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Reverse Engineering Human Brain Evolution Using Organoid Models

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Abstract

Primate brains vary dramatically in size and organization, but the genetic and developmental basis for these differences has been difficult to study due to lack of experimental models. Pluripotent stem cells and brain organoids provide a potential opportunity for comparative and functional studies of evolutionary differences, particularly during the early stages of neurogenesis. However, many challenges remain, including isolating stem cell lines from additional great ape individuals and species to capture the breadth of ape genetic diversity, improving the reproducibility of organoid models to study evolved differences in cell composition and combining multiple brain regions to capture connectivity relationships. Here, we describe strategies for identifying evolved developmental differences between humans and non-human primates and for isolating the underlying cellular and genetic mechanisms using comparative analyses, chimeric organoid culture, and genome engineering. In particular, we focus on how organoid models could ultimately be applied beyond studies of progenitor cell evolution to decode the origin of recent changes in cellular organization, connectivity patterns, myelination, synaptic development, and physiology that have been implicated in human cognition.

INTRODUCTION

Evolutionary changes have repeatedly transformed brain structure during the diversification of primates (Striedter, 2005). In particular, the human brain has expanded threefold since our divergence from a common ancestor with chimpanzee and over fifteen-fold since our divergence from old world monkeys (Herculano-Houzel, 2012). This expansion has been

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accompanied by changes in cortical microcircuits, an increased proportion of upper cortical layer neurons, increased connections between higher order association areas, more monosynaptic connections to motor neurons involved in dexterous movements, delayed myelination, and protracted periods of synaptic plasticity (Bianchi et al., 2013; Preuss, 2011; Sousa et al., 2017a; Striedter, 2005). Many of these differences in growth rate, brain size, and neuron number are apparent prior to birth (Sakai et al., 2013). Importantly, many of these recently evolved human traits may also be particularly vulnerable to dysfunction (Song et al., 2018; Varki, 2000; Van den Huevel et al., 2019). However, direct comparisons of gene expression, signaling pathway activation, and cell behavior during human, ape, and old world monkey brain development that could help to isolate the mechanisms underlying these recent evolutionary changes have been challenging due to the inaccessibility of developing ape brain tissue. Studying the cellular and genetic origin of these evolved differences requires an experimental system in which developmental processes can be compared and candidate sequence changes can be examined in a suitable genetic and cellular context.

Mouse models have enabled pioneering studies of how genes that changed in human evolution could influence brain development (Boyd et al., 2015; Charrier et al., 2012; Dennis et al., 2012; Florio et al., 2015). However, ancestors of mouse and human diverged roughly 90 million years ago. At the genomic level, this divergence makes it difficult to reconstruct many human-specific genetic changes because these changes often occur in regions with poor homology, and even when changes occur in homologous sequence, the broader genomic context of the region may differ. Similarly, at the cellular level, some cell types that are important for human brain development and function, such as outer subventricular zone radial glia, are rare or absent in the mouse brain (Fietz et al., 2010; Hansen et al., 2010; Wang et al., 2011). Finally, at the tissue level, the human brain is over 1000 times larger than the mouse brain, and special challenges associated with large brains, such as the formation of long-range connections, may be difficult to model in mouse. Therefore, despite the many strengths of mouse models in reproducibly capturing developmental patterning and cellular interactions, this model system alone may not be sufficient to identify evolved differences in developmental cell behavior or to identify the genetic and molecular changes that explain these differences.

Cultured cells from diverse species could offer a non-invasive window into the mechanisms underlying evolutionary differences provided they could be coaxed into undergoing similar developmental processes. Indeed, cultured skin cells and immune cells have revealed species differences between humans and other great apes (Barreiro et al., 2010; Lawlor et al., 1990). With the discovery that somatic cells from mouse and human could be reprogrammed to induced pluripotent stem cells (iPSCs) (Meissner et al., 2007; Takahashi et al., 2006; Yu et al., 2007), it became conceivable to imagine creating panels of iPSCs from diverse mammalian species that could be used to study how genetic changes underlie differences in developmental processes. This comparative iPSC approach has recently been applied to study human and chimpanzee iPSCs themselves (Gallego Romero et al., 2015; Marchetto et al., 2013; Wunderlich et al., 2014), iPSC-derived neural crest cells (Prescott et al., 2015), cardiomyocytes (Pavlovic et al., 2018), endoderm lineage (Blake et al., 2018), and forebrain lineage (Field et al., 2019; Mora-Bermúdez et al., 2016; Otani et al., 2016; Pollen et al., 2019; Kanton et al., 2019).

Organoid models, in particular, may further capture cell diversity and cellular interactions that normally occur during development. In principle, organoid models could help to bridge the gap between genome sequence differences and recently-evolved specializations of the human brain by providing an experimentally tractable system with the appropriate cellular and genetic background. Because experimental studies of development are otherwise inaccessible in apes, we know little about evolved differences in developmental cell behavior, as well as the tolerated variation in such processes within a species. Comparative analysis between species and chimeric co-culture in organoids could isolate these developmental differences and determine which differences are driven by cell intrinsic changes. Similarly, genome engineering in iPS-derived cell lines provides the opportunity to determine the extent to which genome sequence changes, including accelerated substitutions (Pollard et al., 2006), duplications (Sudmant et al., 2013), deletions (McLean et al., 2011), and insertions (Hellen and Kern, 2015; Kronenberg et al., 2018), contribute to specific evolved differences.

In this review, we describe the importance of expanding our representation of ape genetic diversity in a dish using iPSCs, the opportunity for harnessing organoid models to represent the diversity of cell types and communities, and general opportunities for improving the health and organization of organoid models. We then transition to discussing the first comparative studies of neural progenitor cell behavior and gene expression in human and ape organoids, and we close by examining how improvements in organoid models could enable mechanistic studies of evolutionary changes thought to be essential for human cognitive specializations.

REPRESENTING APE GENETIC DIVERSITY IN A DISH

Collections of human and ape iPSC lines have the potential to represent divergence of developmental traits between species and variation within species. Characterizing these properties of divergence and variation is crucial for identifying adaptive changes in the human lineage, as well as for revealing both constrained processes and neutral variation tolerated during human and ape development. However, ape primary cells as a source for reprogramming are scarce and difficult to access and transfer given the endangered status of all non-human apes. In addition, although reprogramming of ape cells with protocols optimized for human cells is possible, that reprogramming fibroblasts from non-human primates is not as straightforward, likely due to species-specific differences in optimal culturing conditions (Nakai et al., 2018; Navara et al., 2018). Therefore, early studies that demonstrated proof of principle used only a few individuals and performed reprogramming with integrating retroviruses that may increase efficiency, but could have later consequences on cell behavior. Panels of integration free human and chimpanzee iPSC lines have now been generated and distributed (Gallego Romero et al., 2015), but there is limited representation of additional ape species (Figure 1; Table 1), and most studies have focused on divergence between human and chimpanzee.

Additional great ape stem cell lines would not only help to pinpoint key evolutionary specializations on the lineage leading to humans, but these lines could also help to interpret candidate disease-causing processes in humans. Humans underwent a population bottleneck

to fewer than 10,000 individuals in the last 65,000 years, reducing the common genetic variation segregating in our population (Prado-Martinez et al., 2013). Recent studies have taken advantage of the greater extent of tolerated sequence variation naturally occurring in other primate species to efficiently predict de novo coding mutations that are likely to be pathogenic in humans (Sundaram et al., 2018). Using ape iPSC lines to characterize tolerated variation in gene regulation and developmental processes within primates species may further support interpretation of candidate human disease mechanisms, including those being studied using stem cells and organoid models. In addition, analyzing phenotypic divergence on additional branches of the great ape phylogeny will help to better resolve which changes on our own lineage are related to human traits and which are related to general patterns of divergence.

Finally, additional lines may support conservation for endangered primate species. Unfortunately, approximately 75% of non-human primate species' populations are decreasing in size, and about 60% are under the risk of extinction in the near term (Estrada et al., 2017). Genome sequencing studies can assist conservation efforts to maximize genetic diversity retained in future generations. The extent to which repositories of cultured cells can also contribute to conservation efforts remains to be seen (Benirschke, 1984), but there are some promising avenues. Cell lines from apes have long been useful for understanding immunological differences (Lawlor et al., 1990), and recent studies of Zika virus in human and chimpanzee organoids suggest that organoids may be useful for studying species specific viral tropism and screening for protective drugs (Cugola et al., 2016). Similarly, pluripotent stem cells and organoids could support toxicology screens in other primates to identify compounds that may have species-specific influences on development (Walker et al., 2015). In the more distant future, iPSCs may also support the production of gametes for conservation purposes (Stanton et al., 2019), as proposed for the Northern rhinoceros (Ben-Nun et al., 2011; Hildebrandt et al., 2018). Thus, generating and distributing larger collections spanning many individuals from all ape species may support conservation efforts and represents an opportunity to learn a tremendous amount about our own specializations and vulnerabilities.

ORGANOID MODELS OF BRAIN DEVELOPMENT

Organoid models can capture cellular diversity, tissue organization, and aspects of function across diverse organ systems (Clevers, 2016). These models can be applied to characterizing how genetic diversity segregating within humans and diverging across species influences brain development. This section considers the status of brain organoid models and their fidelity to the developing brain.

