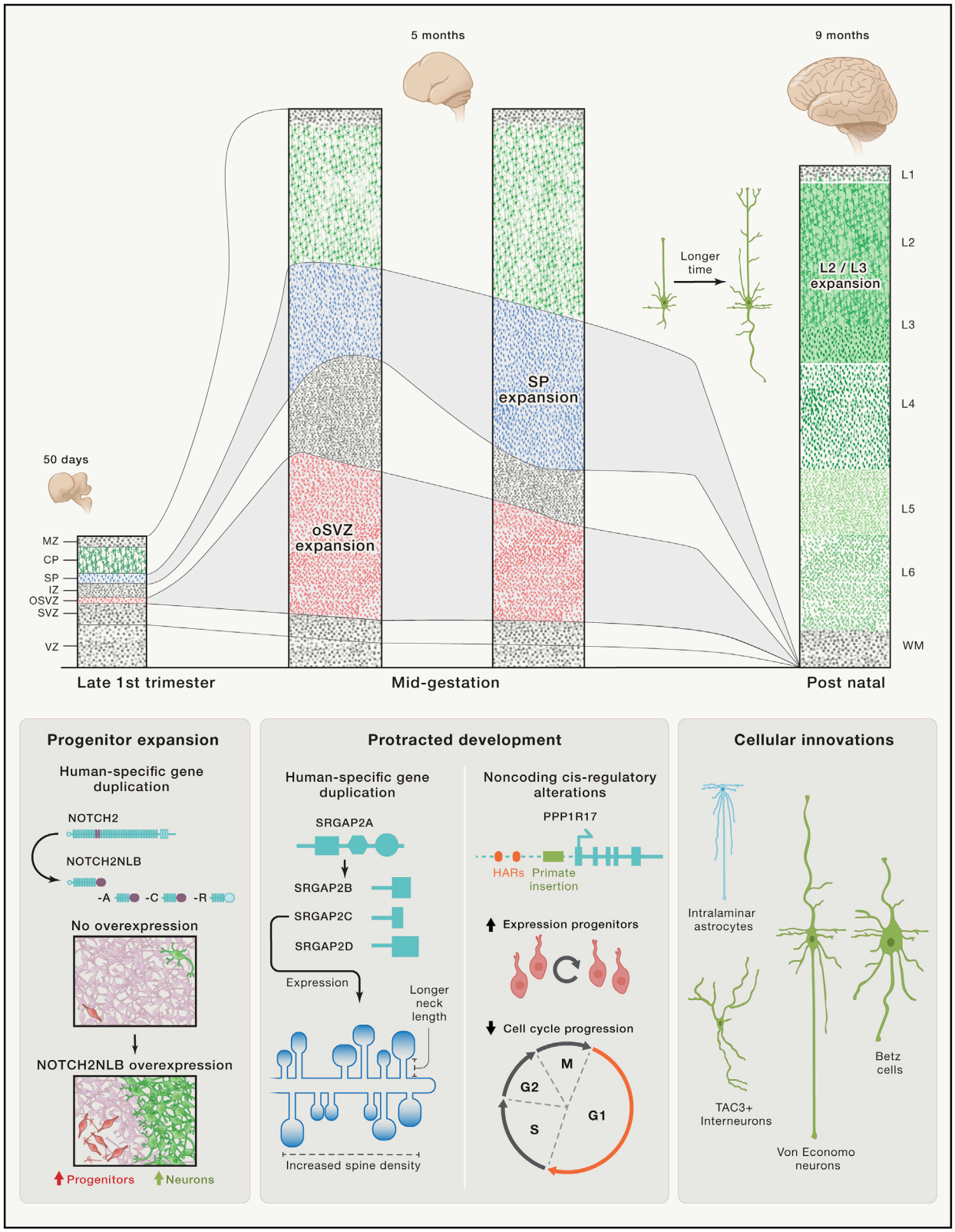

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through the human neurogenic period (i.e., around 17–24 post conceptional weeks, PCW), the RG scaffold becomes discontinuous: vRG detach from the pia surface and retain their VZ attachment, thereby becoming truncated (tRG), and oRG serve as the primary scaffold for neuronal migration (Nowakowski et al., 2016).

The application of single-cell RNA sequencing (scRNA-seq) to primary human fetal tissue has led to an exciting race to uncover the molecular identify of progenitors across regions of developing human cerebral cortex (Nowakowski et al., 2017; Zhong et al., 2018; Polioudakis et al., 2019), hippocampus (Zhong et al., 2020), striatum (Bocchi et al., 2021), midbrain (La Manno et al., 2016), and cerebellum (Aldinger et al., 2021; Cao et al., 2020). These efforts have revealed molecular markers for cells, such as oRG cells, that were previously defined by their morphology, migration, and cellular divisions (Pollen et al., 2015; Camp et al., 2015; Johnson et al., 2015). Subsequent studies have identified molecular features of early neuroepithelial progenitors, proliferative vRG, neurogenic oRG, IPCs, and gliogenic oRG cells (Nowakowski et al., 2017; Zhong et al., 2018; Polioudakis et al., 2019; Eze et al., 2021). Similarly, single-cell variation in oRG morphology (Kalebic et al., 2019), progenitor chromatin states (Ziffra et al., 2021; Trevino et al., 2021), and physiologic responses to neurotransmitter stimulation (Mayer et al., 2019) are beginning to reveal greater heterogeneity than previously appreciated in the progenitor compartment. In contrast, progenitors sampled from early time points of the first trimester across the telencephalon, diencephalon, midbrain, hindbrain, and cerebellum appeared to have largely conserved gene expression patterns (Eze et al., 2021).

Another fundamental question that has been addressed in recent years is how increased neuron numbers and expanded cortical surface area arises in primate evolution. An enlarged SVZ appears to dramatically distinguish humans from rodents with a correlation between oSVZ thickness and cerebral cortex size across mammals (Figure 2) (Lui et al., 2011). An enlarged oSVZ has also been described in the human ventral pallium progenitor zones where cortical interneurons are generated (Hansen et al., 2013). Notably, this expansion of progenitor subtypes is not restricted to the telencephalon, as the developing human cerebellum, where the majority of human CNS neurons reside (Herculano-Houzel, 2010), contains an SVZ with oRG-like cells that are not observed in mouse (Haldipur et al., 2019). These findings suggest that the evolutionary expansion of the human cerebral cortex and cerebellum may be the result of an increase in the number of oRG progenitor cells located in the SVZ. More broadly, the increased proliferative capacity of oRG cells in humans appears to be a key driver of expansion.

Neurogenesis, gliogenesis, and the generation of cellular diversity

Neurons are generated at different rates and times along a rostro-caudal axis throughout the human CNS beginning around 4 PCW in the brainstem and spinal cord, and continuing postnatally in certain regions such as the cerebellum (Silbereis et al., 2016). Single-cell transcriptomics has provided a more in-depth characterization of the molecular progression of newly born excitatory neurons in the developing human prefrontal cortex, from cellular migration to differentiation, and is beginning to identify differential maturation rates of distinct neuronal cell types (Zhong et al., 2018). For example, hippocampal glutamatergic neurons appear to mature more quickly than in the cerebral cortex (Zhong et al., 2020), whereas cerebellar granule cells are more immature and are only just starting to emerge at mid-gestation (Kiessling et al., 2014; Aldinger et al., 2021). At these immature stages, neuronal cell subtypes are less transcriptionally defined compared to their adult counterparts (Li et al., 2018).

