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# Building on a Solid Foundation: Adding Relevance and Reproducibility to Neurological Modeling Using Human Pluripotent Stem Cells

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The brain is our most complex and least understood organ. Animal models have long been the most versatile tools available to dissect brain form and function; however, the human brain is highly distinct from that of standard model organisms. In addition to existing models, access to human brain cells and tissues is essential to reach new frontiers in our understanding of the human brain and how to intervene therapeutically in the face of disease or injury. In this review, we discuss current and developing culture models of human neural tissue, outlining advantages over animal models and key challenges that remain to be overcome. Our principal focus is on advances in engineering neural cells and tissue constructs from human pluripotent stem cells (PSCs), though primary human cell and slice culture are also discussed. By highlighting studies that combine animal models and human neural cell culture techniques, we endeavor to demonstrate that clever use of these orthogonal model systems produces more reproducible, physiological, and clinically relevant data than either approach alone. We provide examples across a range of topics in neuroscience research including brain development, injury, and cancer, neurodegenerative diseases, and psychiatric conditions. Finally, as testing of PSC-derived neurons for cell replacement therapy progresses, we touch on the advancements that are needed to make this a clinical mainstay.

**Keywords: human pluripotent stem cells, neural stem cells, regenerative medicine, human brain development, disease modeling**

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## INTRODUCTION

The brain is the most complex organ in the human body, estimated to contain over 85 billion neurons and a similar number of non-neuronal cells (Azevedo et al., 2009). Specialized neuron subtypes continue to be identified and characterized at an increasing rate (Chen et al., 2017; Zhong et al., 2018; Hodge et al., 2019; Ximerakis et al., 2019; Polepalli et al., 2020; Bakken et al., 2021; Berg et al., 2021). The diverse non-neural populations that populate our brains include astrocytes, oligodendrocytes, neural stem and progenitor cell subtypes, and those with mesenchymal (microglia), epithelial (choroid plexus) and endothelial (vasculature) origins. Cell

types of the brain are highly varied molecularly and functionally, displaying unique signatures of electrical activity, neurotransmitter release, inflammatory responses, synaptic pruning and blood-brain barrier mechanisms. Our understanding of these complex features, how they're driven and how their intersection is important for brain function, remains quite limited. The plasticity of neural networks as they respond to and process stimuli is therefore only beginning to be elucidated. Understanding the remarkable synergy by which the vast number of cells in the brain connect together to produce human emotion and behavior is one of the richest and most exciting frontiers of science.

The pursuit of decoding the mysteries of the nervous system has led to a wealth of current tools that are enabling us to investigate increasingly transformative research questions. It goes without saying that human brain tissue is not easy to obtain from the source. While some behavioral studies performed in human subjects have provided insights, performing such experiments in a scientifically rigorous and ethical manner is challenging. Innovative neuroscientists over the decades have found new strategies to explore the answers they seek that balance physiological complexity with increasing relevance to the human brain (**Figure 1**). The simplest neural circuits can be modeled in fruit flies, zebra fish and roundworms (Guo et al., 2019; El-Daher and Becker, 2020; Singh and Aballay, 2020). Inbred rodents and chick embryos allow for straight-forward genetic manipulation and permit studies to link DNA sequence with consequent effects on cell biology, tissue development, maintenance and function, and organismal behavior (Keynes and Cook, 2018; Gulinello et al., 2019). Primates, like chimpanzees and macaques, can model some aspects of more complex social behaviors and disorders when rodents fall short (Shen, 2016). Traditional animal models used to dissect brain form and function have provided critical insights, informing our current understanding of the basic mechanisms of neurobiology. For example, work done in mice and rabbits was essential to our understanding of how acetaminophen acts as an analgesic (Swierkosz et al., 2002). Our knowledge of neural tube formation and closure was borne from work with frog embryos (for one example see Lutz et al., 1999). The neurotoxic effects of 1-methyl-4-phenyl-1,2,5,6-tetrahydropyridine (MPTP) in creating Parkinson's-like symptoms were not fully resolved until the drug was administered to squirrel monkeys (Langston et al., 1983).

Despite these advances, it's clear that research using human cell sources is critical to overcome significant knowledge gaps that remain about the human brain (**Figure 1**). This is underscored by the existence of basic foundational differences in brain evolution across species. The fact that rodent cortices are smooth (lissencephalic) while the human cortex is highly folded (gyrencephalic) illustrates the point. Recent single cell RNA-sequencing of the middle temporal gyrus from both mouse and human tissue found that while most cell types are broadly conserved, their relative proportions, laminar distributions, morphologies and gene expression patterns are vastly different between the two species (Hodge et al., 2019). A similar conclusion was drawn from analyses of single cells derived from the primary motor cortex of humans, the non-human primate marmoset, and mice (Bakken et al., 2021). While the broad molecular identities of cell types in this region were found to be conserved, their relative proportions, transcriptional and chromatin states, and expression patterns of cell type marker genes were quite distinct between the three species. Additionally, the mouse brain lacks certain defined structures and cell types that are found in humans. These include a greater number of glutamatergic neuron subtypes in the supragranular region of the human cortex (Hodge et al., 2019; Berg et al., 2021) and the human outer subventricular zone (oSVZ). The oSVZ is populated with basal radial glia cells, a neocortical stem cell type important for neuronal expansion and cortical folding in gyrencephalic species; in line with the expanded oSVZ in humans, basal radial glia are abundant in humans and rare in mice (Pollen et al., 2015). Additionally, at the sub-cellular and physiological levels, several proteins known to be involved in human disease, such as amyloid precursor protein, don't display the same pathological features in mice.

A formidable constraint, the vast complexity of human behaviors cannot be accurately modeled in other species. For example, stress has been shown to be one key indicator of depression in humans. Several types of behavioral tests have been established to measure stress in mice, however it has been difficult to meaningfully link the results of these standardized tests to depression-related coping mechanisms typical of humans (see Commons et al., 2017 for one key example). The consequences of inter-species evolutionary and physiological differences are exemplified by research into many developmental and age-related neurological disorders, including Alzheimer's disease, in which rodents are still the prevailing model. The most telling outcome of this has been the large numbers of successful drugs that reverse characteristic biochemical hallmarks and memory defects in mice which subsequently fail in human clinical trials (Ransohoff, 2018). One report mentions that a single strain of mice used to model Alzheimer's Disease was "cured" over 300 times, though not one drug reported clinical success (Zahs and Ashe, 2010). Thus, many questions related to human specific mechanisms of development and disease for which animal models have yielded insights will inevitably require replication and additional study in relevant human models.

## CULTURE MODELS FOR NEUROSCIENCE RESEARCH

### Primary Human Tissue

Advances in brain mapping are increasingly providing a better understanding of the firing patterns of neuronal networks within distinct brain regions. With these rich data sets, correlations can be made between human behavior, disease states, and the functional areas of the brain that are affected (Mitchell et al., 2019). Though to achieve the widely sought goal of successful pharmacological intervention, deeper investigation into these processes at the cellular and biochemical levels is needed. While many current efforts in neuroscience research are providing deep knowledge of molecular and functional neurological mechanisms, correlating *in vitro* and *in vivo* observations remains a significant challenge. Human cellular