Developmental Processes in Brain Organoids

Normal development occurs through natural processes of self-assembly and signaling between neighboring cells. Although these processes may be difficult to fully recapitulate in cell culture, pioneering studies demonstrated that mouse pluripotent stem cells aggregated together and exposed to minimal patterning cues could produce organoids containing structures resembling embryonic forebrain, optic cup, and pituitary epithelium (Eiraku et al.,

2008; 2011; Suga et al., 2011; Watanabe et al., 2005). The forebrain organoids contained germinal zone-like structures, diverse neuronal cell types, and even spatial gradients (Eiraku et al., 2008), and the optic cup organoids further mimicked morphogenesis by invaginating in the absence of a lens to form neural retina and adjacent pigment epithelium (Eiraku et al., 2011). Subsequently, these protocols were adapted to human (Kadoshima et al., 2013; Lancaster et al., 2013) and extended to contain cells from many brain regions in individual whole brain organoids (Cederquist et al., 2019; Renner et al., 2017) or to resemble specific brain regions, including hippocampus and choroid plexus (Sakaguchi et al., 2015), spinal cord (Ogura et al., 2018), pituitary gland (Ozone et al., 2016), ventral telencephalon (Kim et al., 2019; Sloan et al., 2018; Xiang et al., 2017), thalamus (Xiang et al., 2019), hypothalamus and midbrain (Jo et al., 2016; Qian et al., 2018) and cerebellum (Muguruma et al., 2015).

Many studies have focused on cortical organoids and cortical-like regions that emerge in whole brain organoids. Because organoids are a relatively new model system, it is crucial to address what processes of normal development organoids recapitulate and what the opportunities are for improvement (Table 2). The appearance of a polarized neuroepithelium is one of the most striking features of cortical organoid models. Early in the patterning process, ventricular zone-like rosette structures containing radial glia spontaneously emerge in organoids. The arrangement of cells around these rosettes resembles the lateral ventricle of the embryonic telencephalon (Subramanian et al., 2017). Apical proteins localize to the inner lumen of these rosettes and radial glia-like cells undergo interkinetic nuclear migration dividing at the apical surface (Bershteyn et al., 2017; Lancaster et al., 2013). By 10 weeks of differentiation, radial glia also migrate away from the ventricle, express markers of outer subventricular zone radial glia (Qian et al., 2016), and undergo mitotic somal translocation (Bershteyn et al., 2017). These germinal zone structures and cell behaviors match observations of normal primate brain development (Hansen et al., 2010; Pollen et al., 2015), and have been used to study how patient genetic mutations influence cell behavior (Bershteyn et al., 2017; Blair et al., 2018; Iefremova et al., 2017).

Neurons migrate from the ventricular zone-like structures of organoids outwards to the periphery. The outer zone of neurons in organoids has been described as a cortical plate-like region. Indeed neurons expressing multiple markers of deep and upper layer subtypes have been described in the periphery of cortical organoids at around 10 – 15 weeks (Qian et al., 2016). Nonetheless, the organization of neuronal migration and layer formation differs from primary tissue at comparable stages (Pollen et al., 2019). Organoid size is generally constrained to less than 5 mm in diameter, which is less than the typical span from the ventricle to the cortical plate during middle stages of neurogenesis (Pollen et al., 2019), and organoids often contain many rosettes, further limiting the migration distance of neurons. Within this compressed space, there is not a clearly demarcated intermediate zone or subplate, and markers of cortical layers often appear disorganized. In addition, non neuroectodermal-derived cells, including endothelial cells, pericytes, and microglia are missing as well subcortical targets of efferent connections and incoming afferent connections. Thus, tissue organization outside of the germinal zone and the representation of subcortical and non-neuroectodermal cell communities are active areas for improvement (Giandomenico et al., 2019).

Fidelity of Organoids to Normal Development

Single cell RNA sequencing of primary tissue samples and organoid models provides the opportunity to further evaluate the fidelity organoid models to developmental processes for the cell types that are present. Early single cell RNA sequencing studies indicated that the trajectory of neuronal differentiation observed in primary human samples (Pollen et al., 2014; Pollen et al., 2015) is well conserved in organoid models (Camp et al., 2015) and identified molecular signatures of outer radial glia-like cells (Bershteyn et al., 2017). More recently, several studies, across many additional individuals, indicate strong correlations of gene expression signatures between organoid and primary tissue and cell types (Pollen et al., 2019; Velasco et al., 2019; Yoon et al., 2019). For example, we found that the majority of gene co-expression modules present in primary samples independently emerge in organoid models, and that area-specific excitatory neurons appear spontaneously (Pollen et al., 2019). Nonetheless, several studies have observed variability in patterning across individuals (Osafune et al., 2008; Pollen et al., 2019; Quadrato et al., 2017). Some of the observed variability may be related to more permissive protocols (Velasco et al., 2019; Yoon et al., 2019), but this variability in composition does not appear to have a strong effect on gene expression in individual cell types. However, the observation that specific lines can be biased in their differentiation, regionalization, or patterning potential is an important consideration for comparative studies across species or of disease genotypes, particularly when phenotypes may involve differences in cell fate potential. Thus organoids already provide a useful model for examining how genetic changes influence gene expression in specific cell types, but the patterning of these types in reproducible combinations remains an active area of research (Velasco et al., 2019).

Importantly, strong differences in gene expression also exist between organoids and primary samples. Across three major protocols that vary in the extent of cortical patterning, organoid cells commonly exhibit increased glycolysis pathway gene expression and increased signatures of endoplasmic reticulum stress compared to primary cells of the same type (Pollen et al., 2019). These glycolysis signatures extend to newborn neurons that normally undergo oxidative phosphorylation (Zheng et al., 2016) and the signatures also include the unfolded protein response, but the influence of these ectopic pathways on neuronal maturation has not been examined in detail. Interestingly, the unfolded protein response is also induced in organoids after acute hypoxia (Paşca et al., 2019). Inhibiting the integrated stress response pathway by blocking EIF2A phosphorylation (Sidrauski et al., 2013) was discovered to be protective in hypoxic organoids, particularly in intermediate progenitor cells, which tend to be underrepresented in organoids relative to primary samples (Paşca et al., 2019). The same approach, or the use of other small molecules, could potentially be protective in organoids cultured at normoxia, and might bring gene expression and cell composition closer to normal development. Despite these ectopic stress pathways, it is often assumed that organoid cell types more faithfully adhere to normal development than cell types differentiated in monolayer. However, systematic comparisons of 2D and 3D approaches for the same lines by single cell RNA sequencing have not yet been performed. In addition, single cell RNA sequencing has been the main modality for comparison due to recent technological advances, but additional comparisons from chromatin accessibility (Amiri et al., 2018; Kanton et al., 2019) to microRNA (Nowakowski et al., 2018) to full

isoform sequencing and proteomics (Bauernfeind et al., 2015) may be useful to further reveal aspects of organoid fidelity.

Overall, single cell RNA sequencing studies show that broad neuroectodermal derived cell types, gene co-expression networks, and trajectories spontaneously appear in organoids, but that patterning and metabolic differences may still lead to limitations. These limitations could be especially relevant to protein folding and neurodegenerative disorders in which the organoid stress response may already be sensitizing organoids (Halliday and Mallucci, 2015; Pollen et al., 2019). Thus, current forebrain organoid models recapitulate many features of early cortical germinal zones, but there are opportunities for histological and metabolic improvements.

GENERAL OPPORTUNITIES FOR IMPROVING ORGANOID MODELS

This section reviews general opportunities for improving organoid models. In particular, providing organoids with vasculature and microglia and incorporating a realistic extracellular matrix environment represent opportunities for improving tissue health and organization. In addition, both metabolism and the extracellular matrix represent properties that likely changed during primate brain expansion and diversification. Ultimately, a foundation of improved tissue health and organization will be required to further model many evolved traits and cellular interactions, as well as their dysfunction in disease.

Vascularization and microglia

Inclusion of non-neuroectodermal cell types may be crucial for improving organoid tissue quality. Vascularization, in particular, could improve the richness of developmental cell interactions and could potentially alleviate metabolic stress. In the developing brain, blood vessels invading from the pia play a role in both scaffolding and signaling in addition to supporting blood flow (Adams et al., 2015; Schmid et al., 2019). For example, vascular endothelial cells express proteins that can influence pyramidal neuron position, radial glia pial attachment, and astrocyte behavior (Segarra et al., 2018). However, generating vascularized tissues in a dish has been challenging.

Transplantation studies have long demonstrated that human pluripotent stem cell derived tissue can be vascularized in a mouse (Rossant and Papaioannou, 1984) and have recently highlighted that the transplant process reduces the cell death observed in cultured brain organoids (Mansour et al., 2018). Recent studies also indicate that it may be possible to mimic the developmental interplay between vasculature and neuroectoderm lineage cells by fusing brain organoids to blood vessel organoids (Wimmer et al., 2019), by seeding organoids with vascular cells (Pham et al., 2018; Schwartz et al., 2015), or by *in situ* generation of endothial cells via ETV2 overexpression within organoids (Cakir et al., 2019). These vascular cells may permit increased diffusion. However, without pumping oxygenated blood through organoid vessels, it remains unclear whether vasculature alone can alleviate the metabolic abnormalities of organoids. Alternatively, bioengineering approaches may support nutrient and oxygen delivery in organoids, using hydrogels (Grigoryan et al., 2019), subtractive fabrication (Sarig-Nadir et al., 2009) or carbohydrate glass as a sacrificial network scaffold (Miller et al., 2012a; Therriault et al., 2003). Of note, the human brain

utilizes a large proportion of the body's energy, and humans may have higher energetic demands than the other ape species (Pontzer et al., 2016; Preuss, 2011). Whether or not human-specific metabolic adaptations have recently evolved in brain cells has been difficult to study without access to primary tissue. Thus biologically and engineering inspired approaches to organoid vascularization could improve tissue health and organization and could potentially enable comparative studies of neurometabolism.