How are distinct neuronal cell types across different brain regions generated from similarly appearing progenitor cells? Over the years, multiple models have been proposed to explain the emergence of neuronal diversity, particularly within the neocortex where neurons destined for deep cortical layers are generated prior to upper layer neurons in an inside-out manner (Figure 1). One model suggests that precursors of distinct neuronal subtypes are generated early in development. Another model proposed that distinct neurons are gradually specified from relatively homogeneous progenitor cells (Telley and Jabaudon, 2018). Moreover, extrinsic signals such as thalamic input and morphogen gradients have been implicated in specifying neocortical areal identity (Cadwell et al., 2019; Dehay et al., 2001; Shibata et al., 2021). Single-cell profiling is beginning to provide fresh perspectives on this topic. For example, recent work found individual RG progenitors that appeared transcriptionally "primed" to distinct fates prior to cell division (Polioudakis et al., 2019). In addition, work in rodents suggests that progenitors may change over time to generate neuronal diversity by becoming more receptive to external inputs later in development (Telley et al., 2019). Comprehensive lineage tracing studies, which in principle can be performed by tracking somatic mutations that accrue stochastically during human development (Bizzotto et al., 2021), may help clarify the relative importance of intrinsic and extrinsic signaling pathways and the extent of developmental plasticity in distinct progenitor subtypes.

Non-neuronal cells comprise around half of all cells in the adult human CNS (Azevedo et al., 2009) and consist of oligodendrocytes and astrocytes, in addition to non-CNS-derived microglia and blood vessel cells. A notable feature of the adult human cerebral cortex is the large cerebral white matter consisting of myelinating oligodendrocytes, astrocytes, and endothelial cells

Figure 2. Human-enriched cellular organization

Top: schematic of the developing human neocortical wall highlighting the prenatal enlargement of the outer subventricular zone (oSVZ) and subplate (SP) as well as the expansion of layer 2 and 3 postnatally. Abbreviations: ventricular zone (VZ), intermediate zone (IZ), cortical plate (CP), and marginal zone (MZ). Lower left: overexpression of the human-specific *NOTCH2NLB* gene duplication leads to increased human cortical progenitors and neurons (Suzuki et al., 2018); overexpression of the human-specific *SRGAP2C* gene duplication in mice induces protracted development during spine maturation characterized by increased density of longer spines (Charrier et al., 2012); and *PPP1R17* expression is increased in primate progenitors via putative human accelerated region (HAR) noncoding mutations and regulates neuronal progenitor cell-cycle progression (Girskis et al., 2021). Lower right: schematic of putative human-enriched cell types.

(Rilling et al., 2008; Sousa et al., 2017a). Oligodendrocyte precursor cells (OPCs) and astrocytes are generated from RG cells following neurogenesis in a protracted process that continues postnatally (Jakovcevski et al., 2009; Yeung et al., 2014; Silbereis et al., 2016; Zhu et al., 2018). In developing primate cerebral cortex, the evolutionarily expanded oSVZ appears to produce oligodendrocytes and astrocytes following neurogenesis later in prenatal development (Rash et al., 2019). Are there similar mechanisms for neural and oligodendrocyte cell expansion in the enlarged human cerebral cortex? Single-cell studies combined with *ex vivo* time lapse imaging revealed that in humans, but not in rodents, oRG progenitors are a source of increased OPCs in late second trimester via a pre-OPC state that shares molecular features with oRGs (Huang et al., 2020). Moreover, human OPCs undergo several rounds of symmetric cell division to exponentially increase the progenitor cell population prior to differentiation (Huang et al., 2020). This suggests coordinated cellular mechanisms for augmenting oligodendrogliogenesis to meet the increased neurogenesis demand of an enlarged human brain.

Microglia, the resident macrophages of the human CNS, populate the human cerebrum starting around 4 PCW (Menassa and Gomez-Nicola, 2018) and are increasingly recognized as developmental regulators of synapse formation and neuronal circuit assembly (Thion et al., 2018). Work in embryonic human samples demonstrated that human microglia originate from yolk sac-derived primitive macrophages in a process similar to that observed in rodents (Bian et al., 2020). Developing human microglia are transcriptionally diverse and include a putative transient phagocytic population identified during mid-gestation that was not observed in adult microglia (Kracht et al., 2020). Phagocytic microglia have been associated with myelination in mice and share similarities to transcriptional programs active in neurodegenerative disease (Li et al., 2019; Hammond et al., 2019), hinting at possible ways in which dysfunction in developing microglia could be involved in neuropsychiatric disorders.

Neuronal maturation and circuit formation

The formation of mature neuronal circuits depends on the proper targeting of axons, formation of synapses and their subsequent pruning, as well as axonal myelination (Figure 1). In the developing human neocortex, these processes are remarkably extended in time (Figure 2). Newly born neurons begin to extend axons and dendrites around mid-gestation with continued growth and synaptogenesis occurring well into the first years of postnatal life, followed by myelination, which is ongoing for decades in certain CNS regions (Huttenlocher, 1979; Huttenlocher and Dabholkar, 1997; Koenderink et al., 1994). High-resolution transcriptomic trajectories have demonstrated broad conservation in the dynamic progression of these major neurodevelopmental processes between human and non-human primate neocortex, albeit at protracted rates in humans (Zhu et al., 2018).

In early cortical developmental, immature circuits are generated by intrinsic developmental programs containing spontaneous activity patterns that form the foundation of subsequent mature neuronal networks (Molnár et al., 2020). Pioneering work in cats described the requirement of these early born neurons, located in the subplate, for proper cortical targeting of visual input via thalamocortical axons (Ghosh et al., 1990). Interestingly, these initial subplate neurons are a major site of early prenatal synaptogenesis but are only temporarily present during development, guiding the transition from spontaneous to sensory-driven activity (Molnár et al., 2020). For example, work in mice has shown that subplate neurons form transient synapses that direct radial migration of newly born neurons to the cortical plate (Ohtaka-Maruyama et al., 2018). Subplate neurons display bursts of coordinated electrical activity through gap junction coupling (Moore et al., 2011, 2014) and are morphologically and physiologically mature compared to cortical plate neurons (Zhong et al., 2018). Human thalamic and cortical axons first accumulate in the subplate around the beginning of second trimester followed by infiltration into the cortical plate (Krsnik et al., 2017), which occurs after dendrite formation (Mrzljak et al., 1988). Notably, a distinguishing feature of primate neocortical development is an enlarged subplate (Figure 2), suggesting possible primate specializations in circuit formation (Molnár et al., 2020). Dendritic spines start to form later in the second trimester after thalamic input has been established, with rapid growth continuing between 3 to 24 months postnatally depending on the cortical region (Huttenlocher and Dabholkar, 1997). Subsequent synaptic pruning, morphologic refinement, and circuit maturation occurs in the ensuing years to decades in the prefrontal cortex (Huttenlocher, 1979).

Cellular innovations in human brain function

The cognitive abilities of humans are arguably what most distinguish us from other mammals. Yet the cellular adaptations that have arisen along the human lineage, which could include human-specific cell types, functional specializations in conserved cell types, and/or differences in the abundances of shared cell types, remain largely uncharacterized (Sousa et al., 2017a). Recently, comparative single-cell molecular and electrophysiological approaches across humans, nonhuman primates, and rodents have begun to shed light on these differences in a comprehensive manner.

One of the earliest large single-cell transcriptomic comparative studies (Hodge et al., 2019) demonstrated remarkable conservation across all major cell types between adult human and mouse neocortical regions. This and subsequent work revealed frequent species specializations in the compositional makeup of conserved cell types with greater differences among more evolutionarily distant organisms. One striking example is the relative increase in the proportion of cortical GABAergic interneurons compared to glutamatergic neurons in humans compared to nonhuman primates and mice (Krienen et al., 2020; Bakken et al., 2021). Interestingly, this may reflect a human expansion of CGE generated cortical interneurons (Hansen et al., 2013), which primarily populate the upper cortical layers. This is also intriguing given that upper cortical layers are enlarged in primates compared to other species (Figure 2) (Marín-Padilla, 2014; Berg et al., 2021). Alternatively, this increase in interneurons may also be explained by a late migrating population of human interneurons, not observed in rodents, that enters the cortex in the early postnatal period (Paredes et al., 2016). Compositional changes have also been observed in more evolutionary ancient structures such as the cerebellar nuclei, which underwent a massive expansion in humans of a conserved excitatory cell

class that projects to the lateral frontal cortex in mice (Kebschull et al., 2020).