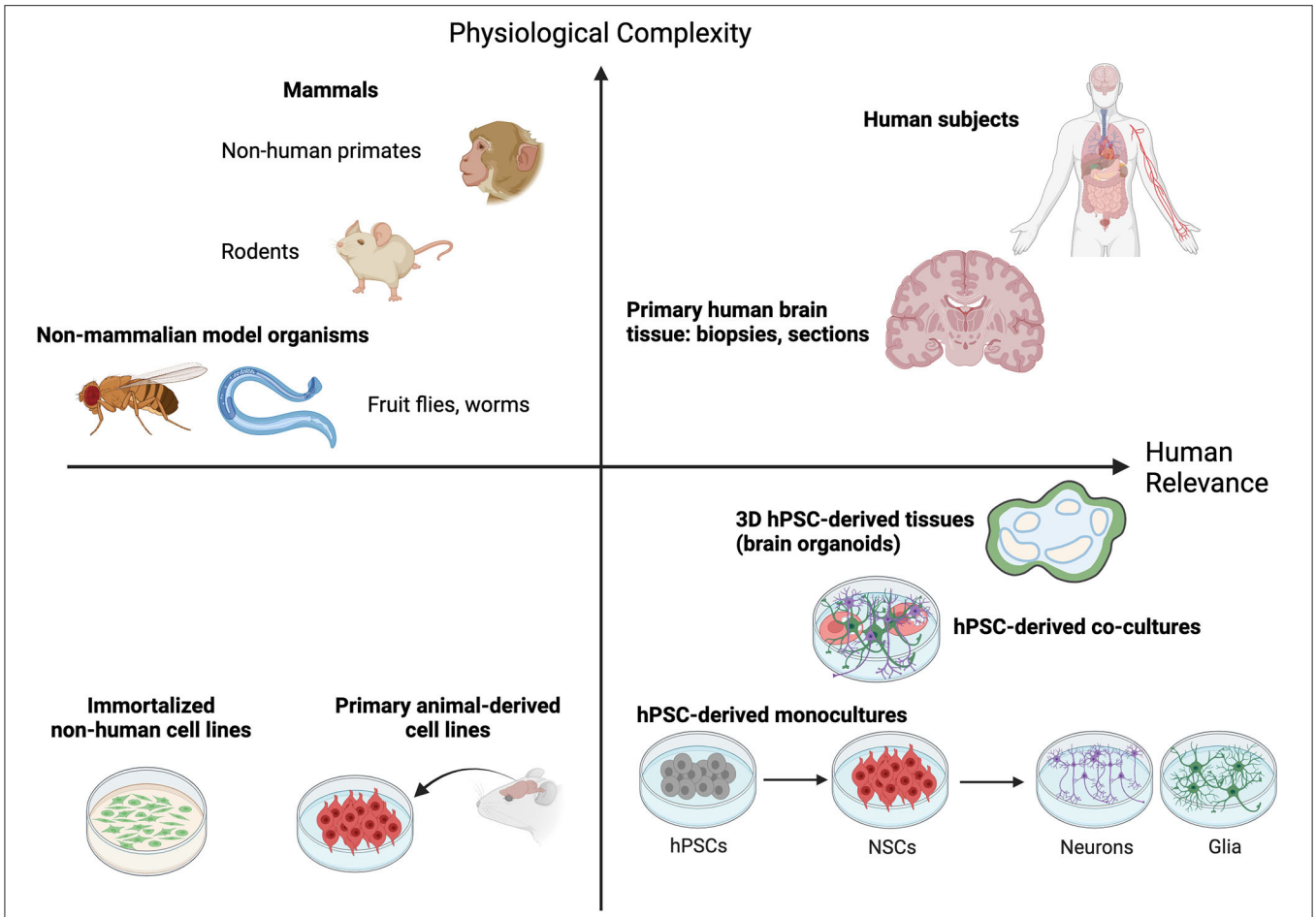


**FIGURE 1 |** Animal and cell culture models fall on a continuum that balances providing physiological complexity with human brain relevance. Non-mammalian model organisms like worms (*C. elegans*) and fruit flies (*D. melanogaster*) allow molecular and functional investigation of simple neuronal circuits and rational dissection of critical regulatory pathways in a physiologically relevant setting. Rodent and non-human primate models provide additional physiological complexity by permitting quantitative analysis of complex behavioral traits and simultaneously enrich relevance to the human condition. Human subjects and dissected human brain tissues fall high on both measures, but their procurement is rare or ethically impossible. The emergence of human pluripotent stem cell (hPSC)-derived 2D cell and 3D tissue models offer a highly needed source of neurological modeling tools with high relevance to the human brain. Though they offer less physiological complexity than animal models this capacity is ever increasing as technology advances (defined cell co-cultures and 3D tissue engineering). Furthermore, their relevance to the human condition is greatly enhanced compared to traditional culture of immortalized or non-human primary cell lines. A combination of hPSC-based and animal model approaches offers the best opportunity to capture both physiological complexity and relevance to the human brain.

models, especially those that mimic the associated *in vivo* tissue environment, provide the best bridge to form that link.

The most relevant source of *in vivo* neural cells is the human brain itself (**Figure 1**). On rare occasions, access to living patient tissue can be granted, though most studies rely on donation of post-mortem tissue. When access to tissue is possible, biochemical analysis of organotypic slice culture allows for the characterization of human-specific cell and molecular phenotypes of disease. In a recent example the existing *in vivo* structures present in organotypic slice culture made it possible to examine the spreading of α-synuclein aggregates between neurons (Elfarrash et al., 2019). Having direct access to the living tissue allowed the authors to show that spreading of this pathogenic protein can occur even in the absence of disease characteristic α-synuclein Serine 129 phosphorylation. Neural stem cells have also been isolated from human embryonic brain tissue and can be expanded in culture and differentiated to downstream neural cell types (Dos Reis et al., 2020). In fact, dopaminergic neurons obtained directly from human embryos were some of the first to be used for cell transplantation experiments in Parkinson's Disease patients. For primary human brain-derived cultures time is the enemy, as most neurons begin to die after a few hours in culture and many neuronal cultures cannot be maintained for longer than 14 days (Croft et al., 2019).

## Pluripotent Stem Cells

Pluripotent stem cells (PSCs) are a powerful tool that have allowed us to overcome the limits of tissue availability and low viability of primary human tissue in culture (for a comparison

**TABLE 1 |** Comparison of typical neural culture systems.

| Culture System | Application | Pros | Cons |
|---|---|---|---|
| Organotypic slices | - Complex neural circuit modeling<br>- Tumor invasion analysis | Retain *in vivo* organization | - Limited culture life<br>- Genetic manipulation difficult |
| Primary neurospheres | - Neural stem and progenitor cell expansion<br>- Tumor cell expansion | - Isolation of mature (active) cell types and pure cell populations possible<br>- Co-culture of distinct cell types possible<br>- Low variability between experiments<br>- Ease of expansion for high cell yield | - Limited passage number<br>- Technically difficult to passage<br>- Need sufficient starting cell source<br>- Difficult to establish from adult tissues<br>- Lacks *in vivo* organization and diverse cell types |
| Primary cell adherent culture | - Separating cell autonomous from non-autonomous effects | - Isolation of mature (active) cell types and pure populations of some cell types<br>- Co-culture of distinct cell types possible<br>- Low variability between experiments | - Isolating pure cell types difficult and time consuming<br>- Difficult to establish from adult tissues<br>- Genetic manipulation difficult outside of proliferating NPCs<br>- Limited culture life-span for mature cell types<br>- Lacks *in vivo* organization and diverse cell types |
| hPSC-derived adherent culture | - Separating cell autonomous from non-autonomous effects<br>- Human genetic studies (background variability and single gene mutations)<br>- Neurogenesis and gliogenesis | - Accessible human relevant cells<br>- Pure culture and co-cultures possible<br>- Can generate specific cell subtypes<br>- Easy to produce multiple cell types from one initial source<br>- Early proliferating cell types (PSCs or NPCs) are expandable and bankable<br>- Genetic manipulation protocols well established | - Cells produced are typically fetal-like<br>- Lacks *in vivo* organization<br>- Long differentiation protocols and maturation times<br>- Mastery of hPSC culture a prerequisite<br>- High cell line to cell line variability |
| hPSC-derived spheroids and organoids | Tumor cell expansion, Modeling brain development and neurodevelopmental disorders | - High efficiency of neural differentiation<br>- Observe interactions between multiple cell types<br>- *In vivo*-like organization<br>- Spontaneous (unpatterned) or directed (patterned) differentiation possible | - Similar to adherent culture<br>- Lacking some critical non-CNS cell types (e.g., microglia and vasculature), though these can be added by co-culture |

of typical neural culture systems see **Table 1**). Now researchers with access to readily available somatic cell sources such as blood, skin and urine from normal or disease donors, can establish induced pluripotent stem cell (iPSC) lines to study their disease of interest through the introduction of core pluripotency transcription factors (such as OCT4, SOX2, c-MYC, Lin28, NANOG, and KLF4). These iPSC cultures can then subsequently be directed to differentiate into almost any cell type of interest using well-established protocols, permitting the derivation and analysis of distinct cell types along major lineages (Chambers et al., 2009; Nolbrant et al., 2017; Tcw et al., 2017). Publically available cell banking initiatives, through which researchers can acquire already established iPSC lines for study, are also now widely available [e.g., International Stem Cell Banking Initiative (https://www.iscbi.org/), Coriell Institute for Medical Research (https://www.coriell.org/), European bank for induced pluripotent stem cells (www.ebisc.org), StemBANCC (www.stembancc.org), HipSci (www.hipsci.org), National Institute of Neurological Disorders and Stroke (NINDS) Human Cell and Data Repository (https://stemcells.nindsgenetics.org/), Simon's Foundation (https://www.sfari.org/resource/ips-cells/)]. The flexibility of human PSC culture systems is well-suited to studies aiming to discern cell autonomous vs. non cell autonomous effects by comparing phenotypes in pure cultures vs. heterogeneous co-cultures, lineage tracing across multiple cell types, and genome editing to permit comparison of control and isogenic edited lines (**Figure 2**).