Microglia are also important for tissue homeostasis, acting not only as immune cells, but also contributing to the clearance of dead cells through phagocytosis, and the regulation of cell proliferation, differentiation, synapse formation, and pruning (Ginhoux and Prinz, 2015). Thus, microglia may respond to the increased apoptosis described in organoids by mitigating the necrotic core (Mansour et al., 2018), in addition to participating in normal developmental processes. However, microglia are derived from the yolk sac during embryonic development (Ginhoux and Garel, 2018), and have only rarely been observed in organoid protocols that pattern for neuroectoderm (Ormel et al., 2018). Three studies demonstrated that transplanted microglia migrate and integrate into brain organoids and physiologically respond to insults (Abud et al., 2017; Muffat et al., 2018; Song et al., 2019). Importantly, organoids containing transplanted microglia appeared to lack a necrotic core and displayed differential intracellular calcium signaling and increased response to pro-inflammatory stimuli compared to untransplanted organoids (Song et al., 2019). Together, these results suggest that brain organoids may provide the necessary microenvironment to support the long-term incorporation of other cerebral cell types, and hint that non-neuroectodermal cell types may improve tissue health and organization.

Extracellular Matrix and Bioprinting

Extracellular matrix (ECM) proteins play an important role in progenitor expansion and organization of developing brain circuits, providing both scaffolding and signaling sources (Pearlman and Sheppard, 1996). Fibronectin, for example, is present in the ventricular zone of the developing cortex and may promote cell division (Pearlman and Sheppard, 1996). Similarly, integrins play a key role in maintaining the high proliferative capacity of progenitors in the outer subventricular zone (Fietz et al., 2010). Indeed, primate outer subventricular zone radial glia strongly express a module of ECM genes that potentiate growth factor signaling, suggesting these cells may contribute to a self-sustaining progenitor niche away from the ventricle (Pollen et al., 2015). Organoid cells may endogenously produce important ECM genes that support self-organization. For example, organoid radial glia preserve expression of many ECM genes, and organoids contain a mesenchymal cell population that expresses DCN, BGN, OGN, and collagen genes found in meningeal fibroblasts (Pollen et al., 2019). However, the extent to which endogenous production of ECM proteins in organoids resembles that of primary tissue in protein types and localization has not been examined in detail.

Supplementing cultures with species- and tissue-specific ECM molecules could potentially improve the fidelity of organoid cell behavior and architecture. Many protocols already embed organoids in droplets of matrigel secreted by mouse sarcoma cells to induce extension of ventricular zones during early patterning (Lancaster et al., 2013). Whether the

matrigel acts through scaffolding alone to induce ventricle expansion or also through other signaling pathways is unclear. Interestingly, the effects of ECM molecules may differ by species. For example, addition of HAPLN1, Lumican, and Collagen I, rapidly increases stiffness and induces folding in primary human organotypic cultures, but not in mouse or ferret cultures (Long et al., 2018). More generally, the encapsulation of neurons into gels derived from natural ECM molecules also leads to neurite extension and formation of active networks (Ma et al., 2004; Mooney et al., 2010). Distinct ECM molecules promote the survival of different cell types. Fibrinogen and thrombin gels, for example, promote the survival of neurons over glia cells (Mooney et al., 2010). Consistent with the importance of tissue-specific ECM signals, supplementing human brain organoids with porcine fetal brain, but not adult brain ECM extracts, results in higher baseline synchrony of neuronal activity, accelerated maturation, and long-term survival of projection neurons (Sood et al., 2019). New protocols that consider a combinatorial approach to ECM composition during aggregation and long-term culture could potentially improve the survival of neurons and the study of neuronal networks beyond modeling early circuit development. Similarly richer cell communities that include vasculature and meningeal fibroblasts may support further endogenous production of key ECM proteins.

Alternatively, the use of artificial hydrogels could provide scaffolding support and replace xenobiotic materials like matrigel with defined culture conditions. Such artificial hydrogels could potentially reduce cost and increase reproducibility, but may lack important bioactive molecules (Broguiere et al., 2019; Tsou et al., 2016). For example, polyethylene glycol hydrogels provide a particularly inexpensive and scalable platform for culture and can be augmented with bioactive components for reproducible organoid generation containing NPCs, microglia and endothelial cells (Barry et al., 2017a; Schwartz et al., 2015). As a proof of principle, encapsulation of primary neurons in polyethylene glycol and polysaccharide-based macroporous hydrogels showed a remarkably high level of neurite outgrowth and neuronal activity, despite the fact that the hydrogel scaffold lacked laminin peptides (Broguiere et al., 2019). Thus, ECM proteins likely play key roles in scaffolding and signaling during development that may shape cell fate potential and tissue organization, and there are encouraging signs that these can be harnessed endogenously and through engineering approaches to improve organoid models.

Finally, the proper cytoarchitecture of neuronal tissue is crucial for the correct function of the organism. For example, the reeler mouse, in which cortical layers are inverted, shows strong behavioral phenotypes, as well as impaired motor coordination, ataxia and tremors (D'Arcangelo et al., 1995; Salinger et al., 2003; Trommsdorff et al., 1999). While brain organoids recapitulate the sequential generation of deep layers followed by upper layer projection neurons, achieving proper cortical lamination has been challenging. 3D bioprinting could potentially enable engineering of cortical organization. In this approach, cells of interest are embedded into hydrogel filaments or other biomaterials and printed in a preprogrammed manner to obtain the desired cytoarchitecture (Grebenyuk and Ranga, 2019). Importantly, cell viability after bioprinting is high, reaching up 90% survival (Christensen et al., 2015). As a proof of principle, human neuronal stem cells have been bioprinted in bioink containing alginate, carboxymethyl-chitosan, and agarose (Gu et al., 2016). These cells form complex structures that upon *in situ* differentiation form synapses,

neuronal networks and have spontaneous activity. Alternatively, the use of 2-photon driven polymerization of biocompatible materials has been used to directly induce the formation of thick, porous and complex scaffolds (Worthington et al., 2017). Neurons grown using this approach extend long processes through the pores and integrate as a system. Overall, there are many tradeoffs between guided self-assembly and engineering approaches, but combinations of the two may be crucial for further advances in tissue health and organization.

ORGANOID MODELS ENABLE COMPARATIVE STUDIES OF ORGANOGENESIS

Comparative studies between human and primate brain organoids have only just begun, but could ultimately link causal genome sequence changes to changes in chromatin state, gene expression, and cell behavior underlying recent evolutionary changes (Figure 2A). This section outlines challenges facing comparative studies and provides two general approaches for determining the cellular origin of species differences and the causative genetic changes underlying developmental differences. Finally, this section highlights the first wave of comparative organoid studies that have focused on differences in progenitor cell behavior, gene expression and signaling pathway activation.

Experimental Approaches and Challenges for Extending Comparative Studies

Performing comparative studies on the evolution of development in stem cell culture shares special challenges with disease modeling studies. In particular, there are many possible sources of variation in stem cell lines beyond genetic differences between source individuals and species, including cell culture derived mutations, epigenetic changes, possible differences in pluripotency state, and patterning differences between species (Carcamo-Orive et al., 2017). Therefore, ideal experimental designs could involve two paths (Figure 2B). In the first path, cross-species comparative studies should include a large number of individuals, while in the second path analysis of candidate mutations in isogenic lines can mitigate source of line to line variation. Both paths, to the extent possible, should anchor differences observed between species to changes also observed in primary brain tissue from human and non-human primates. Because ape primary samples are not available during early developmental stages, either developing macaque tissue or adult chimpanzee tissue has been used for validation in early studies. For example, two studies recently revealed that species differences in gene expression observed in iPSC-derived brain organoids or in cardiomyocytes across at least 8 individuals in each species recapitulate species-specific and tissue-specific differences observed in primary samples (Pavlovic et al., 2018; Pollen et al., 2019). Similarly, the duration of mitotic phases observed in organoids resembles that observed in primary human samples (Mora-Bermúdez et al., 2016). In addition to validating results in primary tissue, chimeric culture approaches, described further below, may be especially powerful to create a common garden environment that reduces batch effects and reveals cell intrinsic species differences (Otani et al., 2016).

Cross-species comparisons must consider several sources of biological variation that may be confounded with species differences in order to draw conclusions. Comparisons within

species are generally safe in assuming that noise due to line-specific patterning or heterochrony is random and centered, but between species, these sources of variation can lead to systematic bias. This bias could involve systematic differences in patterning or maturation rate between species in a standardized protocol due to interspecies differences in the interaction between culture conditions and signaling pathways. These differences in patterning or maturation rates can then drive apparent differences in gene expression and other phenotypes that mainly relate to composition rather than to differences between cells of the same type (Barry et al., 2017b). Therefore, it is crucial to account for composition differences and systematic patterning biases in comparisons between species.

In principle, single cell RNA sequencing analyses enable comparison of molecularly defined cells of the same type between species. However, identifying cells of the same type between species through unbiased clustering approaches is challenging because species differences are reliably the major source of transcriptional variation, likely due to a combination of biological species differences, experimental confounds, and genome annotation artifacts. Recent analytical advances seek to overcome systematic sources of batch variation by enabling "alignment" and clustering of cells from multiple species in a shared low-dimensionality space (Haghverdi et al., 2018; Johansen and Quon 2019; Pola ski et al., 2019; Stuart et al., 2019; Welch et al., 2019). Nonetheless, these alignment methods involve heuristics, non-linear data transformations, distortions, and corrections that could over-fit putative orthologous cell types or depend on arbitrary user-defined parameters, without statistics describing the absolute or relative quality of the alignment output. Thus, in its current state, the field appears to need models that can "say no". These models conceivably would provide an assessment of whether or not cells and clusters should be aligned in the first place by distinguishing and reporting variance due to technical artifacts, evolutionary changes, and underlying cell type homology. Current best practices should include evaluating how alignment results change between independent methods and evaluating whether co-clustered cells in joint analyses are indeed the most correlated groupings between independent analyses in each species. As a complementary approach, clustering-independent methods, such as comparing gene co-expression networks that independently arise between species (Pollen et al., 2019) may provide additional measures of homology.