Are there primate- or human-specific cell types? A surprising result from recent comparative transcriptomic studies is the identification of primate innovations in the more evolutionarily ancient basal ganglia. For example, single-cell transcriptomics identified an abundant, TAC3-positive striatal interneuron population with no apparent mouse homolog that represents approximately one-third of all sampled interneurons in marmosets and humans (Krienen et al., 2020). In a separate study, utilizing bulk transcriptomics across humans, chimpanzees, and rhesus macaques, a cortical cell type expressing tyrosine hydroxylase, previously associated with human disease (Paşca et al., 2011), was found to be enriched in human striatum and cortical regions compared to nonhuman primates (Sousa et al., 2017b). Similarly, single-nucleus transcriptomics coupled with detailed morphological and electrophysiological analyses identified a "rosehip" shaped GABAergic interneuron located in human cortical layer 1 without a mouse homolog (Boldog et al., 2018). Non-neuronal cells also contain human-specific morphological features as well as substantial gene expression differences when compared to rodents (Kelley et al., 2018). Interlaminar astrocytes, which are much larger and have longer processes in humans compared to non-human primates (Figure 2) (Oberheim et al., 2009), are now beginning to be molecularly characterized in adult humans (Hodge et al., 2019).

Multimodal measurements combining morphology, physiology, and single-cell transcriptomics are starting to provide the first comprehensive molecular description of long-observed but minimally characterized primate cellular specializations (Figure 2). For example, von Economo neurons (VENs) are large cells found in the deep layers of the cingulate and insular cortices of primates, cetaceans, and elephants, but absent in rodents (Seeley et al., 2012; von Economo and Koskinas, 1926). Loss or dysfunction of VENs has been proposed to contribute to the behavioral impairments seen in some neuropsychiatric disorders, such as frontotemporal dementia (Kim et al., 2012). Nearly 100 years after their initial identification, single-cell multimodal measurements demonstrated that VENs are likely subcortically projecting excitatory neurons by homology mapping to rodent cells, which suggests that VENs arose from diversification of conserved deep layer projection neurons (Hodge et al., 2020). Analogous experiments conducted on Betz cells (Betz, 1874), an unusually large deep layer corticospinal neuron found in primate and carnivore motor cortex, have molecularly identified them as layer 5 subcortical projecting glutamatergic neurons (Bakken et al., 2021).

Are there functional differences that are unique to human neurons? Technological advances in the preservation and *ex vivo* interrogation of neurosurgically derived adult brain tissue is also leading to an unprecedented characterization of human neuron physiology. Compared to rodents, human supragranular pyramidal neurons are larger, have more complex dendritic branching (Deitcher et al., 2017; Mohan et al., 2015), and display a type of dendritic calcium wave that has not been previously observed (Gidon et al., 2020). Electrophysiological differences in passive membrane properties in these cells were found to be mediated through h-channels, with human enriched expression of HCN1 compared to mouse (Kalmbach et al., 2018). Similarly, electrical recordings from layer 5 pyramidal neurons suggest that the increased dendritic length in human cells alters input-output properties and cortical computation (Beaulieu-Laroche et al., 2018). Single unit recordings obtained in behaving humans and macaques also revealed functional differences between species, with more efficient information encoding in human cingulate and amygdala neurons (Pryluk et al., 2019). Overall, the extent of observed human functional differences, even among putatively similar cell types, suggests the importance of cross-species investigations for a complete understanding of human physiology.

Molecular regulation of human brain development

The human genome provides the foundational blueprint that governs the molecular processes underlying brain organogenesis. The past decade has seen the development of high-throughput sequencing technologies that have enabled the initial characterization of the precise timing and location of human genome regulation during brain development, as determined by epigenome and transcriptome profiling. These molecular atlases are providing important foundational groundwork for refining our understanding of human brain evolution and disease.

Bulk tissue transcriptome profiling across human cortical development has highlighted large-scale molecular changes that occur in distinct time windows (Colantuoni et al., 2011; Kang et al., 2011; Jaffe et al., 2018; Werling et al., 2020; Li et al., 2018). How many genes are dynamically regulated across time? It seems that approximately two-thirds of all protein coding genes are developmentally regulated (Li et al., 2018). Remarkably, the majority of this temporal variation in the human neocortex occurs during two periods: an early fetal transition from PCW 6–10 that is associated with the establishment of regional identity and a late fetal transition that continues through infancy that corresponds to a critical transition point (Werling et al., 2020). Convergent evidence from these distinct gene expression cohorts, in addition to methylation data (Lister et al., 2013; Jaffe et al., 2016), and alternative splicing analyses (Li et al., 2018), point to the late-fetal period as a highly coordinated and massive molecular transition that distinguishes prenatal and postnatal development. Transcriptomic data from the developing rhesus macaque brain (Zhu et al., 2018) has also identified a similar molecular transition around birth, suggesting conserved mechanisms in primate neurodevelopment. Cellular compositional changes, such as astrogliogenesis and myelination, in addition to neuronal maturation processes, such as synaptogenesis and dendritogenesis, could explain the observed molecular changes during this period. It remains unclear whether this molecular transition is driven by birth and its environmental changes or by pre-programmed intrinsic developmental processes.

Are there human-specific molecular differences during neurodevelopment? Comparative analyses of orthologous genes across mammalian organogenesis have indicated that the evolution of gene expression in the brain might occur more slowly than in other organs (Brawand et al., 2011; Cardoso-Moreira et al., 2019). This is consistent with the general conservation of broad neurodevelopmental expression programs observed between humans and non-human primates (Bakken et al., 2016; Zhu

et al., 2018). However, it is difficult to infer cell-type-specific transcriptional changes from bulk tissue analyses and it remains challenging to match different species across developmental time (Workman et al., 2013). For example, comparing single-cell gene expression data between prenatal human and macaque cortex revealed that many of the interspecies differences identified at the bulk tissue level were most likely a result of variation in cellular composition rather than molecular differences between cell types (Zhu et al., 2018). This contrasts with comparative adult brain single-cell gene expression analyses, which have identified substantial changes among homologous cell types in human, non-human primates, and rodents (Hodge et al., 2019; Krienen et al., 2020; Bakken et al., 2021). Given the larger sample sizes and higher coverage sequencing in the adult studies, it remains unclear whether species differences within similar cell types are less pronounced during prenatal development. A complementary avenue is the search for evolutionary changes in the noncoding gene regulatory architecture of developing human neural tissue (Reilly et al., 2015; Won et al., 2016; de la Torre-Ubieta et al., 2018). This molecular layer of regulation will be essential for determining the downstream functional consequences of the human-specific genetic elements discussed below.

Molecular innovations in human brain development

Identifying the genetic features responsible for human brain evolution is a significant challenge. The search for causal molecular mechanisms underlying human brain evolution can broadly be described by two general approaches: (1) utilizing comparative transcriptomics as an entry point to deconstructing human-enriched molecular pathways and (2) investigating downstream biological consequences of human-specific genetic features. The second approach has focused on protein coding genes with human-specific duplications (Dennis et al., 2017) or deletions (McLean et al., 2011), as well as conserved non-coding genomic regions with increased human substitutions, known as human accelerated regions (HARs) (Pollard et al., 2006).