In addition to iPSC reprogramming and subsequent directed lineage differentiation, transdifferentiation using cell-type specific transcription factors allows direct reprogramming of one somatic cell type into another (**Figure 2**; for example, fibroblasts to neurons: Vierbuchen et al., 2010). This approach has the advantage of maintaining the epigenetic signature of the cell type of origin by avoiding transition through an embryonic iPSC state, which is especially useful when modeling diseases of aging (Huh et al., 2016). Similarly, forward programming of PSCs using transcription factor overexpression can produce highly pure populations of neurons (Zhang et al., 2013), astrocytes (Li X. et al., 2018) and oligodendrocytes (García-León et al., 2018). The speed and ease of use of these protocols has made them especially attractive for large scale screening applications (Sridharan et al., 2019). The most direct clinical application of somatic cell reprogramming is the potential for *in vivo* transdifferentiation of resident astrocytes into functional neurons for neuronal cell replacement therapy (Liu et al., 2015; Aravantinou-Fatorou and Thomaidou, 2020; Ma et al., 2021). This strategy, which has been achieved with both neuronal transcription factor expression and more recently targeted small molecules, could importantly circumvent the need to generate large numbers of cells for transplant and the worry of immunocompatibility. Questions remain, however, about the true identity, functionality, and

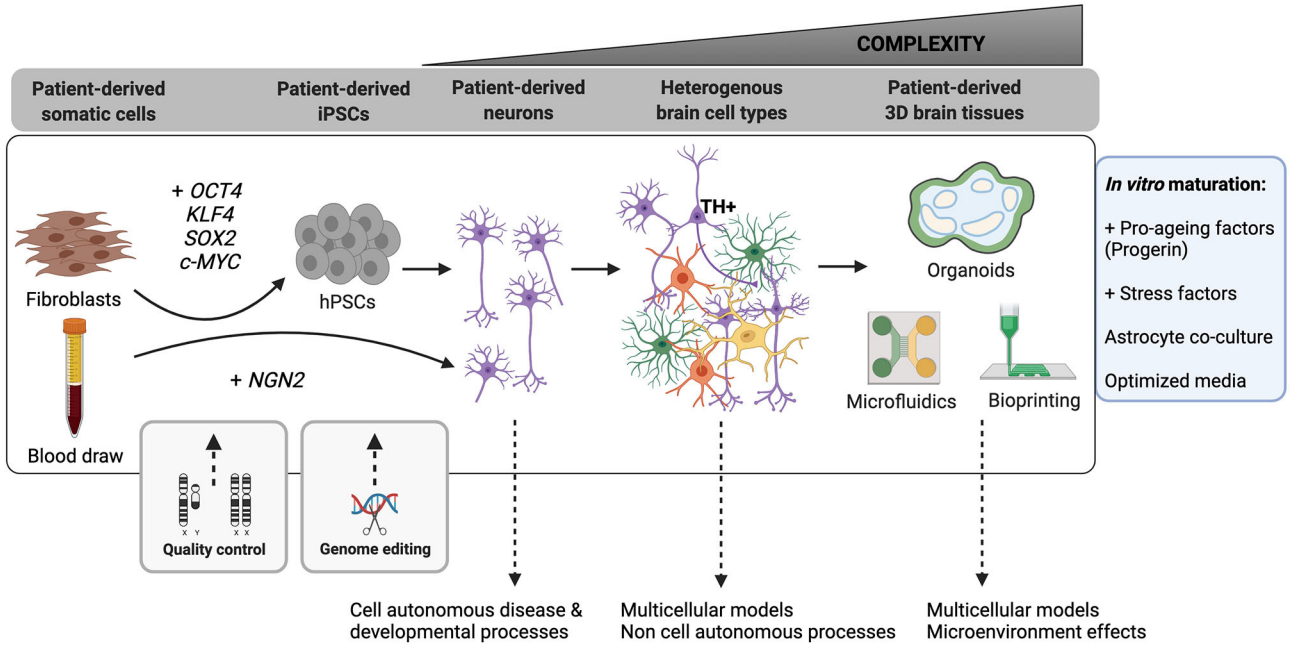


**FIGURE 2 |** Advances in hPSC-based neurological modeling are increasingly improving the complexity of human neural cell types and circuits we can model. Somatic cells from multiple human patient sources (fibroblast skin cells, blood, urine) can now be used to produce induced human pluripotent stem cells (hPSCs) by over-expression of typically four key pluripotency transcription factors (*OCT4, SOX2, KLF4, c-MYC*). Similarly, induced neurons can be directly established by over-expression of neuronal identity genes like *NGN2*. It is essential to regularly perform quality control assays (detailed in the main text) to ensure maintenance of a high-quality pluripotent state in reprogrammed hPSCs. This stage also offers a critical period for genome editing (e.g., CRISPR-Cas9) in which to introduce mutations of interest or create isogenic control lines. As a field, we can reproducibly generate fetal-stage excitatory and inhibitory neurons in 2D and 3D cell cultures. These cultures permit analysis of cell-autonomous mechanisms of development and disease, especially in the presence of a disease-causing mutation. Recent efforts have increased our ability to generate additional specialized brain-resident cells ("Heterogenous brain cell types") and to produce more mature neurons (purple = excitatory and inhibitory neurons with elaborate neurite networks; green = astrocytes; orange = oligodendrocytes; yellow = microglia). These heterogeneous cultures permit modeling of non-cell autonomous processes. Offering the most advanced level of complexity in hPSC models are 3D brain tissue engineering approaches, including organoids (rounded enclosed objects = ventricular zones; blue shading = neural stem and progenitor cells; green shading = neurons and other differentiated cell types) and bioprinted constructs, which can incorporate microfluidic channels for advanced microenvironmental control. Studies have also begun to incorporate vasculature and components of the blood-brain barrier into microfluidic and organoid models. The box to the right outlines recent and developing strategies for promoting neuronal maturation and aging in 2D and 3D models.

origin of the resulting cell types if developmental programs are not strictly followed and when artificially high levels of cellular factors are expressed (Nehme et al., 2018; Wang et al., 2021). In fact, a recent group pointed out that neurons that were presumed to be reprogrammed from astrocytes were actually, in fact, derived from other endogenous neurons (Wang et al., 2021). This highlights the need for a closer look at the consequences of somatic cell reprogramming, including a necessity to incorporate careful lineage tracing analyses to ensure we understand which cell identity transitions are in fact taking place.

A more recent advance in stem cell research has been the development of differentiation protocols in three-dimensional cultures (**Figure 2**; see Tian et al., 2020 for a recent review), including both spheroids (aggregates of differentiated cells) and organoids (structures that form out of aggregated progenitors). The first neuroectoderm-derived organoids (Eiraku et al., 2011) were able to recapitulate many features of the developing eye. Later protocols exploited the intrinsic self-organizing potential of aggregated neural precursors to activate endogenous cell signaling mechanisms to promote spontaneous differentiation into cerebral cortex tissue, mimicking similar developmental stages and tissue architecture as the developing human brain (Lancaster et al., 2013). Similar to 2D differentiation protocols, cytokines and small molecules can be added to pattern the organoids into specific cortical regions (Muguruma et al., 2015; Jo et al., 2016; Birey et al., 2017; Sloan et al., 2018; Marton et al., 2019; Eura et al., 2020; Hor and Ng, 2020). The primary advantage of organoid culture technology is the ability to replicate cell organization and maturation during development while simultaneously maintaining cell viability in long term culture (see **Table 1**). Using these sophisticated human tissue models, researchers have also been able to fuse organoids that represent adjacent anatomical regions. This permits modeling of the connections between neurons from different areas of the brain (Bagley et al., 2017) and between the eye and brain (Fligor et al., 2018). These same principles have also been applied to cancer research, such that organoids derived from patient biopsies more closely mimic the attributes of the parent tumor than do traditional immortalized cancer cell line models (Hubert et al., 2016; Jacob et al., 2020).