Comparing gene expression between species is also challenging because of the superior genome assembly and transcriptome annotation in human, despite recent improvements in ape assemblies (Kronenberg et al., 2018). Approaches to overcome these biases include aligning reads only to orthologous exons, aligning reads to a consensus alignment between species with SNPs, indels and copy number variants masked, and aligning reads to the native genome of each species and mapping human annotations to other primates (Blekhman, 2012; Marchetto et al., 2013; Zhu et al., 2014, Pollen et al., 2019). Currently, each approach likely has strengths and weaknesses with respect to controlling for differences in gene models and read mapping rates between species versus capturing information from the greatest number of genes, including species-specific genes. Future studies may incorporate the best of each approach, considering consensus alignments for orthologous sequences, while also using higher quality de novo assembled genomes and improvements in gene annotations based on isoform sequencing across species and tissues to more fully account for molecular distinctions (Kronenberg et al., 2018). Overall, the sources of biological and

technical variation that can confound species comparisons make validation of differences in available primary tissue samples particularly important.

As a second approach to reduce variation, human and chimpanzee iPSC lines may be directly modified to recreate ancestral or derived mutations respectively (Figure 2B). These modified lines can be compared directly to the unmodified (isogenic) version of the same line as a control to examine the necessity and sufficiency of human and chimpanzee mutations for particular phenotypes. For example, a recent study of human-specific NOTCH2NL duplications used CRISPR/CAS9 to ancestralize three human-specific functional paralogs of NOTCH2NL. Organoids with two homozygous and one heterozygous ancestral-like NOTCH2NL deletion showed premature neuronal differentiation, supporting a role for the NOTCH2NL duplicate genes in potentiating NOTCH signaling and maintaining proliferative divisions (Fiddes et al., 2018). Future studies may apply this isogenic approach more systematically to evaluate the quantitative contribution of candidate human-specific mutations to developmental cell behaviors. Together, both the comparative and the isogenic line approaches can now be applied across modalities from chromatin state, gene expression and proteomics, to cell behavior, morphology, and physiology, and these approaches will be particularly effective when anchored in species differences observed in primary tissue samples (Figure 2B).

Discovering Evolved Differences in Neural Progenitor Behavior

Studies of normal brain development have long predicted that changes in the proliferation of radial glia neural stem cells could underlie the evolutionary expansion of primate brains (Fish et al., 2008; Kriegstein et al., 2006; Rakic, 2009). Given the importance of radial glia in brain expansion and the preservation of radial glia cell behaviors and germinal zone structure in organoids, early studies have focused on this cell type. Comparisons of cell behavior in 2D, 3D, and chimeric co-culture suggest that human radial glia have an intrinsically greater proliferative capacity during deep layer neurogenesis than their macaque counterparts (Otani et al., 2016). The duration of cell cycle phases may also vary between species and could influence whether divisions are proliferative or neurogenic. Interestingly, time lapse imaging of cell cycle in neural progenitors of day 30 organoids revealed that the duration of metaphase, the portion of mitosis during which spindle fibers attach to chromosomes, lasts longer in human than in chimpanzee and orangutan neural stem cells (Mora-Bermúdez et al., 2016). However, by day 52, the duration of metaphase shrinks in human organoids to match that of other apes. Although these studies involved a small number of individuals from each species, the results hint that changes in human neural progenitor cell cycle behavior may influence early stages of cortical development when proliferative divisions are more common (Mora-Bermúdez et al., 2016).

Changes in signaling pathway activation could influence radial glia cell divisions and morphology. For example, human outer subventricular zone radial glia display increased activation of the LIFR-STAT3 self-renewal pathway compared to ventricular radial glia (Pollen et al., 2015). This signaling pathway is necessary for normal radial glia divisions in organotypic slice culture (Pollen et al., 2015) and activation of the pathway substantially amplifies the number of outer subventricular zone radial glia and the formation of upper

layer neurons in organoids (Watanabe et al., 2017). Thus, this signaling pathway could contribute to the expansion of outer subventricular zone radial glia and their progeny among primates. Nonetheless, key genes in this pathway show conserved expression between human and macaque (Pollen et al., 2015). However, further analysis of gene expression patterns in human and chimpanzee radial glia across many individuals revealed divergent expression of genes involved with PI3K-AKT-mTOR signaling (Pollen et al., 2019). The mTOR pathway influences many aspects of cell growth, division, metabolism, and fate potential (Baser et al., 2019; Blair et al., 2018; Takei and Nawa, 2014) and displays enriched expression in outer subventricular zone radial glia compared to other cell types (Nowakowski et al., 2017). Immunohistochemistry and western blot analysis in organoids suggested that human radial glia undergo a quantitative increase in mTOR activation compared with chimpanzee radial glia that could be driven in part by increased expression of INSR and ITGB8. Analysis of primary tissue from human and macaque across stages of neurogenesis supported these findings and indicated that the changes in pathway activation may be derived on the human lineage (Pollen et al., 2019). Characterizing the upstream drivers and influence of these changes in pathway activation on radial glia cell behavior and morphology will require further study, but these early findings indicate that organoid models combined with analysis of primary tissue make it possible to compare the activation of major signaling pathways during neurogenesis.

Together, these early studies provide hints that human and ape organoid models can help us to decode cellular and molecular changes that recently evolved in the human lineage. While, current forebrain organoid models may be well suited to study how changes in radial glia of the germinal zone contribute to human brain expansion, possible improvements in organoid models discussed below may enable studies of richer phenotypes also associated with changes in human cognition.

IMPROVING ORGANOID MODELS TO STUDY TRAITS LINKED TO HUMAN COGNITION

Careful histological comparisons (Bianchi et al., 2013; Raghanti et al., 2009) and transcriptomic studies in primary tissue (He et al., 2017; Konopka et al., 2012; Liu et al., 2012; Sousa et al., 2017b) have revealed several evolved postnatal differences between humans and other apes that influence cell composition, connectivity, and plasticity may be particularly important for human cognition beyond the quantitative increase in human brain size. However, studying the genetic and developmental sources of these evolved differences requires that organoid models mimic more sophisticated aspects of development beyond progenitor behavior in the germinal zone (Figure 3). In this section, we review promising opportunities in organoid modeling that could be applied to study specialized traits linked to cognition that recently changed in human evolution, including changes in cell type organization, connectivity relationships, protracted myelination, dendritic complexity, and physiology.

Cell type reorganization

Classical anatomical comparisons have suggested many evolutionary changes in cell type organization between primate and rodent brains. These differences may include changes in gene expression in homologous cell types, changes in the distribution of cell types, and possibly novel cell types in the adult brain. Comparative single cell genomic and spatial transcriptomic studies are poised to systematically reveal these quantitative and qualitative changes in available adult brain samples (Krienen et al., 2019). In parallel, organoids and *in vitro* models can help to evaluate the physiological relevance of species-specific changes in cell organization and to reveal the underlying developmental mechanisms, provided the cell types and brain regions can be modeled.

Upper layer cortical neurons provide connections across cortical areas. These cells have undergone changes in gene expression and abundance in primate evolution. For example, comparisons by *in situ* hybridization revealed that many markers specific to layer 5 cortical neurons in mouse are enriched in human upper layer cortical neurons (Zeng et al., 2012). Among these genes, the h-channel, HCN1, regulates subthreshold activity, and may contribute to the propagation of theta band frequency signals linked to learning and memory (Kalmbach et al., 2018; Rutishauser et al., 2010). Organoids containing mature upper layer neurons could support systematic studies on the functional consequences of these gene expression shifts. In addition to altered gene expression, the proportion of upper layer neurons has also expanded in humans (Aboitiz and Montiel, 2003; DeFelipe, 2011). Recent studies suggest that it is possible to compare fate potential and timing of neurogenesis between species (Kanton et al., 2019; Otani et al., 2016). Thus, the relative production of upper layer neurons and the cell intrinsic bias of radial glia clones to produce neurons of each subtype could be quantitatively examined in each species using *in vitro* models to identify the developmental basis for upper layer expansion, provided such protocols are reproducible across individuals.

As a subtle example of cell type reorganization, gene co-expression network analysis revealed increased expression of dopamine pathway related genes in human cortex and striatum compared to African apes (Sousa et al., 2017b). These findings support histological analyses suggesting changes in the distribution of tyrosine hydroxylase-expressing cells (Raghanti et al., 2009). The expression of tyrosine hydroxylase and DOPA decarboxylase does not confirm physiological dopamine production, but investigating the physiology of these cells in adult human and ape brains was impossible. Nevertheless, a subset of iPSC-derived inhibitory interneurons generated in a forebrain induction protocol resembled the cell types observed to have a divergent distribution in human forebrain. These *in vitro*-derived cells possessed the capacity to secrete dopamine, suggesting the observed change in cell organization could have important physiological consequences including a local source of dopamine in human cortex (Sousa et al., 2017b). Importantly, directly matching *in vitro* derived cells to primary cell types in cortex or striatum will be required to further establish the physiological relevance. Nonetheless, improvements in single cell RNA sequencing and impending comprehensive cell atlases across humans and apes should make it realistic to more precisely anchor *in vitro*-derived cell types and their measurable phenotypes to

changes in organization and cell-type-specific gene expression whose consequences would otherwise be difficult to study *in vivo*.