The characteristic enlargement of the human neocortex is thought to partially be dependent on expanded progenitor zones, particularly an enlarged oSVZ (Lui et al., 2011). What is the molecular basis for human cortical progenitor expansion? Interestingly, several groups have implicated new human-specific gene paralogs that have arose from segmental duplications as drivers of cortical progenitor expansion. For example, three human-specific paralogs of the NOTCH2 receptor are expressed in primary human fetal RG, including oRG cells located in the oSVZ, during corticogenesis (Fiddes et al., 2018; Suzuki et al., 2018). Overexpression of *NOTCH2NLB* in cortical progenitor cultures led to clonal expansion of progenitors through activation of the Notch pathway via cell-autonomous inhibition of Delta/Notch interactions (Suzuki et al., 2018). Deletion of *NOTCH2NL* in human cortical organoids resulted in premature neuronal maturation (Fiddes et al., 2018), altogether suggesting *NOTCH2NL* may have evolved to support progenitor self-renewal in humans (Figure 2). In support of the involvement of Notch signaling in neocortical primate evolution, recent work has identified a link between the periodicity of neural progenitor Notch signaling and gyrencephaly (Han et al., 2021). Similarly, ectopic expression of the human-specific *ARHGAP11B* in mice (Florio et al., 2015) or marmosets (Heide et al., 2020) led to increased oRG numbers and neocortical expansion. In a third example, gain- and loss-of-function experiments of human duplications of *TBC1D3* modulated oRG generation and cortical expansion (Ju et al., 2016). Whereas *NOTCH2NL* appeared to have more general effects on cortical progenitor expansion, *ARHGAP11B* and *TBC1D3* were more specific to oRG expansion phenotypes, hinting at non-mutually exclusive molecular innovations in human progenitor expansion.

Human-specific gene duplications have also been implicated in the evolution of other neurodevelopmental processes. Partial duplications of the gene encoding the ancestral SLIT-ROBO Rho GTPase-activating protein 2 (SRGAP2) are also implicated in human brain development (Charrier et al., 2012; Dennis et al., 2012). Ectopic expression of *SRGAP2C* in developing mice surprisingly extends the period of dendritic spine formation, mimicking some aspects of protracted neuronal development seen in humans. SRGAP2C appears to inhibit the ancestral SRGAP2, which has roles in dendritic spine maturation (Figure 2). Intriguingly, mice modified to express *SRGAP2C* in cortical glutamatergic neurons displayed increased intracortical circuit connectivity and enhanced sensory learning, further suggesting that the emergence of *SRGAP2C* in humans has functional consequences in brain evolution (Schmidt et al., 2021).

Another driver of phenotypic evolution is divergent expression of functionally conserved proteins, presumably through alterations in non-coding gene regulatory structure (Carroll, 2008). Comparative bulk transcriptome analyses identified the platelet-derived growth factor D (PDGFD) as being highly enriched in human compared to mouse RG cells and functionally important for progenitor cell expansion in human but not mouse cortex (Lui et al., 2014). More recent comparative scRNA-seq between non-human primate and human fetal cerebral cortex and organoid models suggested increased mammalian target of rapamycin (mTOR) signaling in human RG as a candidate mechanism for human-enriched progenitor expansion (Pollen et al., 2019). What are the regulatory mechanisms that drive these expression differences? For the vast majority of differentially expressed genes described in comparisons with humans, the suspected underlying non-coding genetic alterations are unknown. One notable example, however, is the identification of a human accelerated regulatory enhancer of *FZD8*: a WNT pathway modulator that altered neuronal progenitor dynamics and increased brain size when ectopically expressed in mice (Boyd et al., 2015). High-throughput reporter assays are further elucidating the regulatory activity of thousands of human-specific genomic sequences in human neuronal progenitor cells (Girskis et al., 2021; Uebbing et al., 2021). One such approach identified *PPP1R17* as a putative HAR-regulated gene in primate cortical progenitors and revealed that *PPP1R17* modulates neuronal progenitor cell-cycle progression, mirroring the increased cell-cycle length and protracted development observed in humans (Figure 2) (Girskis et al., 2021). Novel comparative model systems, such as the generation of human-chimp hybrid pluripotent stem cells and their differentiation into cortical lineages, could aid in efforts to untangle the *cis* regulatory factors leading to human-enriched gene expression (Agoglia et al., 2021).

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Modeling human brain organogenesis *in vitro*

Our understanding of human brain development has traditionally come from descriptive observation or has been inferred from findings in model organisms. The mechanistic principles of human brain organogenesis have been constrained by limited tissue access and lack of functional *in vitro* preparations. Advances in stem cell technology provide an alternative avenue for systematically deciphering the developmental rules that govern human organogenesis (Sasai, 2013; Clevers, 2016; Shahbazi and Zernicka-Goetz, 2018). Exploiting the remarkable abilities of stem cells to form self-organizing 3D cultures, known as organ spheroids or organoids, is driving the generation of better models of human brain development (Paşca, 2018).

Historical overview of three-dimensional cellular models

It has been known for decades that isolated mammalian fetal brain cells can be maintained *in vitro* to form highly organized aggregates, and co-aggregates, that resemble *in vivo* differentiated structures such as the cortex, hippocampus, and neuromuscular junction (DeLong, 1970; Crain et al., 1970; Garber, 1972). However, breakthroughs in the ability to both culture embryonic stem cells (Evans and Kaufman, 1981; Thomson et al., 1998) and create iPS from somatic cells (Takahashi and Yamanaka, 2006) along with improvements in generating neural lineages from pluripotent stem cells (Chambers et al., 2009) have ushered in a new era of modeling human brain development and disease. The early applications of this technology used directed differentiation of human stem cells into two-dimensional (2D) neuronal cultures, which could be patterned with small molecules and growth factors to generate various neuronal types, including cortical, striatal, or midbrain like cells (Mertens et al., 2016). Protocols to rapidly generate large numbers of 2D human neuron cultures by forced expression of transcription factors (Zhang et al., 2013) enable large-scale pharmacologic screens (Wang et al., 2017). However, intricate cell-cell and cell-extracellular matrix (ECM) interactions are challenging to recapitulate in 2D cultures, leading to *in vitro* morphological artifacts, such as the flattening of cells and alterations in the polarity and proliferation rate of progenitors (Paşca, 2018). Moreover, 2D cultures tend to be more homogeneous in cellular composition and are difficult to maintain for the timescales necessary to model maturing human neuronal properties, circuit formation, and other late developmental processes, such as gliogenesis.

Three-dimensional cultures that rely on the inherent self-organizing properties of progenitors (Sasai, 2013) have emerged in recent years as an alternative approach to 2D systems to better recapitulate the cellular interactions, diversity, and tissue architecture of early development. One of the first examples of *in vitro* complex tissue construction was the generation of optic cup structures around a decade ago (Eiraku et al., 2011). The resulting aggregates demonstrate cytoarchitectural similarity to human organs and were later termed organoids. Protocols to generate human brain organoids from human iPS (hiPS) cells have been refined over the last decade and can be generally categorized following two different approaches: unguided and guided differentiation (Figure 1). Unguided cultures take inspiration from development's own intrinsically regulated progression, allowing human pluripotent stem (hPS) cells to differentiate in an embedded ECM without exogenously added cues (Lancaster et al., 2013). These organoids exhibit a stunning amount of cellular diversity, forming various forebrain, diencephalon, and hindbrain structures, in addition to retina and non-ectodermal cells. However, undirected organoids exhibit large variability in cell composition across differentiations due to the stochasticity and inconsistency of neural induction, making disease modeling more difficult (Camp et al., 2015). In contrast, guided differentiation approaches rely on the exogenous enforcement of known developmental signals, starting with early neuroectodermal induction by SMAD inhibition (Chambers et al., 2009) and subsequent specification into regionalized organoids with the addition of patterning morphogens. This directed differentiation approach can also be started in 2D and subsequently continued in 3D cultures (Mariani et al., 2015) or initiated in 3D followed by plating in 2D (Kadoshima et al., 2013). Our laboratory introduced a straightforward, matrix-free approach for deriving dorsal forebrain in 3D by neuralization and culture exclusively in suspension (Paşca et al., 2015). This approach demonstrated high reproducibility across lines (Yoon et al., 2019) and experiments with progression to early post-natal developmental stages in long-term cultures (Sloan et al., 2017; Trevino et al., 2020; Gordon et al., 2021). Subsequently, other approaches have been used to grow region specific organoids in mini-reactors (Qian et al., 2016) or in slice preparations (Qian et al., 2020).