**TABLE 2 |** Considerations for PSC culture.

| Source material | Study design | Reprogramming method | Replicates |
|---|---|---|---|
| **In-house derived hPSCs:**<br>- Dependent on access to somatic cell sources (skin, blood, urine), ease and efficiency of reprogramming (e.g., urine is easily accessible, but more laborious than other sources to reprogram)<br>- Age (younger cells reprogram more efficiently)<br>- Access to patients with mutations of interest can be challenging<br>**Purchased cell lines** (e.g., Coriell, EBISC, StemBANCC, HipSci, NINDS):<br>- Dependent on price, availability of desired mutation, patient medical data<br>- Assess restrictions on use (expansion, banking, differentiation, commercial use)<br>- Verify appropriate patient consent and institutional review forms available (ICF and IRB review and approval) | - Biological replicates are critical: collect from multiple donors where possible<br>- Non affected familial control or isogenic wild-type lines are necessary<br>- For case-control study designs: multiple clones required<br>- Consider genetic engineering if controls or lines with mutation of interest are unavailable | **Integrating vs. non-integrating reprogramming**:<br>- Genomic integration of vector can lead to unwanted secondary mutations and reactivation of reprogramming factors<br>- Certain vectors are more effective with certain cell types (Okita et al., 2013)<br>- Labor cost: non-integrating methods often require specialized equipment (e.g., electroporator)<br>- Financial cost: increasing efficiency typically comes with higher cost<br>- Selection of reprogramming factors (e.g., OCT4, SOX2, c-MYC, KLF4)<br>- Consider published protocols to increase reprogramming efficiency (Huangfu et al., 2008; Esteban et al., 2010)<br>- Selection of high efficiency reprogramming medium | **Accounting for inherent variability among hPSC lines:**<br>- Include hPSCs from at least 3 similarly affected subjects. If not possible, use at least 3 hPSC clones per donor<br>- Ensure all experimental lines are of a similar age<br>- Sex balance: use an equal number of female and male hPSC lines<br>- Consider other potential mitigating health factors from sample (other health issues, diet, potential genetic modifiers) |
| **Culture Considerations** | **Quality Control** | **Maintenance** | **Differentiation/Maturation Method** |
| - Dedicated Biological Safety Cabinets and safety protocols for human tissue work<br>- Separate space for human vs. other mammalian culture preferable<br>- Asses incoming human samples for presence of infectious viruses<br>- Mycoplasma testing of all new cell lines<br>- Cryogenic storage essential for biobanking viable hPSCs | - Daily morphological assessment: visibly differentiated cells can be manually removed<br>- Karyotype all new lines, genetic analysis (QPCR for common abnormalities) for new thaws and before important experiments<br>- Assess pluripotency (teratoma or trilineage differentiation) of all new lines<br>- Assess markers of pluripotent state (e.g., OCT4, TRA-1–60) for all new lines | - Choose between defined (feeder and serum free) or undefined (feeders and serum/serum replacement, sometimes more efficient for differentiation) culture conditions<br>- Clump vs. single cell passaging (single cell passaging can increase chromosomal abnormalities)<br>- Matrix: defined (vitronectin, laminin) or undefined (matrigel)<br>- Low protein, robust or stabilized growth media<br>- Regulatory compliance of hPSC growth media<br>- Feed, passaging and banking schedule | - Developmental patterning to drive lineage induction vs. forward programming (e.g., NGN2 overexpression)<br>- Pure populations or co-cultures<br>- 2D (largely homogenous cell types) vs. 3D (e.g., heterogeneous organoids) architecture<br>- Transdifferentiation (retain epigenetic signature of source by skipping PSC stage)<br>- Identify best time-points to freeze and biobank cells, with high post-thaw viability<br>- Maturation strategy (e.g., increased culture time, physiological maturation medium, addition of cell stressors) |

## Considerations for Starting PSC Cultures

Starting a project using PSCs for the first time is not without a learning curve (see **Table 2** and Ernst, 2020 for an in-depth review). However, advances in the field have lowered many barriers to adoption. Robust reagents and methods exist for embryonic stem (ES) and iPSC culture, as well as neural differentiation. While it may never be possible to fully recapitulate every neural subtype that exists in both space and time, protocols have been established to generate most major cell types (Chambers et al., 2009; Falk et al., 2012; Douvaras et al., 2014; Tcw et al., 2017; Marton et al., 2019). Addition of protein and small molecule patterning factors specifies regionality to these protocols to generate even more specialized cell types like dopaminergic (Kirkeby et al., 2012), hippocampal (Sarkar et al., 2018), serotonergic (Lu et al., 2016), and motor neurons (Hu and Zhang, 2009). Protocols for neural crest cell differentiation (Hackland et al., 2017; Tchieu et al., 2017) enable the generation of peripheral neurons (Prince et al., 1991) as well as their mesenchymal derivatives (cartilage, bone (Leung et al., 2016), fat (Gomez et al., 2019) and smooth muscle (Serrano et al., 2019; Delaney et al., 2020; Li X. et al., 2020). Even non-ectodermal contributors to the nervous system can be produced and incorporated into brain tissue models including microglia (Abud et al., 2017; McQuade et al., 2018), brain microvascular endothelial cells (Qian et al., 2017) and pericytes (Stebbins et al., 2019).

Standardized media and protocols have made it easier than ever for labs that have traditionally focused on animal work to adopt hPSC-based culture into their pipelines. Though despite the advances mentioned above, there are limits to the current applicability of stem cell-derived neural cells. Many labs new to stem cell culture, even when experienced in

traditional primary or immortalized cell culture, struggle to maintain quality human stem cell lines. This is especially acute when the project involves generating novel iPSC lines from multiple patients. The choice of starting cell type, reprogramming method, clone selection, culture medium and passaging protocol can all influence the efficiency of downstream differentiation (see **Table 2** and Ernst, 2020). Knowing how crucial starting cell quality is for downstream differentiation, several large consortia have agreed on a minimum set of criteria required for assessing stem cell quality (Sullivan et al., 2018). Several can be routinely monitored in the lab without specialized training or equipment (morphology, marker expression, trilineage differentiation potential, epigenetic signature). Tracking and maintaining genetic stability is more difficult. qPCR kits exist that will give basic information on common small chromosomal amplifications and deletions known to exist in pluripotent cells (for review see Halliwell et al., 2020). This needs to be combined, however, with G-banding and possible copy number variation analysis to truly understand genetic stability. These later two methods are costly and are therefore usually only done when starting a new cell bank or before a critical experiment. Several publications suggest that even small chromosomal gains or losses influence downstream differentiation efficiency (reviewed in Henry et al., 2018). Labs ignore karyotyping at their peril. For example, gain of chromosome 20 frequently occurs in PSC culture, driven by *BCL2L1*, a gene associated with reduced apoptosis and cancer (Enver et al., 2005; Baker et al., 2007). Cells with amplifications like this have a growth advantage, but with a potential increase in tumorigenic potential (Blum and Benvenisty, 2008; Ben-David and Benvenisty, 2011).

## Addressing Long Time Frames for *in vivo* Maturation

An important consideration is the immature fetal-like nature of PSC-derived neural cell types. Several approaches are being taken to increase the speed at which neurons are produced and become functionally mature *in vitro*. Applying compounds, such as inducers of reactive oxygen species, to induce age-associated cellular stressors has been effective in revealing phenotypes associated with progressive neurodegenerative disease (Mertens et al., 2018) genetic over-expression of pro-aging factors (i.e., progerin) have also been applied and result in increased molecular signatures of aging (Miller et al., 2013; Cornacchia and Studer, 2017). However, to date no single approach has been conclusively proven to generate adult-like functionally mature neurons.