More generally, the proportion of inhibitory interneurons has also changed substantially in cortical and thalamic evolution. However, studying these evolutionary changes using organoids may require modeling both the progenitors that give rise to inhibitory neurons and the behavior of interneurons in their ultimate destination. In rodents interneurons encompass ~15–20% of cortical neurons, while in primates they can represent over 40% of the neurons of the supragranular layers (DeFelipe, 2011). Similarly, interneurons represent about 5% of all neurons in the mouse thalamus compared to 30% in human thalamus (Arcelli et al., 1997). In both brain regions, the inhibitory neurons are not generated locally, but migrate in from a distinct brain region. In cortex, the inhibitory interneurons mainly migrate from the ventral telencephalon (Jones, 2009). In thalamus, less is known, but recent studies suggest the inhibitory neurons in sensory relay nuclei migrate from midbrain (Jager et al., 2016), while inhibitory neurons in higher order thalamic nuclei may also migrate from ventral telencephalon (Jager et al., 2019; Letini and Kostovi, 1997; Letini and Rakic, 2001).

Cortical and thalamic organoids protocols mainly yield excitatory neurons (Pollen et al., 2019; Quadrato et al., 2017; Velasco et al., 2019; Xiang et al., 2019). However, new protocols enable production of organoids that resemble the medial ganglionic eminences, and fusion of these ventralized organoids with cortical organoids resulted in extensive interneuron migration towards the excitatory cells (Bagley et al., 2017; Birey et al., 2017; Xiang et al., 2017), resembling the *in vivo* context (Lim et al., 2018). Upon migration, the cells differentiated in all interneuron lineages, albeit at different than expected frequencies. For example, while parvalbumin-positive interneurons encompass ~40% of cortical interneurons (Rudy et al., 2010), they were observed at a low frequency and only after long culturing periods (>200 days in culture) (Bagley et al., 2017; Birey et al., 2017). This is in agreement with previous observations that parvalbumin-positive interneurons are rarely observed *in vitro*, and not observed *in vivo* until late stages of gestation (Maroof et al., 2013; Nicholas et al., 2013). Midbrain organoids have mainly been analyzed for dopaminergic neurons (Monzel et al., 2017; Qian et al., 2016) but do contain inhibitory neurons (Jo et al., 2016), although it remains unclear whether fusion with thalamic organoids would result in similar inhibitory neuron migration to thalamic organoids. Thus, region-specific organoids could enable comparative studies of evolutionary changes in inhibitory neuron production, but understanding the reorganization of these cell types in cortical and thalamic regions using organoids would still require improvements in modeling differentiation, maturation, and migration.

Overall, changes in cell type organization are challenging to model using organoids that lack the reproducibility of cell composition and tissue architecture of animal models. Nonetheless, organoids may enable modeling the physiological consequences of novel cell types or gene expression patterns whose role may be difficult to determine in comparisons of post-mortem primary tissue or in mouse model systems. Additionally, recent fusion experiments between organoid types hint that modeling migratory processes contributing to evolutionary changes in cell distribution may also now be tractable.

Increased subcortical and intracortical connectivity

Connectivity has also changed substantially in primate species. Cortical connections to motor neurons are thought to contribute to improved primate dexterity, while connections between cortical areas and through thalamus are thought to contribute to human cognition and behavioral flexibility (Rikhye et al., 2018; Striedter, 2005). Modeling evolutionary changes in these connections will require proper specification of source neurons and their targets. Although challenging, preliminary studies suggest organoids may permit modeling these important evolutionary differences.

Anterograde labeling experiments indicate that several large-brained primates have direct monosynaptic connections between corticospinal neurons and motor neurons of the spinal cord (Bortoff and Strick, 1993; Lemon, 2019; Lemon et al., 2002; Porter, 1987). These connections may contribute to the delicate and dexterous movements of primates (Bortoff and Strick, 1993; Porter, 1987). In particular, direct and possibly human-specific monosynaptic corticobulbar connections may contribute to human vocal dexterity and language production (Kuypers, 1958). However, we still do not know whether these connectivity differences are caused by intrinsic changes in the cortical projection neurons themselves, changes in target-derived factors, or changes in activity patterns that favor enlarged cortical networks in competition with subcortical nuclei (Herculano-Houzel, et al., 2016). Interestingly, rodents transiently develop cortico-motoneuronal connections (Maeda et al., 2016; Murabe et al., 2018; Ohno et al., 2004), and blocking Sema6D-PlexA1 signaling in mice prevents elimination of these transient cortico-motoneuron axons, leading to increased dexterity (Gu et al., 2017). Recent studies demonstrate that organoids grown in an liquid-air interface generate nerve tracks reminiscent of subcortical and intracortical projections (Giandomenico et al., 2019). Co-culture of these organoids with mouse spinal cord showed that these "organoid-fugal" axons can innervate spinal cord explants and elicit responses in adjacent muscles (Giandomenico et al., 2019). Similarly, recent studies found that organoids positioned either in two wells separated by a custom made chamber, or at two ends of an artificial hydrogel column, extended reciprocal axons to each other forming strong fasciculated bundles (Cullen et al., 2019; Kirihara et al., 2019). Strikingly, electrical stimulation of one organoid elicited a calcium response in the axonal fascicle and the distal organoid, suggesting the formation of complex networks (Cullen et al., 2019; Kirihara et al., 2019). Although the specificity of these connections has not been probed in detail, these studies hint that organoids may eventually enable comparative studies of cellular and genetic mechanisms contributing to subcortical and intracortical connectivity.

Importantly, connectivity patterns vary substantially across cortical areas. For example, human vocal dexterity may in particular depend on subcortical projection neurons from laryngeal motor cortex that specifically project through the corticobulbar tract to laryngeal motor neurons in the nucleus ambiguus (Simonyan, 2014). Similarly, connections between higher order association areas and between association areas and dorsal thalamus also have stereotyped areal patterns (Buckner and Krienen, 2013). Recent studies indicate that cortical area-specific excitatory neurons emerge prior to sensory experience and are further diversified in adult cortex (Cadwell et al., 2019; Nowakowski et al., 2017; Tasic et al., 2018). Thus, organoid based studies of connectivity evolution may require precise specification of

appropriate cortical areas. Recently, we developed a classifier based on primary cell data and observed that excitatory projection neurons with area-specific molecular identities spontaneously emerge in human and ape organoids (Pollen et al., 2019). However, their organization within organoids and the fidelity of their connections has not yet been explored. In addition, we do not yet have a full catalog of area-specific signatures in primary tissue as early studies compared anterior and posterior cortical areas, and we do not know the extent to which molecular distinctions are arranged in discrete boundaries or gradients. Delivery of patterning molecules such as FGF8 to cortically specified organoids may permit further control of organoid arealization. Indeed, recent studies have directly engineered a patterning center-like structure to achieve dorsoventral gradients (Cederquist et al., 2019) and advances in microfluidics (Brassard and Lutolf, 2019) or in optical control of gene expression (Repina et al., 2019) could further support more reliable control over organoid arealization.

Properly specified areal organoids could then be examined for their connection ability to *ex vivo* mouse tissue (Giandomenico et al., 2019), to other region-specific organoids (e.g., medulla, spinal cord, dorsal thalamus, or higher order association areas) (Kirihara et al., 2019), or to *in vivo* targets through transplantation into mouse (Espuny-Camacho et al., 2013; Mansour et al., 2018; Mostajo-Radji and Pollen, 2018; Real et al., 2018; Stenevi et al., 1976). Early proof-of-principle studies using all three approaches indicate that mechanistic comparisons of connectivity development and evolution in humans and apes may soon be possible through control of areal, regional and subtype identity of pre- and postsynaptic neurons.

Protracted Myelination

Changes in myelination patterns may also be crucial for human cognition. The formation of a myelin sheath surrounding axons supports rapid communication of electrical signals across long distances (Rosenbluth, 1999). In the neocortex, myelin is gradually acquired throughout postnatal development following the activation of oligodendrocytes. Delayed myelination may support extended periods of plasticity (Fields, 2015; McGee et al., 2005), but may also contribute to multiple sclerosis susceptibility (Bove, 2018). In addition, disrupted myelination and white matter may contribute to other disorders including schizophrenia and bipolar disorder (Davis et al., 2003; Mighdoll, et al., 2015; Tønnesen et al., 2018; Van den Huevel et al., 2019). Importantly, the myelination process is particularly protracted in humans compared to other primates, continuing past age 25 (Miller et al., 2012b). In contrast, in chimpanzees, myelination density reaches its peak at the time of sexual maturation, around age 10 (Miller et al., 2012b). Similarly, at birth the human neocortex has almost no myelination while the chimpanzee cortex is ~20% myelinated (Miller et al., 2012b). These myelination differences could contribute to the accelerated divergence of lipid content reported in the human brain compared with other primates (Bozek et al., 2015).

Whether these species differences in myelination are controlled by general hormones, by neurons that signal to developing oligodendrocytes, or by cell intrinsic differences in oligodendrocytes themselves remains unknown. The heterogeneity of myelination across cortical layers and areas suggests the potential for local regulation. For example, humans

contain a greater proportion of upper-layer excitatory neurons, which undergo myelination later than the deep cortical layers (Tomassy et al., 2014), and prefrontal cortex myelinates much later than primary visual cortex (Turner, 2019). Interestingly, pyramidal neurons may have the capacity to directly instruct their own myelination, as different neuronal subtypes have distinct myelination patterns (Micheva et al., 2016; Tomassy et al., 2014; Zonouzi et al., 2019), and there is limited molecular heterogeneity in oligodendrocytes across brain regions compared with neurons (Zeisel et al., 2018). In addition, when myelinated sheaths are ablated from individual axons, the axons ultimate remyelinate in their original patterns (Auer et al., 2018). Thus divergence in gene expression and composition of pyramidal neurons could potentially contribute to species-specific myelination timelines, but the source of these differences has been difficult to identify.