Regional specification and the generation of cellular diversity

The patterning of the vertebrate brain into distinct territories and regions depends on the precise timing and combination of key signaling molecules as ectodermal cells acquire specialized identities (Rowitch and Kriegstein 2010; Beccari et al., 2013). Work in model systems has demonstrated the presence of conserved signaling centers, such as the roof plate and floor plate, which are the sources of morphogen gradients that establish positional identity along the neuraxis. Bath application of morphogens to mimic normal development has been used to generate regionalized human organoids (Figure 3) resembling the cerebral cortex (Paşca et al., 2015; Mariani et al., 2015; Qian et al., 2016), striatum (Miura et al., 2020), hippocampus (Sakaguchi et al., 2015), choroid-plexus (Pellegrini et al., 2020), thalamus (Xiang et al., 2019), hypothalamus (Qian et al., 2016; Huang et al., 2021), midbrain (Jo et al., 2016; Monzel et al., 2017; Qian et al., 2016), cerebellum (Muguruma et al., 2015), and spinal cord (Ogura et al., 2018; Andersen et al., 2020). Similarly, the creation of precisely controlled WNT gradients can also model the emergence of rostral-caudal neural identity (Rifes et al., 2020). The combinatorial addition of morphogens (Tchieu et al., 2017) and small molecules (Qi et al., 2017) in an unbiased fashion has the potential to further elucidate developmental rules for cellular specification and to generate regions and subnuclei that have thus far eluded 3D *in vitro* construction.

The cellular composition of cortical organoids has been studied using both bulk and scRNA-seq in combination with histological approaches. At early time points, cortical organoids reproduce aspects of human neuroepithelium physiology, including basal process retraction during mitosis (Subramanian et al., 2017), and contain progenitor cell clusters that resemble those found

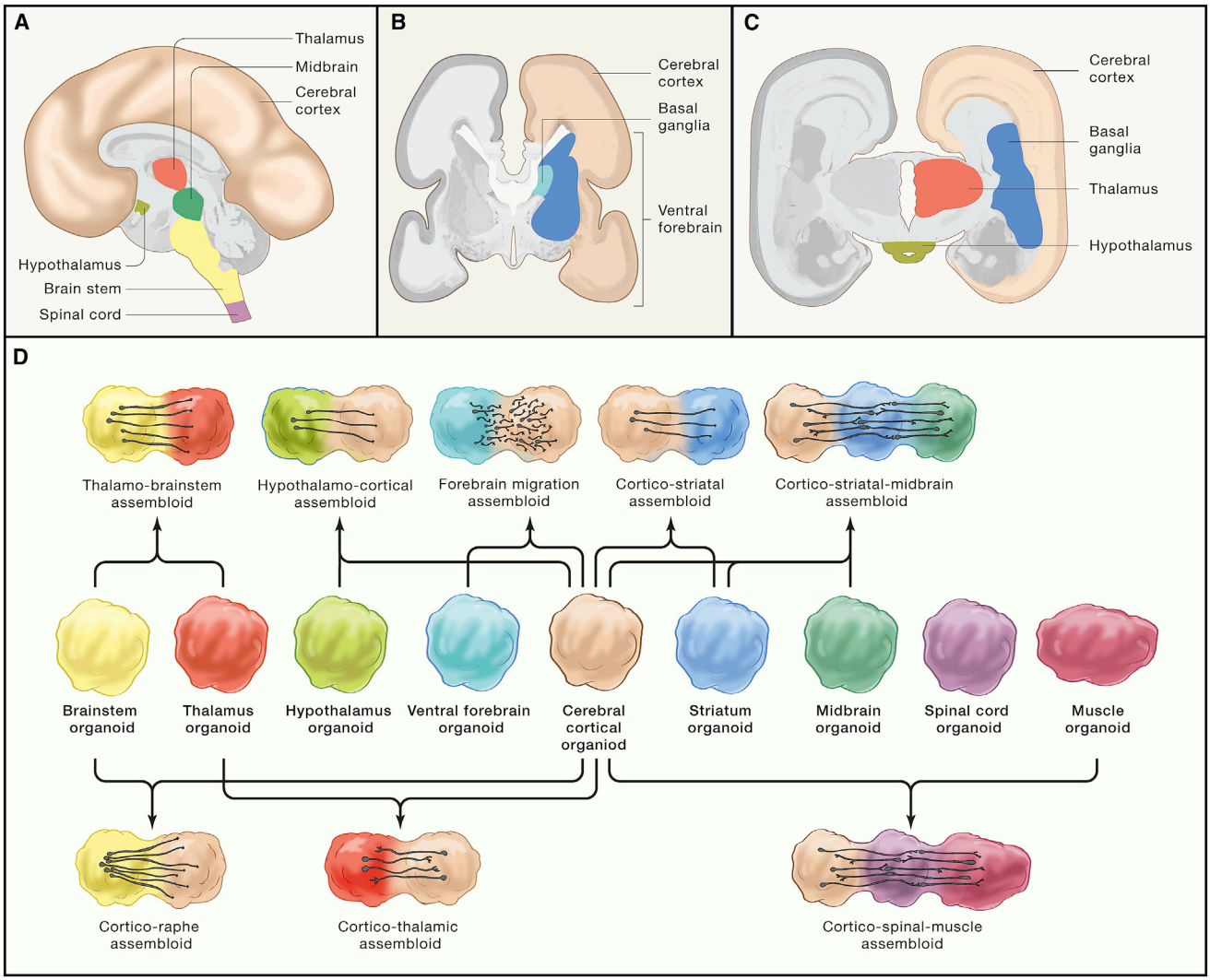

Figure 3. Building human neuronal circuits in assembloids
(A–C) Schematic of the human prenatal brain at around PCW 23 at (A) sagittal, (B) anterior coronal, and (C) more posterior coronal sections.
(D) Schematic of various assembloids.

in primary human fetal samples (Eze et al., 2021). At ~2–3 months *in vitro*, cortical organoids contain cellular clusters of neural progenitors, including vRG, oRG, and IPCs in addition to deep and superficial layer excitatory neurons (Paşca et al., 2015; Qian et al., 2016). As cortical organoids further develop, astrocytes are born that, if maintained for hundreds of days, can mature transcriptionally and functionally to resemble postnatal human astrocytes (Sloan et al., 2017). To promote OPC expansion and oligodendrocyte maturation *in vitro*, the addition of supplemental signaling factors, such as PDGF and T3, is required (Madhavan et al., 2018; Marton et al., 2019). Importantly, as oligodendrocytes in 3D cultures form near neurons, the myelination of human axons has also observed in forebrain organoids.

Although cortical organoids are currently the most well characterized 3D system, cellular diversity has also been observed in unguided organoid protocols and regionalized cultures. scRNA-seq of unguided whole-organoid samples demonstrated early expression of markers of multiple regions, including forebrain, midbrain, hindbrain, retina, and non-ectodermal cells (Quadrato et al., 2017). A challenge inherent in these comparisons is how to robustly establish the cellular and regional identity of scRNA-seq-derived clusters given relatively limited ground truth primary data. A newly developed approach (Fleck et al., 2021) shows promising data-driven annotations by mapping scRNA-seq clusters to a high-resolution mouse developmental *in situ* atlas, and this can, in principle, be deployed to future gene expression references. This approach has demonstrated the presence of telencephalon, midbrain, diencephalon, and hindbrain regional signatures in undirected organoids (Kanton et al., 2019). Similarly, directed striatal organoids contain convincingly map to developing striatum (Miura et al., 2020) and spinal organoids recapitulate some of the specific dorsal and ventral progenitor domains seen in primary tissue (Andersen et al., 2020).