Modifying neural cell culture protocols to increase maturation *in vitro* has, however, yielded some degree of success. Co-culture of neurons with astrocytes has long been shown to increase neurite outgrowth and activity, and this approach is now yielding more physiologically relevant iPSC-derived neuronal cell disease models (Odawara et al., 2014; Kuijlaars et al., 2016; di Domenico et al., 2019; Enright et al., 2020; Leventoux et al., 2020). Cell culture media formulated to mimic human cerebral spinal fluid (Bardy et al., 2015) increases neuron maturation and generates neurons which become active at a much earlier stage in culture. Organoids appear to provide the necessary tissue structure and cell non-autonomous support needed for increased neuronal cell maturation, demonstrating complex oscillatory firing patterns in neurons derived from cerebral organoids (Trujillo et al., 2019; Fair et al., 2020; Giandomenico et al., 2021). However, there is still some debate as to what molecular or activity readouts define a "mature" neuron. These important phenotypic parameters first need to be defined before the field can move forward with improving neuron maturation in culture (Livesey et al., 2016; Ghanavatinejad et al., 2019).

## Dealing With Genetic and Technical Variability

Many recent efforts have focused on identifying and addressing key technical issues to increase the biological relevance and decrease the variability of PSC-derived models (see Volpato and Webber, 2020 for a recent review). Genetic variability between cell lines can not only mask phenotypes but also influence differentiation efficiency (Ortmann and Vallier, 2017). One interesting method to overcome this involves "normalizing" signaling pathways by simultaneous activation and inhibition during the first phases of differentiation (Hackland et al., 2017). Other groups have reported that ES/ iPSC with naïve gene expression profiles are better able to differentiate into neural lineages (Park et al., 2018; Watanabe et al., 2020). Further, there is some evidence to suggest that addition of something as simple as low concentrations of DMSO can increase differentiation to neural lineages in difficult to differentiate PSC lines (Li J. et al., 2018); this has been attributed to activation of the Retinoblastoma signaling pathway. While standardized culture media and protocols can overcome much inter-cell line variability, there needs to be a deeper understanding of the signaling pathways involved during the earliest cell fate decisions that take place during differentiation. Normalizing these pathways before differentiation is induced could help increase reproducibility in differentiation efficiency across cell lines, especially PSC lines that are generated using slightly different methods and by different researchers. Increased access to and decreased pricing for single cell genomic studies is continually improving our understanding of variation among human neuronal cells and tissues, and is providing insight into the genetic vs. technical basis of developmental and disease phenotypes observed *in vitro* (van den Hurk and Bardy, 2019).

Traditionally, the main advantage of using animal models has been the ease of genetic manipulation and control over the genetic background. Due to the extensive genetic variability that exists between humans, stem cell-based studies aiming to determine the neurological impacts of mutations in even a single gene have traditionally required a large number of cell lines derived from patients with the same disease (and with multiple clones per patient), as well as multiple cell lines from unaffected individuals to serve as controls. Though more recently, for diseases caused by known genetic variants or with established genotype-phenotype correlations, genome editing technologies such as CRISPR/Cas9 (reviewed in Rehbach et al., 2020) can efficiently establish paired gene variant and isogenic control

cell lines. Thus, inbred animals are no longer the only way to model disease-related mutations across isogenic backgrounds. The relative ease by which isogenic control lines can now be engineered in hPSC also greatly increases the opportunity for stem cell-derived models to yield significant phenotypic changes, by limiting genomic variability among experimental samples without the need to establish and analyze a cost-prohibitive number of patient and control cell lines.

For diseases with complex genetics or combined environmental and genetic contributions, human cell-based studies still need to rely on genetically related or cohort matched unaffected controls (see Riemens et al., 2020 for a recent review). In some cases, it can also be argued that to develop effective disease treatments study designs need to reflect the inherent genetic variability in the population they are aiming to treat. In animal models this is accomplished by testing different strains and stratifying study subjects by sex, age, diet, and environment. With stem cell-derived models this same level of control can be accomplished by incorporating samples from multiple patients, deriving multiple clones from each line, creating multiple sets of isogenic variant and control lines (in the case of single gene mutations), and adopting a standardized cell culture protocol with carefully implemented quality control measures while maintaining diverse hPSC lines. When modeling phenotypes that underlie development or disease states, assessing variability between cell lines established from different donors is particularly critical; increasing access to and streamlined strategies to analyze iPSC lines is continually making these endeavors more possible (Schuster et al., 2015). An effective way to establish the validity of an observation in the face of high biological variability is to test it across multiple experimental models and methods. In one study (Yuva-Aydemir et al., 2019), the authors modeled Amyloid Lateral Sclerosis (ALS) by producing Drosophila mutants with expanded GGGGCC repeats in *C9ORF72*. With this approach, axonal and locomotor defects that reflect those observed in ALS patients were reproduced. The simplicity of Drosophila genetics allowed them to perform an unbiased genetic screen in which they discovered that partial loss of Lilliputian activity can suppress the toxicity of the repeat expansion. Using CRISPR-Cas9 genome editing they were then able to knockout one of the mammalian homologs of Lilliputian in iPSC derived from ALS patients with *C9ORF72* repeat expansions, in which they observed a decrease in TDP-43 pathology and axonal degeneration. Subsequently, Anastasaki et al. used CRISPR-Cas9 to engineer seven pairs of isogenic iPSC lines covering a range of mutations in *NF1*, the causative gene in the developmental disorder Neurofibromatosis Type 1 (Anastasaki et al., 2020). As expected, some phenotypes were common across all cell lines (increased proliferation, RAS pathway activity) while some varied according to the particular mutation (dopamine levels, apoptosis and neuron differentiation). In order to determine which phenotypes were likely to be disease related the authors compared their results to those collected in *Nf1*-mutant mice (which also represented a range of mutations). Notably they were able to replicate the finding that dopamine levels are reduced as a result of some, but not all, *NF1*/*Nf1* mutants in mice/humans.

Both of these studies demonstrate how replication of phenotypes across model systems increases the confidence in disease-associated observations. Pinpointing phenotypes that persist across such wide variability in genetic background will help to identify treatment targets with the most wide-spread effect. In the next section we will discuss disease specific examples of this strategy.

## COMPLEMENTING AND BUILDING ON ANIMAL MODELS WITH HUMAN STEM CELL MODELS

Uncovering disease-causing mechanisms and effective therapeutic strategies are expensive, high stakes undertakings. Collecting multiple data sets from orthogonal model systems to support these efforts greatly enhances the likelihood of success. Below are some recent examples where combining human and animal models of disease have resulted in new discoveries of disease mechanisms and promising treatments.

### Neurodegeneration

Neurodegenerative diseases, particularly those with a protein aggregation component, have been especially hard to model accurately in animals like mice. As such, PSCs have become a very attractive model for this field (for more detailed discussions on the topic see: Riemens et al., 2020; Valadez-Barba et al., 2020; D'Souza et al., 2021). Alzheimer's disease, Parkinson's disease, ALS and Huntington's disease are complex disorders with the vast majority being sporadic, but with familial cases caused by known mutations that provide evidence of strong genetic risk factors, such as ApoE4 (Corder et al., 1993). Neuronal cell death is a hallmark of most of these disorders, making cell replacement therapy an attractive treatment option. Replacement of dopaminergic neurons for treatment of Parkinson's disease has been advancing rapidly with notable recent success (Duma et al., 2019; Takahashi, 2020; Piao et al., 2021). Stem cell-based models have been used to recapitulate most, if not all, disease phenotypes including immune and vascular pathologies (Blanchard et al., 2020; Liu et al., 2020; McQuade et al., 2020; Zhao J. et al., 2020). Organoids are particularly attractive models as some protein aggregate pathologies are only observed in 3D culture systems (see Venkataraman et al., 2020; Cenini et al., 2021 for recent reviews). Incredibly, this is despite the fact that PSC-derived neurons are fetal in nature; though this may be helpful in detecting pre-clinical abnormalities (Malankhanova et al., 2020). Still, age is the primary indicator for the majority of these conditions. Although approaches such as transdifferentiation (see Mertens et al., 2021 for a recent example) to generate "aged" neurons are being developed, combining animal models and pluripotent stem cell technology is especially useful in modeling the breadth of phenotypes in these diseases.

An approach taken by Höing et al. (2012) combined mouse ES cell-derived motor neurons and astrocytes with a transformed human microglia cell line (BV2). This co-culture model was

developed as a cost-efficient way to scale up for a high-throughput screen. The microglia were activated to mimic a pro-inflammatory environment, which the authors hypothesized was a contributing factor in sporadic ALS. The activated microglia induced cell death in the neighboring motor neurons and the authors screened for compounds that could reduce this death. One compound they found to be effective, termed E4, was then tested on human PSC-derived neurons. E4 was found to increase neuron survival in the presence of DeltaNO (a known inducer of cell death), with no adverse effects in the absence of DeltaNO. This co-culture approach of mouse and human cell types provided a quick and convenient way to screen compounds in a high-throughput manner, and to assess any potential cell non-autonomous mechanisms of action.