Comparing oligodendrocyte lineage cells or pyramidal neurons from a range of primate species in common garden environments could help to reveal the cellular source of species differences in myelination rates. For example, it would be interesting to examine whether human and chimpanzee neurons transplanted to a common garden environment in the mouse cortex undergo different rates of myelination instructed by species-specific differences in neuronal maturation and signaling. In addition, several protocols now exist to generate oligodendrocytes from embryonic stem cells, which could be used to examine cell intrinsic changes in oligodendrocyte maturation across species (Douvaras and Fossati, 2015; Numasawa-Kuroiwa et al., 2014; Stacpoole et al., 2013). These oligodendrocytes can myelinate neurons and also micropillar nanofiber arrays that mimic axons for increased experimental control (Mei et al., 2014).

Modeling myelination in organoids has been challenging, but recent studies demonstrated that the addition of specific growth factors, nutrients and hormones to organoid culturing media resulted in the expansion and differentiation of oligodendrocytes, and ultimately *in vitro* myelination in the organoid (Madhavan et al., 2018; Marton et al., 2019). In addition, the use of media with physiological levels of glucose accelerated the appearance of oligodendrocyte precursor cells by several weeks (Kim et al., 2019). Although these studies showed the progressive acquisition of myelin basic protein and the compaction of the myelin sheath, fine structural refinement including the presence of nodes of Ranvier was not reported in these organoids, even at 30 weeks in culture (Madhavan et al., 2018; Marton et al., 2019). The decades long timeline of human *in vivo* maturation, the long term nature of these *in vitro* experiments, and the uncertainty of the underlying cellular source of species differences makes future comparative myelination experiments challenging, However, the importance of the phenotype to cognition and to neurological disorders makes future studies worthwhile. Co-transplant studies of human and ape iPSC derived neural progenitors and oligodendrocyte precursors to mouse could provide an avenue for current studies as organoid models of these later stages continue to improve.

Increased Synaptogenesis and Dendritic Complexity

Rates of synaptic maturation, pruning, and overall synapse number also vary across primates. Comparative histological and transcriptomic studies across developmental stages suggest that cortical synapse number continues to increase in humans until age four to eight,

but already peaks in the first year of chimpanzee and macaque postnatal development (Huttenlocher and Dabholkar, 1997; Liu et al., 2012; Petanjek et al., 2011; Rakic et al., 1986; Somel et al., 2009), while other studies also suggest an early acceleration of synaptogenesis in humans (Zhu et al., 2018). Continued developmental remodeling of synapses occurs well beyond adolescence in human prefrontal cortex, and the prolonged retention of early features of synaptic plasticity in humans, termed synaptic neoteny, has been implicated in learning and cognition (Petanjek et al., 2011). Synapse number varies dramatically across cortical areas within a species, with increased synaptic density and dendritic complexity in higher order association areas of cortex compared with primary processing areas (Bianchi et al., 2013; Elston et al., 2011; Hrvoj-Mihic et al., 2014). Despite substantial variation among cortical areas, comparisons between species of upper layer pyramidal neurons in prefrontal and temporal cortex, reveal that human excitatory neurons have larger dendritic arbors, longer dendritic segments and more dendritic spines than their chimpanzee and macaque counterparts in homologous areas (Bianchi et al., 2013; Deitcher et al., 2017; Elston et al., 2001; Mohan et al., 2015; Semendeferi et al., 2011; Teffer et al., 2013). This expansion is thought to increase the potential for integration of signals and abstraction of information in microcircuits of human prefrontal cortex, a key feature of human cognition. Interestingly, the increased dendritic complexity of human pyramidal neurons compared to rodent may also create additional neuronal compartments that perform non-linear transformations in individual neurons (Beaulieu-Laroche et al., 2018; Eyal et al., 2018; Tran-Van-Minh et al., 2015), but future work is required to examine whether this structure engenders richer computation and whether this compartmentalization also differs between humans and other primates.

Organoids and iPSC-derived pyramidal neurons may help to further reveal evolutionary changes in synaptic development and dendritic complexity and underlying mechanisms, (Hrvoj-Mihic et al., 2013; Hrvoj-Mihic et al., 2014). Indeed, a recent study indicates that molecular signatures related to early synaptogenesis and neuronal maturation appear later in human organoids than in chimpanzee and macaque organoids, suggesting that early timing differences following neurogenesis are preserved in vitro (Kanton et al., 2019). Nonetheless, neuronal maturation phenotypes are particularly challenging to study because synaptic development occurs over very long time periods, with many distinct phases from exuberant synapse formation through pruning and maintenance, and because the surrounding environment influences key aspects of development. To overcome these challenges, a recent study directly transplanted a mixture of human and chimpanzee iPSC-derived neural progenitor cells into the mouse cortex (Marchetto et al., 2019). The mouse cortical environment may provide connectivity partners crucial for normal synaptic development (Espuny-Camacho et al., 2013; Linaro et al., 2019; Mansour et al., 2018; Mostajo-Radji and Pollen, 2018; Real et al., 2018) and may reduce cell death compared to organoid models over long term experiments (Mansour et al 2018). Importantly transplanting a labeled mix of human and chimpanzee cells to the same cortical area enabled direct assessment of cell intrinsic differences in synaptic development. In this common garden environment, the human pyramidal neurons developed increased dendritic arborization and spine number, but this was visible only 8 – 19 weeks post-transplant. Interestingly, multielectrode arrays revealed that human *in vitro* cultures initially showed a lower mean firing rate, but ultimately

acquired a higher firing rate after 6 weeks in culture (Marchetto et al., 2019). Together, these results are consistent with prevailing ideas about an extended duration of synaptic development in humans. Future studies can expand on this work by directly anchoring measurement of pyramidal neurons to area-specific cell types, by including additional individuals and stages of development, and by measuring phenotypes related to plasticity. The strategy of transplanting chimeric mixtures of human and ape cells into mouse pioneered in this study may be particularly beneficial for analyzing these synaptic phenotypes, but improvements in organoid maturation may also support further *in vitro* analyses.

Towards comparative neurophysiology and circuit activity

The computational function of the brain is an electrochemical property that arises from neuronal activity, cell composition, and connectivity relationships. If we hope to ultimately understand how differences between species' cognition arise, we must examine the evolution of the basic circuitry of brain cells. Only comparative studies of *in vivo* physiology can currently address these questions at the circuit level. For example, recent single cell electrophysiology studies in the adult human and macaque brain suggest increased efficiency of information capacity in human anterior cingulate cortex neurons at the expense of robust coding (Pryluk et al., 2019). However, the capacity to perform *in vivo* human and primate studies at scale and to perform perturbations is limited. New tools, such as those supported by the BRAIN Initiative enabling further studies will ultimately be required to address these questions (Koroshetz et al., 2018).

Comparative *ex vivo* electrophysiology on surgical resections may also represent a powerful method for comparing physiological properties between human and macaque (Ting et al., 2018). For example, recordings of hippocampal slices from human and mouse suggest a possible divergence of spike-timing-dependent plasticity rules (Testa- Silva et al., 2010). Similarly, recordings in upper cortical layers indicate a functional role for HCN1 in dendritic conductance, with a possibly greater contribution of these channels to subthreshold synaptic integration and theta oscillations in human cortex (Kalmbach et al., 2018). However, these ex vivo resections are difficult to access and scale as an experimental model system, particularly at early stages of brain development. Organoids may help to fill these gaps, but the extent to which physiological properties of organoid neurons relate to normal physiological properties of developing brain remains unclear. Several studies have taken the first step of recording extracellular field potentials in brain organoids using multielectrode arrays, and one organoid study suggests a predictive relationship to oscillatory dynamics observed during normal brain development (Kawada et al., 2017; Mansour et al., 2018; Monzel et al., 2017; Quadrato et al., 2017; Trujillo et al., 2019). While organoid neural circuits will never share the receptive fields and precise connections that form in normal development, organoids offer the flexibility to selectively introduce cell types and to combine neurons from specific brain regions. This flexibility may allow organoid studies to complement *in vivo* and slice physiology by enabling reductionist studies of how cell type composition influences circuit properties outside of the normal space of circuits tolerated *in vivo*. In addition, organoids could enable studies of how homologous cell types from humans and apes perturb similar circuits in different ways. Speculatively, it may also be

possible to construct novel architectures of neural circuits outside of the constraints of a normal brain (Hofman, 2014) that possess interesting properties and functions.

Both *ex vivo* brain slices and organoid cultures are particularly amenable to simultaneous recording and stimulation of large pools of neurons. One approach would involve viral delivery of cell-type-specific reporters that enable optical analysis of calcium signaling or voltage changes in specific cell types (Dimidschstein et al., 2016; Förster et al., 2017; Graybuck et al., 2019). Recently, genetically-encoded voltage indicators coupled with high speed cameras have been applied *in vivo* to support optogenetic manipulation and recording of neuronal activity in small pools of neurons (Adam et al., 2019; Piatkevich et al., 2019), and this approach could likely be extended to larger populations and timescales *ex vivo*. Following physiological analysis, reconstruction of the molecular identity of constituent cell types can be performed using spatial transcriptomic approaches or through interpretation of reporter gene expression. Ultimately, such longitudinal observation of circuit activity may reveal further species differences and could be used to study the cellular and molecular origin of these differences.