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How accurately do human organoids reflect early prenatal *in vivo* development? Despite the relative simplicity of cortical organoids, which lack sensory input and non-ectodermal cells like microglia and vascular cells, transcriptional comparisons with prenatal primary human samples have demonstrated remarkable preservation of major cell classes and gene expression programs (Camp et al., 2015; Pollen et al., 2019; Sloan et al., 2017; Velasco et al., 2019; Tanaka et al., 2020; Gordon et al., 2021). A recent report has suggested the presence of an *in vitro* upregulated metabolic signature in organoids (Bhaduri et al., 2020). Notably, some of these changes were not present in all protocols, and separate experiments with cortical organoids maintained for up to 21 months *in vitro* have suggested these gene expression differences reflected homeostatic alterations from the culture microenvironmental rather than a deleterious state of cell stress (Gordon et al., 2021).

Human brain assembloids

Human brain construction depends on the precise spatiotemporal integration of distinct cellular lineages and structures, ultimately resulting in interconnected circuits that can span meters in adults. For example, coordinated movement is controlled by networks of diverse neurons and muscle cells across multiple cortical and spinal systems. Proper brain formation also relies on the migration and incorporation of cell types generated in deep structures of the CNS but also outside the CNS, as is the case for the yolk-sac derived microglia and the mesoderm-derived vascular cells. In addition, certain ectodermal cell types, such as inhibitory neurons and OPCs, are known to migrate widely from their region of birth to their final location. The inability to model these intricate cellular interactions in humans has led to emerging approaches to generate brain assembloids—the controlled assembly of regionalized organoids and multiple cell lineages in 3D (Pașca, 2019).

Initial assembloid efforts focused on modeling cell migration. During development, interneurons are specified in ventral forebrain progenitor domains prior to their migration and integration into the dorsal cortex. This process occurs into infancy, and deficits are hypothesized to result in several neuropsychiatric disorders (Marín, 2012). To model these cellular interactions *in vitro*, our lab developed multi-region assembloids by fusing dorsal and ventral forebrain organoids and allowing GABAergic interneurons to migrate, and functionally integrate into cortical circuits *in vitro* (Birey et al., 2017). A similar approach has been used by other laboratories to model interneuron migration (Bagley et al., 2017; Xiang et al., 2017).

Subsequent assembloid work has attempted to model long-range axonal connectivity by integrating organoids to study thalamo-cortical (Xiang et al., 2019), cortico-striatal (Miura et al., 2020), hypothalamic-pituitary (Kasai et al., 2020), and cortico-spinal-muscle (Andersen et al., 2020) interactions. Interestingly, circuit assembly mimicked *in vivo* axonal pathways with the creation of reciprocal thalamocortical projections and unidirectional projections from cortex to striatal organoids. Moreover, assembly of cortico-striatal and hypothalamic-pituitary organoids appeared to accelerate striatal neuron and pituitary maturation, respectively, suggesting the importance of specific synaptic connections for proper circuit formation. How many regions can be functionally assembled? A three-part system consisting of human cortico-spinal-muscle assembloids has been shown to form a functional di-synaptic human circuit that controls muscle contraction (Andersen et al., 2020). In this preparation, deep layer cortical neurons connected synaptically with spinal motor neurons that subsequently innervated muscle cells, forming circuits that could be manipulated optogenetically to control muscle contraction. Retinal ganglia cells have also been shown to project into thalamic-like organoids that were fused to cortical organoids (Fligor et al., 2021). We envision building on this modular approach to create ever more complex assembloids, including the addition of brainstem nuclei, in order to incorporate neuromodulation of circuit formation and its dysfunction in patient cells (Figure 3).

A major limitation of current region-restricted and unguided organoids is the lack of well-defined positional axes that are essential for the creation of ordered brain structure *in vivo*. Precise gradients of morphogen signaling molecules emitted from organizing centers are key for the establishment of positional identity. An exciting avenue is the assembly of distinct organizer-like structures that can be integrated into organoids to induce positional axes in polarized assembloids. For example, an engineered SHH-expressing stem cell line ventralized forebrain organoids in a distance-dependent manner akin to the *in vivo* dorso-ventral gradient of SHH (Cederquist et al., 2019). We expect that the development and assembly of future *in vitro* organizers, such as floor plate, roof plate, or anterior neural pore organizer cells, will allow for more complex and controlled structure formation, in addition to making accessible novel models of human axon guidance.

Interactions with cell lineages specified outside of the nervous system are becoming increasingly recognized as important contributors to neurodevelopment and disease. To more accurately model the compositional diversity of the developing human brain, non-ectodermal cell types or their progenitors can be directly added to brain organoids at different stages of differentiation to create multi-lineage assembloids. For instance, to promote the vascularization of cortical organoids, introduction of human umbilical endothelial cells (Shi et al., 2020), mesodermal progenitors (Wörsdörfer et al., 2019), or stem cells with forced expression of a lineage converting transcription factor (Cakir et al., 2019) formed organoids with vasculature-like structures. The introduction of umbilical endothelial cells or mesodermal progenitors also provides a method for modeling human neuro-immune interactions in development. An alternative approach to model this crosstalk is the direct addition of microglia-like cells, which was shown to model specific mutations associated with Alzheimer's disease (Lin et al., 2018). Similarly, directly adding pericyte-like cells to cortical organoids was shown to support astrocyte maturation and proved to be an enhanced model of infection with severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2) (Wang et al., 2021). The assembly of three cell lineages to create a 3D blood-brain barrier (BBB) consisting of astrocytes, endothelial cells, and pericyte-like cells provides another example of striking self-assembly properties *in vitro* (Blanchard et al., 2020).

Neuronal maturation and network activity in vitro

The emergence of mature neuronal electrical properties is a long and protracted process in developing humans, raising the

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question of whether neuronal physiology can be appropriately modeled *in vitro*. Because the timing of developmental maturation *in vitro* is similar to that *in vivo*, long-term culture methods (months to years) are necessary to fully recapitulate these molecular trajectories. Several approaches have been developed to promote neuronal cell viability and development, including transplantation into rodents (Espuny-Camacho et al., 2013; Mansour et al., 2018; Real et al., 2018; Linaro et al., 2019), protocols to increase nutrient delivery (Giandomenico et al., 2021; Qian et al., 2020), and the forced expression of lineage promoting transcription factors to accelerate neuronal maturation (Zhang et al., 2013). How long have human neurons been maintained? Human cortical neurons transplanted into mice have been assayed through 11 months post transplantation (Linaro et al., 2019) and cortical organoids have been cultured *in vitro* for as long as 21 months (Gordon et al., 2021).

How well do *in vitro* derived human neurons model key developmental milestones? Electrical activity is rarely observed in earlier stages of cortical organoid differentiation, but spiking has been noted after ~3 months in culture (Paşca et al., 2015; Fair et al., 2020). Morphological and gene expression analysis throughout these ages of differentiation also demonstrated structural features of maturing neurons, including the presence of dendritic spines (Quadrato et al., 2017). It remains unclear whether later stages of neuronal maturation including the development of large complex morphologies, dynamic dendritic remodeling, and synaptic plasticity can be achieved *in vitro*. Longer-term cultures, which have demonstrated some molecular hallmarks of human postnatal development, such as changes in NMDA subunit composition (Gordon et al., 2021), may be sufficient to model some of these aspects. Transplantation of individual neuronal cells into rodent hosts has shown remarkable maturation of human neurons, including large dendritic arbors, and functional integration into mouse visual circuits (Linaro et al., 2019). Human brain organoids have also been transplanted into adult mice, which led to their vascularization and synaptic integration with host cells (Mansour et al., 2018), although more mature neuronal features remain to be investigated.