An alternative approach is to identify phenotypes in human cells, and subsequently validate and expand on these findings in *in vivo* animal models. Laperle et al. (2020) derived iPSCs from three individuals with young onset Parkinson's disease with no known family history or predisposing mutations. Dopaminergic neurons derived from those cells accumulated α-synuclein, which was found to be due to an impairment in the lysosomal degradation pathway. Deriving an additional seven control and nine patient lines confirmed this novel phenotype in 8/9 patient lines. Three lysosomal agonists were added to the cells in an attempt to decrease α-synuclein levels. One of those agonists, PEP005, was able to reduce α-synuclein and increase the median TH intensity in human dopaminergic neurons. The activity of PEP005 was subsequently confirmed by stereotactic injection into the striatum of wild type mice, in which a similar decrease in α-synuclein levels was observed. The combination of *in vitro* human and *in vivo* observations have increased the confidence in PEP005 as a potential therapeutic.

In Alzheimer's Disease models, altered mitochondrial morphology and function which manifest as increased AMPK activity and decreased mitophagy, were recently observed using electron microscopy (Fang et al., 2019). To investigate this compelling phenotype further, iPSCs from one sporadic and one familial Alzheimer's patient with a matched control were derived. A decrease in mitophagy was further confirmed in cortical neurons differentiated from these cells as indicated by decreases in several mitophagy related proteins. One hypothesis posited is that neuron loss in Alzheimer's disease could be caused by accumulation of damaged mitochondria, leading to energy deprivation and inflammation. To test if activation of mitophagy could affect Alzheimer's disease pathogenesis in a simple *in vivo* model, *C. elegans* was used to screen for inducers of mitophagy. The screen identified two potential candidate compounds, urolithin A and actinonin. These candidates were able to improve memory defects and reduce Aβ levels in a transgenic *C. elegans* Alzheimer's disease model. The mechanisms behind the action of these drugs were conserved in multiple species' models, as similar effects were seen in an Alzheimer's mouse model. Further investigation of these mice revealed that decreased mitophagy in microglia resulted in an inability to remove accumulated Aβ plaques and an increase in the release of pro-inflammatory cytokines. The conservation of these mechanisms across species suggests that enhancers of mitophagy are a good target for new Alzheimer's disease drugs and that physiological assessment of their effects can be adequately performed in animal models.

## Neurodevelopment

Stem cell-derived neural cells are especially powerful models of embryonic development since differentiation protocols are typically designed to closely mimic key signaling pathways during early embryogenesis (for recent reviews see Imaizumi and Okano, 2021; Sabitha et al., 2021). Stem cell-derived neurons, whether in 2D or 3D culture, have been found to exhibit gene expression signatures that are most similar to early to mid-gestational stages (Handel et al., 2016; Logan et al., 2020). Recently, organoids have been shown to display electrical firing patterns that are similar to fetal signals detected by electroencephalography, providing a potential connection between *in vitro* models and *in vivo* phenotypes (Trujillo et al., 2019; Fair et al., 2020; Matsui et al., 2020; Giandomenico et al., 2021). This has led to discoveries using organoids to model fetal toxin exposure (Prince et al., 2019; Arzua et al., 2020) and developmental disorders (Gomes et al., 2020). In a novel application of the technology, organoids are being generated from across several species, leading to discoveries about how brain development has changed over evolution (Kanton et al., 2019; Benito-Kwiecinski et al., 2021; Chan et al., 2021). Additionally, protocols have recently been developed to generate human neural crest cells and their derivatives. A transient population in the embryonic ectoderm, neural crest cells are difficult to isolate and study even in animals. Neural crest cell culture allows for the study of neurocristopathies (Barrell et al., 2019) and neural tube development (Rifes et al., 2020; Libby et al., 2021). These features make stem cell-derived ectoderm cell types including neurons ideal tools for studying neurodevelopmental disorders, and mechanisms of normal brain development and evolution across species.

The Zika virus epidemic of 2015-2016 yielded important advances in modeling vulnerabilities of neurodevelopment using hPSC-derived models (Cavalcante et al., 2020; Pettke et al., 2020; Krenn et al., 2021). Gabriel et al. (2017) took advantage of the ability of cerebral organoids to model microcephaly, a main phenotype in infants born to ZIKA infected mothers. Using two novel and one established strain of Zika virus, the authors found productive infection in 2D cultures of neural progenitor cells (NPCs). While all three strains decreased NPC proliferation one of the novel strains had a far more pronounced effect, showing less spontaneous neuron differentiation and increased apoptosis compared to the other strains. The mechanism of NPC disruption was found to be linked to centriole disruption, with abnormal recruitment of Cep152, PCNT, Cep164, and CPAP proteins. Using infected cerebral organoids the authors linked the centriole disruption to the decrease in proliferation by showing altered planes of division of NPCs in the putative "ventricular zones" of the organoids. As occurs *in vivo*, altered planes of division influence whether NPCs divide symmetrically to self-renew or asymmetrically to generate neurons. The Zika infected NPCs in the organoids skewed toward asymmetric divisions, increasing the number of neurons but consequently depleting the

NPC pool. As the organoids continued to develop in culture the lack of NPCs resulted in fewer overall neurons being produced and smaller organoids, thereby replicating the decreased cortical size characteristic of microcephaly. By combining viral infection and organoid technologies the authors were able to correlate the severity of the strain with mechanism of action in driving aberrant brain development: either an increase in apoptosis or an increase in premature neuronal differentiation.

The previous example illustrates how a combination of homogenous populations of 2D cells with more complex organoid models can help reveal *in vivo* mechanisms of disease. However, while organoids can replicate some important features of human brain development like cortical folding (Li et al., 2017; Karzbrun et al., 2018) and abundant outer radial glia (Bershteyn et al., 2017; Andrews et al., 2020; Eze et al., 2021), complex interconnections, neuron remodeling, and synaptic pruning are still best modeled *in vivo*. Toward this goal, Real et al. (2018) injected human iPSC-derived neurons into the cortices of young mice and followed their integration and maturation at a single-cell level over time using *in vivo* imaging. Real-time tracking revealed that more neurons extend processes than in *ex vivo* culture and that those extensions occur more quickly than retraction of processes. This resulted in a pattern of neurite gains and losses that changed dynamics the older the graft was. When neurons derived from Down Syndrome patients were stereotactically injected into adult mouse brains, fewer extensions and retractions were observed, with an increase in neurite stability and reduced activity. This combination of approaches demonstrates an interesting developmental paradigm for human neurons and a new mechanism for Down Syndrome pathology at the synapse level.

## Psychiatric Disease

The most difficult brain pathologies to model are those with a primary behavioral manifestation, as so many are unique to humans as a species. In these cases, correlating molecular and cellular phenotypes from human cells to specific behaviors in animal models provides our strongest evidence for promising therapeutic targets. When using PSC-based models this connection can be made by linking electrophysiological function of PSC-derived neurons to EEG readings from human patients (see (Wang M. et al., 2020)) for a review and (Naujock et al., 2020) for a recent example). Mechanistic and safety studies for new psychiatric drugs can also be worked out in advance of or aligned with clinical trials (see Marcatili et al., 2020 for a recent example using ketamine as an antidepressant). A recent advance using co-cultured organoids patterned to different brain regions (AssemBloids™) opens the possibility to study connectivity issues between specific areas of the brain (reviewed in Marton and Paşca, 2020). This is of particular interest to psychiatric research as pathology often involves signaling between multiple brain regions. The genetics of psychiatric disease are also very complex (Prytkova and Brennand, 2017).

One group (Sawada et al., 2020) used pairs of discordant twins with psychosis to examine the developmental origins of the disease. Single cell RNA sequencing in cerebral organoids from the affected and unaffected twin revealed that organoids from the affected twin had a decrease in the proportion of SOX2+ progenitor regions with a concordantly higher proportion of GABAergic inhibitory type neurons. This phenotype was reversed when the organoids were treated with LiCl, a GSK3β inhibitor and antipsychotic. This suggested an underlying imbalance in the excitatory/inhibitory network of neurons early in embryogenesis as a mechanism for the development of psychosis. This striking result was confirmed in organoids produced from three additional pairs of discordant twins. A new technique aimed at combating the high genetic variability between patients is the so-called "population in a dish," where many bar-coded PSC lines are combined in a single dish and interrogated together. Single-cell RNA seq can then be used to create phenotypic "sub groups" for further analysis (see Cederquist et al., 2020 for an example).