OUTLOOK

This review has considered human and primate-specific evolutionary differences whose cellular and genetic origin could conceivably be studied using organoid models. Indeed, several comparative studies have already revealed potential changes in progenitor behavior, signaling pathway activation, and gene expression. However, studying these and other traits will require many general improvements in organoid models including improved fidelity to primary tissue, more precise patterning of brain regions down the level of cortical areas or subcortical nuclei, and engineering communities of cell types that enable the study of neuronal migration and connectivity. It is remarkable how far unguided self-assembly can already go to mimic normal development in a dish, but incorporating engineering approaches such as delivering precise morphogen gradients and scaffolds could further add reproducibility to these processes. The possibility of chimeric culture of iPSC lines between species together in the same organoid or in a mouse environment may help to reduce experimental confounds and isolate cell intrinsic species differences across a range of molecular and cell behavior phenotypes. Meanwhile, genome engineering approaches now allow for systematic reconstruction of genetic changes in organoid models that could ultimately lead to a quantitative genetic understanding of evolved human specializations. In addition, organoid models offer the opportunity for high-dimensional phenotyping and longitudinal analysis of neurogenesis and neural circuit formation across genotypes and species that is only just beginning to be exploited. Multielectrode arrays provide one example of long-term analysis, but imaging of organoids that express fluorescent reporters of specific cell types and of processes such as neuronal activity provide a path to rich phenotyping of otherwise inaccessible species differences and to the evaluation of candidate causal mutations and their effect sizes. Thomas Henry Huxley and Richard Owen debated the uniqueness of the human brain and the origin of specialized features over 150 years ago (Gross, 1993; Huxley, 1863). Brain organoids from humans and great apes combined with genome engineering now make it possible to begin to dissect the cellular and genetic origin of these recently-evolved differences.

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HIGHLIGHTS

- Brain organoids offer a window into the mechanisms underlying of evolution.

- Organoids capture cell diversity and interactions that occur during development.

- Organoid modeling could be applied to study specialized traits linked to cognition.
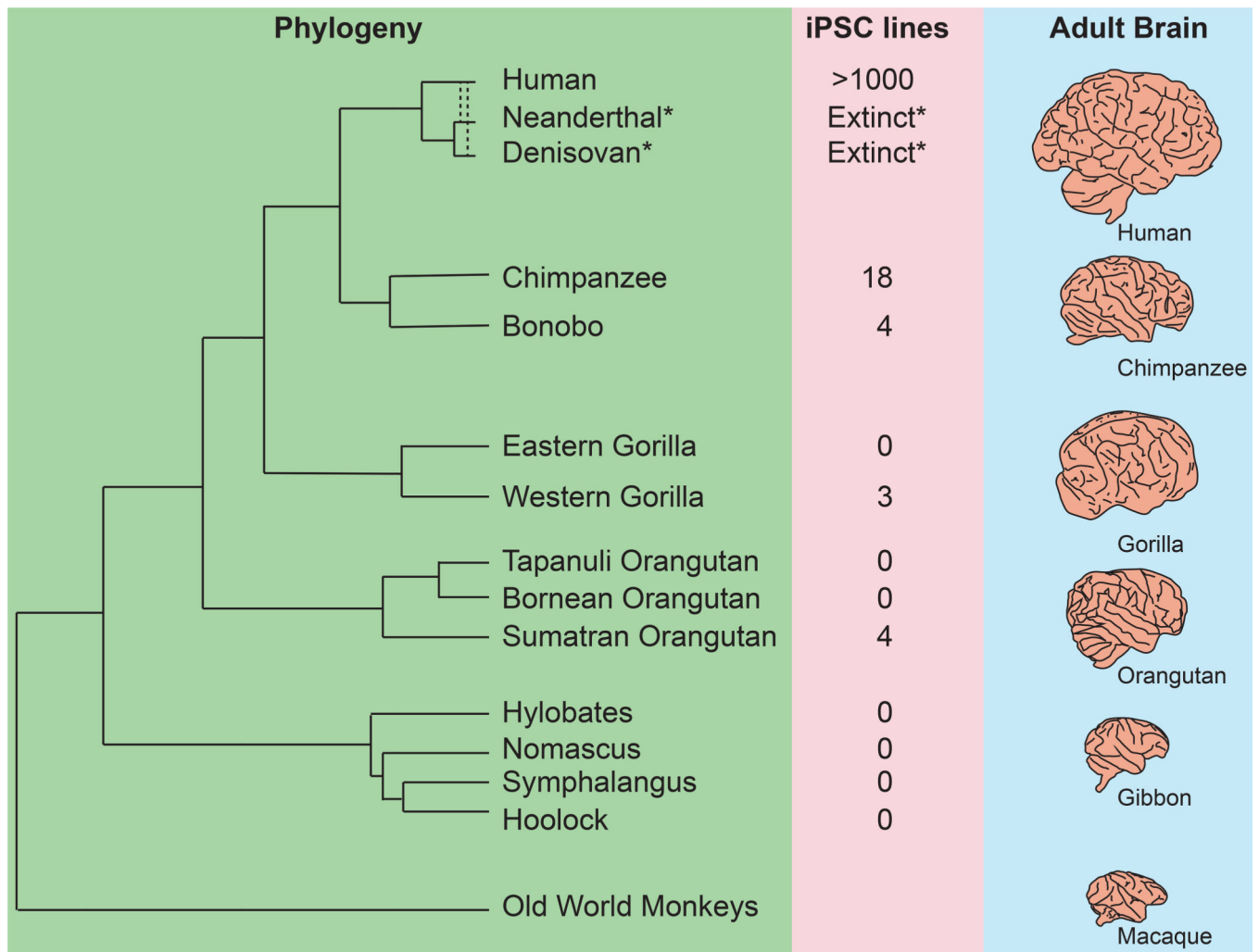
Figure 1. Existing pluripotent stem cell lines sample a limited fraction of ape diversity.
Phylogeny describes the radiation of ape species since divergence from a common ancestor with old world monkeys, with gibbon and orangutan clades updated based on recent studies (Carbone et al., 2014; Nater et al., 2017). Second column indicates the approximate number of individuals currently reprogrammed across published papers. Note that although Neanderthal and Denisovan are extinct, a substantial portion of their genomes exists as introgressed alleles in human populations and is accessible to genetic mapping approaches (Vattathil and Akey, 2015). Third column shows approximate relative brain size across species based on the Comparative Mammalian Brain Collection (http://neurosciencelibrary.org)
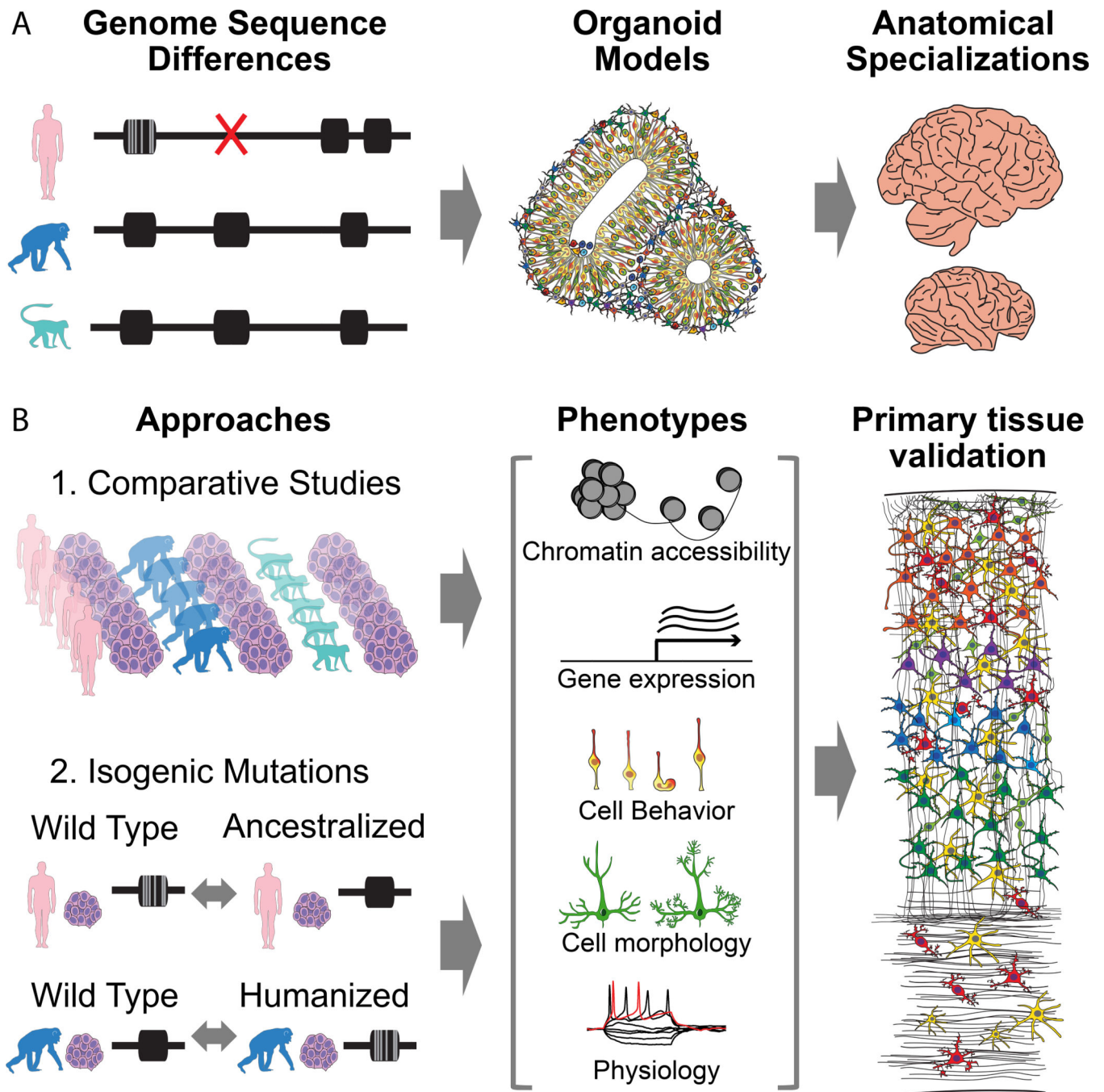
Figure 2. Organoid models help bridge the gap between human-specific genome sequence changes and evolved anatomical specializations of the human brain.