Modeling human neuropsychiatric disorders

Recent progress in gene discovery using large patient cohorts has begun to outline the genetic underpinnings of neuropsychiatric disorders (Sullivan and Geschwind, 2019). Genetic variation in common alleles, as well as rare protein coding and copy number variants (CNVs), is increasingly being implicated in various disorders. For example, dozens of rare protein-altering mutations have been found in individuals with developmental delay, autism spectrum disorder (ASD) or epilepsy (Deciphering Developmental Disorders Study, 2017; Heyne et al., 2018; Coe et al., 2019; Satterstrom et al., 2020). Similarly, large numbers of common risk-alleles have been robustly associated across neuropsychiatric disorders. How do these genetic variants influence disease risk? The downstream molecular consequences of most disease-associated variants remain unknown. Relevant model systems that allow for the direct interrogation of disease variants are key to elucidating the biology of neuropsychiatric disorders (Figure 4).

Cellular pathways altered in disease

In parallel to gene discovery efforts, high-resolution molecular mapping of human brain tissue and *in vitro*-derived cells are beginning to annotate putative cell types and developmental epochs enriched with neuropsychiatric disease variants (Wang et al., 2018; Amiri et al., 2018; Li et al., 2018; Trevino et al., 2020, 2021). ASD genes identified from rare protein coding variants appear to show convergent expression in prenatal cortical samples with enrichment in maturing glutamatergic and GABAergic neuronal cell classes, hinting that dysfunction in the development of these cell types leads to disease (Parikshak et al., 2013; Willsey et al., 2013; Polioudakis et al., 2019; Satterstrom et al., 2020). In contrast to rare coding variants, the biological pathways by which common variants influence disease risk are more challenging to decipher, given that they occur mostly in noncoding regions of the genome. To annotate common variants, there has been some success in identifying putative disease genes by associating variants to gene expression changes (i.e., eQTLs) in adult and fetal brain (Walker et al., 2019; Werling et al., 2020) or by defining regulatory relationships through measuring open chromatin states, histone modifications, and chromatin interactions (de la Torre-Ubieta et al., 2018; Won et al., 2016; Song et al., 2020). These types of analyses can nominate specific cell types and pathways as drivers of disease pathogenesis. For instance, schizophrenia-associated common variants have been linked to synaptic pruning via the complement pathway (Sekar et al., 2016). Novel hypotheses can also be generated such as the surprising finding that common variants associated with schizophrenia converged on placental biology and pathways responsive to environmental stress (Ursini et al., 2018), offering insights into potential gene-environment interactions that increase disease risk. Similarly, associated variants might point to dysfunction in specific cell types such as the unexpected enrichment of Alzheimer's disease genetic signals in regulatory regions of genes expressed in adult microglia (Young et al., 2021; Nott et al., 2019). Linking common variants associated with psychiatric disorders to specific genes was a major goal of the psychENCODE Consortium, which profiled and integrated a large collection of functional genomic measurements from human brain tissue and found that schizophrenia associated common variants were linked via multiple regulatory measurements to putative genes showing enrichment in adult excitatory neuron markers (Li et al., 2018; Wang et al., 2018).

How do disease-associated variants impact nervous system development and function? Molecular mechanisms of disease are generally interrogated by analysis of post-mortem tissue from individuals with pathogenic variants, introduction of variants into model organisms, or the generation of patient-derived 2D or 3D *in vitro* cells (Amin and Paşca, 2018). Genomic measurements from postmortem tissue have provided broad surveys of altered molecular pathways, such as gene expression changes in ASD (Voineagu et al., 2011) and synaptic gene expression differences in schizophrenia (Fromer et al., 2016; Jaffe et al., 2018; Gandal et al., 2018). More mechanistic biological studies of postmortem samples, especially during neurodevelopment, are limited given the inaccessibility of brain tissue. A notable recent example of *ex vivo* primary tissue analysis of individuals with disease

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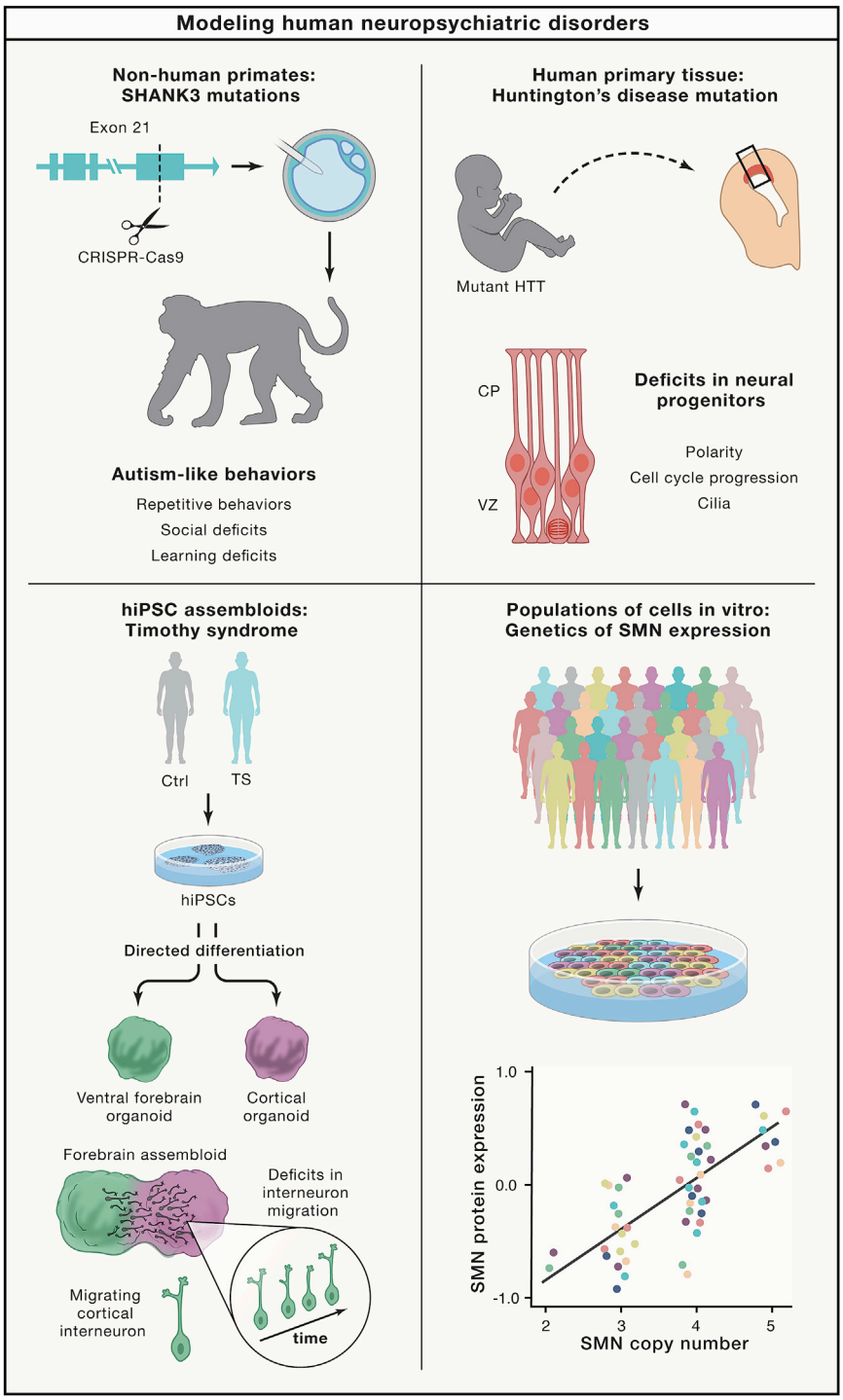

Figure 4. Modeling neuropsychiatric disease.

Top left: monkeys with engineered SHANK3 mutations exhibited social impairments and repetitive behaviors (Zhou et al., 2019). Top right: fetal tissue samples carrying the Huntington's disease mutation display neural progenitor deficits (Barnat et al., 2020). Lower left: forebrain assembloids from patients with Timothy syndrome result in interneuron migration phenotypes (Birey et al., 2017). Bottom right: cells from large numbers of distinct individuals grown together model genetic effects of SMN protein expression (Mitchell et al., 2020).

mutations comes from a study that examined human fetuses caring the Huntington's disease mutation, an adult-onset neurodegenerative disorder (Barnat et al., 2020). Mutant developing cortical progenitors demonstrated surprising deficits, including changes in cell-cycle progression, cell polarity, and ciliogenesis (Figure 4). In another example, analysis of fetuses with trisomy 21, or Down syndrome, identified altered oligodendrocyte maturation and myelination (Olmos-Serrano et al., 2016).