Shao et al. took a different approach by generating iPSC lines from 14 unaffected and 14 Schizophrenia patients (Shao et al., 2019). RNA-seq performed on 8 week old neurons derived from these iPSC lines indicated that neurons from all individuals had very similar gene expression patterns. Only a few genes, including *PCDHA2* and other protocadherin family members previously linked to Schizophrenia, were differentially expressed between affected and unaffected lines. Examination of a *Pcdha* KO mouse model found decreases in VGAT+ staining on parvalbumin positive neurons with a decrease in neurite length and branch number, suggesting a defect in inhibitory neuron development and function. When the control and patient derived neurons were transplanted into 5–7 week old mice via stereotaxic injection, a mild but significant defect in inhibitory synapse formation was observed. This study suggests a novel link between protocadherin gene expression and inhibitory synapse formation.

Together these studies, using 2D and 3D culture of hPSC-derived neural cells in conjunction with animal models, raise interesting questions about the balance that inhibitory and excitatory neurons play in psychiatric disease. These efforts suggest that even more complex physiological models can be developed using stem cell derived 3D cultures.

## Brain Injury

Repair and regeneration of damaged neurons has been a longstanding goal for regenerative medicine (see Farzaneh et al., 2020 for a recent review). In addition to transplanted mesenchymal stromal cells (MSCs), neural progenitors have also been the subject of testing for clinical trials (eight on-going or completed www.clinicaltrials.gov) for brain repair (Nieves et al., 2020) or spinal cord injury (Kamata et al., 2021). Recognizing the key role they play in CNS function, glia are being considered for replacement as well (Balakrishnan et al., 2020). This is particularly relevant to the treatment of myelination disorders like multiple sclerosis (Smith et al., 2021). An emerging field that has developed tangentially to this is the development of biomaterials to increase PSC-derived graft survival and efficacy (reviewed in Lacalle-Aurioles et al., 2020). An interesting alternate approach is the *in vivo* transdifferentiation of glia/glial progenitors into functional

neurons or oligodendrocytes (reviewed in Qian et al., 2021). This approach side-steps the need for host immune matching and graft survival, however more work is needed to fully understand the resulting cell type, it's function and connectivity. Organoids are also being considered as tissue sources for transplantation, though primarily for retinal replacement (Lin et al., 2020), due to the increased maturation of cells in 3D structures. Additionally, organoids are proving a robust injury model, whether it be the consequences of reduced oxygen to the brain after systemic injury (Kim M. S. et al., 2021) or direct traumatic injury to the brain tissue itself (Shi et al., 2021).

Testing PSC-derived cells for cell replacement necessitates safety and efficacy studies *in vivo*, which benefits from even simpler models like Zebrafish (Tayanloo-Beik et al., 2021). To support those studies, Shiga et al. looked at ways to improve autologous transplantation of iPSC-derived neural progenitors (Shiga et al., 2019). The authors tested a previously identified activator of fibrinolysis, an enzymatically inactive tissue-type plasminogen activator (EI-tPA), known to promote neuron growth on iPSC-derived NPCs. EI-tPA increased expression of neural progenitor markers and reduced thrombin-mediated apoptosis *in vitro*. The NPCs were then implanted into a rat model of spinal cord injury either with or without pre-treatment with EI-tPA. The EI-tPA treated NPCs significantly increased motor recovery, body weight and tibialis weight over rats injected with untreated NPCs. The EI-tPA treated NPCs also showed increased survival in the graft (16 weeks) and generated neurons which projected into the ventral spinal T7 segment of the spinal cord with no negative effects on sensory function.

Another group recently built on what was known about the promising effects of MSCs in transplant grafts (Zheng et al., 2021). MSCs don't generate new neural cells in grafts meant to repair brain injuries, but instead secrete extracellular vesicles (EVs) that contain substances which promote cell growth and regeneration. The authors asked whether EVs secreted from NPCs had similar effects. NPCs and MSCs were isolated from newborn mouse subventricular zones and adipose tissue, respectively, in order to collect enough material for the study. EVs were isolated from these cells using two common methods, PEG and ultracentrifugation, and the cargo they contained was characterized. Mass spectrometry revealed the expected cytosolic proteins, transmembrane proteins and EV markers common to most EVs. A selection of miRNAs with known neuroprotective function was also detected by qPCR. To determine their potential protective properties the EVs were added to the culture medium of iPSC-derived cerebral organoids exposed to oxygen-glucose deprivation as a stroke model. The EVs reduced the number of TUNEL+ cells even at lower doses after only 8 h of treatment. In a final experiment, the authors found that EVs from NPCs were able to improve movement in a rodent model of cerebral ischemia to a similar level as treatment with the MSC-derived EVs. Impressively, this improvement persisted for 84 days post-stroke with an increase in neuronal density and proliferating cells. This approach further confirms the therapeutic utility of EVs and opens up new potential sources for producing functional neurons *in vivo*. Treatments could even be considered that induce endogenous NPCs to release EVs without the need for generating cells and EVs in the lab.

## Brain Cancer

The cancer stem cell hypothesis predates human pluripotent stem cell technology though still remains controversial (see Gimple et al., 2019 for a recent review). Methods for isolation and culture of CNS solid tumor stem cells are established and can be used for examining the first steps of cancer formation (see Goranci-Buzhala et al., 2021 for a recent example). Human stem cells (endogenous and PSC-derived) make excellent complimentary models for studying the cell of origin, cellular heterogeneity, and mechanisms of progression of multiple types of brain cancers, particularly since dedifferentiation and re-activation of an embryonic stem-like state is a hallmark of many cancers. This has been particularly true in efforts to model the highly lethal brain cancer glioblastoma multiforme (Gimple et al., 2019) but has also been used for non CNS cancers like medulloblastoma (Xue et al., 2021) and neuroblastoma (Cohen et al., 2020). Researchers have found that 3D models are particularly successful in modeling the lineage progression, cellular heterogeneity and invasive potential of these devastating cancers (see Luo and Li, 2021 for a recent review). 3D modeling approaches for glioblastoma are ever evolving and have included organoids generated from primary patient samples or hPSC derivatives, and more recently bioprinted constructs that aim to more faithfully recapitulate multiple elements of the tumor tissue microenvironment (Gimple et al., 2019; Zhang et al., 2020; Stanković et al., 2021). There are two main approaches to 3D cancer modeling: creating a "tumorsphere" consisting of cancer cells to model growth, signaling and drug response (Lenin et al., 2021; Pinto et al., 2021) or co-culture of cancer cells with "normal" organoids to study invasion and tumor formation (Choe et al., 2020; Azzarelli et al., 2021). Some groups have even reproduced tumor initiating events by introducing known tumor causing mutations into wild type organoids (Kim H. M. et al., 2021). In one compelling example, a streamlined strategy was recently developed to establish organoids from resected patient tumor tissues that closely recapitulate the cell type heterogeneity, histological patterns, mutation spectrum, and gene expression patterns of the parental tumors (Jacob et al., 2020). The authors created a biobanking strategy for these patient-derived models, and eloquently demonstrated their utility in modeling tumor invasiveness following mouse brain xenografts and in high throughput drug testing, including demonstrated sensitivity to CAR-T cell immunotherapy. The next phase of 3D cancer modeling will involve increasing the complexity of organoids to capture the tumor microenvironment (Ruiz-Garcia et al., 2020) by including key aspects such as vascularization and immune components (reviewed in Majc et al., 2021).