A) Human-specific genetic changes, including accelerated nucleotide substitutions, deletions, and duplications, as well as other insertions and inversions have been implicated in human evolution. Organoid models from humans and apes may provide the relevant cellular and genetic background for investigating the developmental consequences of these mutations. **B)** Two approaches for studying human-specific changes using organoids: First, comparative studies across sufficiently large numbers of biological replicates from human,

chimpanzee, and other primate outgroups can reveal species differences during development that would otherwise be difficult to dissect. Second, re-creating evolutionary differences at individual loci in isogenic iPSC lines can directly dissect the function of individual mutations and may overcome some of the variability observed across iPSC lines. This can involve re-creating the ancestral state in human cells or examining the function of the human allele in a chimpanzee context. These iPSC lines can then be differentiated into organoid models or specific neural subtypes to dissect how the entirety of genetic differences across species or individual candidate loci influence phenotypes, including changes in chromatin state, gene expression, cell behavior, morphology, and physiology. Observed differences can then be anchored by validation in primary tissue from human and a non-human primate when possible.
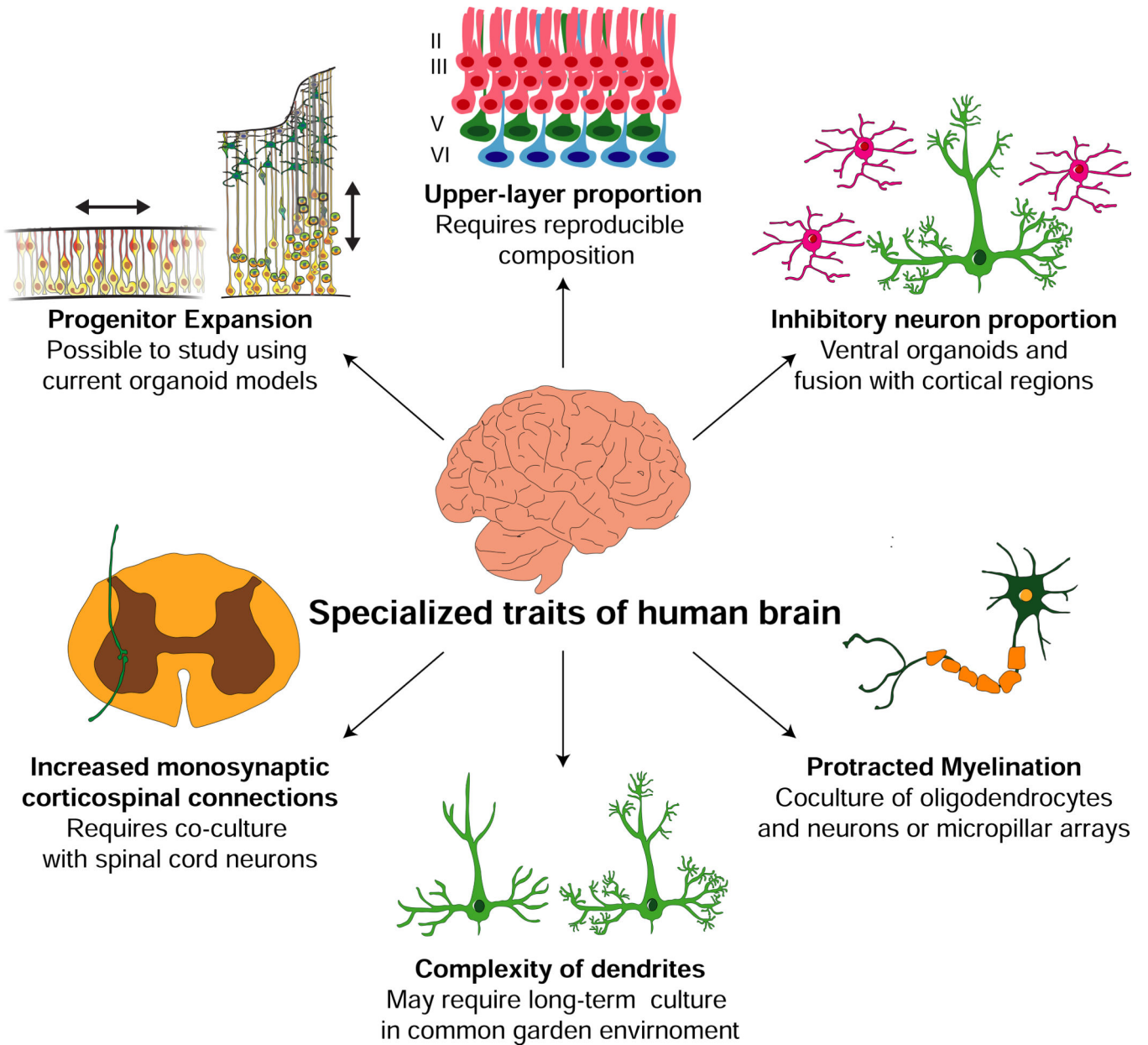
Figure 3. Reverse engineering the genetic and cellular basic of human cognitive specializations using organoid models will require methodological advances.

Comparative studies suggest several traits, including increased brain size, a reorganization of cell types, changes in connectivity patterns, protracted myelination and increased dendritic arborization contribute to human cognition. Current brain organoids recapitulate germinal zone structure and progenitor cell behaviors and have enabled comparative studies. On the other hand, studying the origin of the increased proportion of upper layer cortical excitatory neurons and cortical and thalamic inhibitory neurons will require reproducibility of cell fate potentials in organoid models across lines and species. In addition, studying traits like increased monosynaptic corticospinal connections and protracted myelination in humans depend on interactions between distinct cell types. These traits could be studied by co-

culturing different cell types after differentiating them separately. Finally, the complexity of dendrites is a function of intrinsic properties of neurons, as well as connectivity with the surrounding cells. Studying differences across species therefore requires comparison of cells in the same background environment to determine whether the human increase in dendritic arborization and synaptogenesis are driven by cell-autonomous factors.

Table 1.

Ape iPSC lines analyzed in recent studies, including newly generated lines:

Publication	Number of Individuals	Reprogramming Method	Individuals
Marchetto et al., 2013	2 chimpanzee (new), 2 bonobo (new)	Lentiviral from fibroblast	PR00818, PR01209, AG05253B, PR01086
Wunderlich et al., 2014	1 western gorilla (new), 1 bonobo (new),	Lentiviral from fibroblasts and endothelial cells	B1_i_1/2, G1_i_1/2a/2b/3a/3b,
Gallego Romero et al., 2015	7 chimpanzee (new)	Episomal from fibroblast	3647, 3649, 3651, 4955, 8861, 40210, 40280
Ramaswamy et al., 2015	2 Sumatran orangutan (new)	Retrovirus from fibroblasts	KB10460, KB10973
Otani et al., 2016	2 chimpanzee	Lentiviral from fibroblast,	PR00818, PR01209,
Mora-Bermúdez et al 2016	2 chimpanzee (1 new), 1 Sumatran orangutan (new)	Lentiviral and Episomal from fibroblast and blood	PR00818, Sandra A, Toba
Ramsay et al., 2017	2 western gorilla (new),	Retrovirus from fibroblast	PR00053 and PR00075
Blake et al., 2018	4 chimpanzee (1 new)	Episomal from fibroblast	3647, 3649, 40300, 4955
Pavlovic et al., 2018	10 chimpanzee (2 new)	Episomal from fibroblast	3624, 3647, 3649, 3651, 4933, 4955, 8861, 40210, 40280, 40300
Field et al., 2019	1 chimpanzee (new) 1 Sumatran orangutan (new)	Episomal from fibroblast	S008919 Josephine (11045–4593)
Pollen et al., 2019	8 chimpanzee (4 new)	Episomal from fibroblast	3649, 4955, 8861, 40210, 40290, 3611, PR00226, PR00738
Kanton et al., 2019	3 chimpanzee (1 new) 1 bonobo (new)	Retroviral and episomal from fibroblast and blood	PR00818, SandraA, JoC, Bokela

Table 2.

Assessment of the fidelity of distinct features of cortical development in current organoid models.

Category	Property	Correspondence in Organoids
Architecture	Ventricular and inner subventricular zones	Highly preserved
	Outer subventricular zone	Preserved
	Intermediate zone and subplate	Absent, but cell types are present
	Cortical plate	Excitatory neuron compartment, but limited lamination
	Afferent and efferent projections	Limited, may require assembloids
Cell types	Cell composition	Variable
	Radial glia (ventricular and outer)	Preserved
	Intermediate progenitors	Preserved
	Excitatory neurons	Preserved, but subtypes require further characterization
	Inhibitory neurons	Preserved, but protocol dependent
	Astrocytes	Preserved
	Oligodendrocytes	Preserved, but protocol dependent
	Vasculature	Requires transplant or bioengineering
	Microglia	Likely requires transplant
	Cell type gene expression	Similar
Developmental processes	Radial glia maturation	Present, but variable
	Radial glia interkinetic nuclear migration	Preserved
	Radial glia mitotic somal translocation	Preserved
	Neuronal differentiation	Preserved
	Excitatory neuron migration	Present, requires more characterization
	Inhibitory neuron migration	Present, requires more characterization
	Dendritic spines	Present, requires more characterization
	Spontaneous activity	Present, requires long-term culture
	Myelination	Present, requires long-term culture
Cell states	Cell cycle	Highly preserved
	Gene co-expression networks	Highly preserved
	Glycolysis	Organoid-enriched
	ER Stress	Organoid-enriched
	Apoptosis	Organoid-enriched