Modeling the effect of single-gene mutations and CNVs associated with neurodevelopmental disorders

Animal models are a more commonly utilized system for investigating the function of single genes or rare pathogenic protein coding variants and have the advantage of being able to query impacts on animal behavior and intact functioning neural circuits. Studies in *Drosophila* and zebrafish can relatively rapidly confirm hypotheses that a given gene is broadly involved in nervous system development. Similarly, gene knockouts can be readily generated in rodents for detailed neurodevelopmental studies in mammals. However, large evolutionary distances between humans and common laboratory models raise the issue of the generalizability of these findings to human disease (Nestler and Hyman, 2010). One approach to overcome this is the generation of more evolutionary-related non-human primate models, such as the common marmoset or macaque. Recently, CRISPR-Cas9 was used to generate macaque monkeys with germline transmissible mutations in the *SHANK3* gene (Zhou et al., 2019)—a major synaptic scaffolding protein that causes Phelan-McDermid syndrome and has been linked to ASD (Figure 4). Monkeys with SHANK3 mutations exhibited social impairments and repetitive behaviors, mimicking some aspects of ASD, and may thus serve as a better model system for developing much needed treatments for this disorder.

In vitro 2D and 3D organoid systems offer a powerful alternative approach for modeling cellular phenotypes of specific genetic variants associated with disease and idiopathic patient-derived cells. Neurodevelopmental disorders that lead to severe

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structural malformations early in developmental are particularly well-suited given the presence of robust phenotypes at neural progenitor stages. For example, forebrain organoids derived from patients with Miller-Dieker syndrome, which causes severe lissencephaly resulting from deletion of chromosome 17p13.3, were smaller in size and displayed deficits in progenitor cell division (Iefremova et al., 2017; Bershteyn et al., 2017). Organoids generated from patients with primary microcephaly, which results from recessive mutations in centrosomal proteins, were also smaller in size and displayed neural progenitor cell dysfunction due to altered cilium assembly, including in oRG cells (Zhang et al., 2019). With the advent of CRISPR technologies, genetic screens will allow for the unbiased discovery of disease genes and phenotypes. For example, candidate microcephaly genes were deleted in a CRISPR-Cas9 screen in early-stage brain organoids, providing biological validation of several genes including nomination of non-centrosomal pathways (Esk et al., 2020). In addition to genetic alterations, infections account for up to half of all cases of congenital microcephaly. Human brain organoids have been able to recapitulate some of the structural defects and molecular alterations associated with Zika virus- induced microcephaly (Cugola et al., 2016; Garcez et al., 2016).

Beyond modeling early disease-associated proliferation phenotypes in brain organoids, there have been increasing examples of alterations in later neurodevelopmental processes. Patients with mutations in the cadherin receptor-ligand pair *DCHS1* and *FAT4* develop periventricular heterotopia, defined by a failure of neuronal migration that leads to the formation of clumps of neurons around the ventricles. Organoids derived from these patients demonstrated heterotopia-like features that appeared to be restricted to a specific subpopulation of mutant neurons (Klaus et al., 2019). As described earlier, assembloids of dorsal and ventral forebrain organoids enable the molecular dissection of interneuron migration, a process thought to be altered in several neuropsychiatric disorders. In forebrain assembloids from patients with Timothy syndrome (Birey et al., 2017)—a neurodevelopmental disorder associated with ASD and epilepsy and caused by mutations in the *CACNA1C* calcium channel gene, interneurons displayed more frequent but less efficient migratory steps (Figure 4). Another intriguing example is one of the most highly penetrant mutations to cause psychosis, the 22q11.2 chromosomal deletion syndrome, which offers a unique molecular window into studying neuronal phenotypes of this enigmatic syndrome. In both cortical organoids and 2D cortical cultures, 22q11.2 syndrome-derived neurons demonstrated an increase in neuronal excitability and impaired calcium signaling, which can be at least partly explained by loss of the miRNA-processing *DGCR8* gene (Khan et al., 2020). Can organoid systems model neural circuit functional connectivity? Cortico-striatal assembloids from patients with Phelan-McDermid syndrome demonstrated reduced network synchronization of neuronal calcium co-activity (Miura et al., 2020), which phenocopies striatal hypo-connectivity observed in mutant monkeys (Zhou et al., 2019). Importantly, this phenotype in striatal neurons was only observed after fusion with cortical neurons but not in unfused striatal organoids, which suggests that moving forward, organoid assembly may enable the discovery of disease-relevant phenotypes. Future work incorporating multiple region-specific organoids that resemble more elaborate neural circuits, such as brainstem neuromodulatory systems or CNS circuit loops, are needed to capture the biology of complex neuropsychiatric disorders.

Modeling the effects of common genetic variants associated with neuropsychiatric disorders

Deciphering the molecular alterations of common variants implicated in idiopathic ASD and schizophrenia using *in vitro* systems have proven challenging given the necessity of using isogenic comparisons and the large increasing number of associated variants to test. Despite this, patient-derived cells from cases of idiopathic ASD, without identified protein coding variants, displayed observable cellular phenotypes such as changes in the composition of GABAergic neurons (Mariani et al., 2015). To overcome the challenges of common variant functional modeling, one group has generated a 2D glutamatergic neuron pipeline for generating isogenic lines with disease associated variants using CRISPR editing in combination with biochemical and physiological neuronal assays (Schrode et al., 2019). Although this approach is potentially difficult to scale to the now hundreds of identified variants, it was able to identify neuronal phenotypes and interactions with eQTL genes converging on synaptic function of a putative schizophrenia associated polymorphism in the *FURIN* gene. An alternative approach to capture and model genetic diversity is the development of "village-in-a-dish" methods, in which cells from large numbers of distinct individuals are grown, perturbed, and assayed in a single experiment (Mitchell et al., 2020) (Figure 4). In the future, we envision highly multiplexed experiments investigating the complex interplay of common and rare disease associated variants and their impact on cellular phenotypes.

Future directions

It is an exciting time for human neurobiology with unprecedented tissue access and the ability to apply a myriad of high-resolution molecular and physiological assays. Yet we are far from understanding the cellular mechanisms of what makes us human and of how neuropsychiatric disorders manifest.

An area of particular importance for further study is the transition from specified neuron to mature neural circuit. Current *in vitro* approaches are limited in this regard and have yet to provide a means to probe these important postnatal processes, such as the development of large complex cellular morphologies, synaptic plasticity, experience-dependent neuronal wiring, and more intricate circuit ensembles. Improved methods for long term culturing and the promotion of maturation (from both *ex vivo* brain specimens and organoids) are needed to investigate this key developmental period. In addition, large-scale neuronal activity measurements will further characterize the emergence of coordinated electrophysiologic patterns. The precise construction of neuronal circuits using human assembloids will complement these efforts, allowing for axon guidance and connectivity tracing in ever controlled cell types and disease contexts. Moreover, comparative work involving diverse non-human primates and mammals will help to place these findings in evolutionary context. Lastly, large-scale genomic efforts that systemically perturb risk variants in human tissue will begin to transform our understanding of the molecular pathogenesis of disease.

These combined efforts are needed to fully understand the mechanisms of human-specific neurodevelopmental innovations and of potential increased human susceptibility to neuropsychiatric disease. Ultimately, this may reshape the diagnosis of nervous system disorders onto specific deficits in molecules, cells, and circuits and eventually lead to a new generation of treatments.

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DECLARATION OF INTERESTS

Stanford University holds patents and has provisional patent application covering the generation of brain region-specific organoids and assembloids (S.P.P.).

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