Medulloblastoma is the most common malignant brain tumor. Patients with Gorlin Syndrome, caused by a mutation in *PTCH1*, develop pediatric medulloblastomas at a rate of 5%. *PTCH1* mutations are also common in sporadic medulloblastoma cases, making Gorlin patients a useful model

to study the mechanism of this gene in cancer initiation. Susanto et al. generated iPSC from a patient with a mutation leading to a truncation in PTCH1 protein and differentiated them, along with a control line, to neuroepithelial stem cells (NES cells) (Susanto et al., 2020). They found that patient NES cells showed a decrease in apoptosis compared to the unaffected control NES cells. When injected into the brains of mice only the patient, not control, NES cells grew into tumors with histology reflective of medulloblastoma. Secondary cells isolated from those tumors were able to form new tumors in a second injected mouse, confirming their transformation. These tumors showed expression of genes similar to a common SHH-dependent medulloblastoma type in humans and responded similarly to Vismodegib, a common treatment for SHH-subgroup medulloblastoma. Examining the transcriptional differences between the parent patient NES cells, and those from primary and secondary tumors revealed an increase in *LGALS1* expression, a gene which is novel in medulloblastoma but has been shown to promote invasiveness and immune evasion in neuroblastoma. Using stem cell derived neural cells to model cancer initiating events that reflect *in vivo* biology therefore has great utility for novel mechanistic and therapeutic discovery.

Stem cell derived models are also important tools to validate potential novel cancer drugs discovered in animal models. Atorvastatin is a potential cancer therapeutic that can cross the blood brain barrier; however the systemic dose needed for an effective dose in the brain is cytotoxic. Lübtow et al. (2020) created novel micellar atorvastatin formulations and tested their efficacy against established glioblastoma cell lines and cultured mouse tumor spheroids. The new formulations reduced the growth of both human and mouse cancer cells. To determine if the new formulation could cross the blood brain barrier, and at what concentration, the authors established an iPS cell-derived human blood brain barrier model in a transwell system. With this innovative approach they were able to administer high doses of the drug with no cytotoxic effects and found that the new formulations could cross their engineered blood brain barrier.

## THE NEXT STEPS IN HUMAN CELL MODELS

Cell replacement therapy is often discussed as one of the main applications of PSC-derived neural cells. Much work is being done to perfect differentiation protocols to achieve high yield, purity, functionality and regulatory compliance. Ganat et al. developed one of the first protocols for dopaminergic neuron differentiation to be used for cell replacement therapy for Parkinson's disease (Ganat et al., 2012) (see Parmar et al., 2019 for a recent review of Parkinson's Disease clinical trials). Autologous transplant, where a patient's tissue is reprogrammed and differentiated to the target cell type, is most certainly too costly and too slow for effective therapeutic intervention. Transdifferentiation (as mentioned above) has been a popular avenue of exploration in this regard. Several groups are pursuing transdifferentiation of astrocytes into neurons for cell replacement therapy (Brulet et al., 2017; Gao L. et al., 2017; Zhu et al., 2018; Zhao A. D. et al., 2020). Another approach is that of generating the "universal cell donor" where banks of genetically modified universal cell donor or homozygous HLA matched iPSCs are generated. This promising approach is still actively under development but offers substantial potential for long-term sustained impacts (Han et al., 2019).

Scale-up manufacture and purity of differentiated cultures are key considerations for cell replacement therapy, with labor and financial costs posing major hurdles to overcome (Kim and Kino-Oka, 2020). It will not be enough to be able to produce billions of cells simultaneously if no one can afford the cost to inject them into patients. One Phase I trial injected up to 20 million NPCs into stroke patients (Kalladka et al., 2016), while for other transplanted tissue types 1–10 $\times$ $10^9$ cells per patient are required (Kropp et al., 2017). While stem cells have a high proliferative potential, increased time in culture means more opportunity for genetic instability. Protocols have been developed for large scale iPSC generation and culture (Nathanson, 1989; Ismadi et al., 2014; Otsuji et al., 2014; Pagliuca et al., 2014). Bioreactor scale differentiation of PSCs into NPCs has also been developed (Miranda et al., 2015), providing a second intermediate proliferative cell type for expansion. Methods for cortical neurons generated in suspension culture (Parmet and Berg, 1989) and attachment-based scale up methods are also feasible in "cell-factory" style culture systems (Tirughana et al., 2018).

Toward disease modeling in a dish, brain organoids that mimic more diverse elements of tissue architecture are advancing rapidly. With the recent development of protocols to differentiate microglia, brain microvascular endothelial cells, pericytes, and choroid plexus and to then co-culture them with neural organoids, we are well on our way to building structures that truly mimic the *in vivo* cellular environment. Recent development of choroid plexus organoids allows for complementary blood-cerebrospinal fluid barrier modeling (Pellegrini et al., 2020). True vascularization of organoids is clearly a goal of the field, however, this has not yet been fully established in culture and has required transplant of engineered tissues into recipient animal models (Mansour et al., 2018). The most recent work in this field, however, has shown that co-culture of blood vessel organoids with neural organoids can create vascularization *in vitro* (Ahn et al., 2021). While an exciting advance, additional work will be needed to determine the functionality and long-term viability of these *in vitro* vessels.

Organ on a chip technology (**Figure 2**) allows for regulated flow through microfluidic channels and precise placement of cells in concert with them. This approach is especially promising for developing more complex brain neovascular unit models (Campisi et al., 2021). Blood brain barrier modeling is relevant to disease modeling (Pelkonen et al., 2020) and drug delivery studies (Wang X. et al., 2020). An added advantage over vascularized organoid models is the ability to control flow rate, pressure and compound delivery though the channels (see Lovett et al., 2020 for a review of organoids vs. bioengineered 3D models). Microfluidics has been used recently to examine barrier function upon SARS-CoV-2 infection (Buzhdygan et al., 2020) as well as

to mimic signaling gradients to model neurodevelopment (Rifes et al., 2020). Outside of the brain, microfluidic models can be used to mimic flow through a variety of lumens and linking several tissue chips in succession is an intriguing way to model systemic interactions (see Raimondi et al., 2019 for a review on gut-brain-axis modeling).

The neural contribution of primarily non-CNS diseases can also now be examined given the breadth of stem cell-derived models that have recently been developed (Maiullari et al., 2018). Enteric neurons have been co-cultured with human intestinal organoids (Workman et al., 2017). Spinal motor neurons in co-culture with stem cell-derived muscle cells can form working neuromuscular junctions to permit analysis of processes underlying spinal muscular atrophy (Afshar Bakooshli et al., 2019). Sympathetic and parasympathetic neurons co-cultured with cardiac muscle can be used to test the effect of drugs on heart rate and heart health (Winbo et al., 2020). The next level of these complex co-cultures is 3D tissue printing (**Figure 2**). Using bio-mimic matrix type "inks" cells are layered into precise patterns in pre-set ratios and architectural patterns to accurately reproduce *in vivo* tissue structures. This technology has been used to create trachea (Gao M. et al., 2017), heart (Maiullari et al., 2018), and skin (Lee et al., 2014). Advances in 3D bioprinting of brain tissues are also on the rise with recent studies reported in modeling tumor, spinal cord, and developing cortex microenvironments (Walus et al., 2020). The advantage of bioprinted constructs is the level of control over spatial organization, potentially increasing the reproducibility over spontaneously formed organoids (see Li Y. C. et al., 2020 for an example). In a recent example, printed adipose-derived mesenchymal stem cells were differentiated into dopaminergic neurons displaying electrical response, a step toward functional patient-derived tissue for transplantation (Restan Perez et al., 2021). This technology is particularly suited to cancer modeling as normal brain tissue, cancer stem cells and associated immune cells can be positioned to mimic tumor growth, invasion, drug and immunological response (Tang et al., 2020). Incorporation of drug releasing microspheres into printed constructs can promote patterning during differentiation to generate tissue from multiple regions in the same construct (Sharma et al., 2020). Many of these tissue models now also incorporate microfluidic channels, providing advanced environmental control. With the ultimate goal of engineering functional transplantable tissues for therapeutics, this exciting technology paves the way for the production of rationally patterned heterogeneous full organs and tissue constructs entirely produced *in vitro* from human stem cells.

## CONCLUSION

The most robust neurological phenotypes and clinically impactful drug targets are those that are reproducible across multiple cell lines, biological tissue samples, animal models, and experimental techniques. The power of combining traditional cell culture and animal models with the emerging technologies described here, is the ability to deeply interrogate cellular mechanisms of development and disease and to then translate these findings in clinically relevant ways. The future of translational medicine research will increase the demand for validation in human-relevant models in addition to animal studies. Science done through collaboration between groups specializing in model organisms and those specializing in human stem cell culture will yield the most fruitful research to this end. A focus on the standardization of differentiation procedures and cost-efficient scale-up methods is necessary to drive these technologies closer to *in vivo* modeling and to unlock their full potential for cellular therapy and regenerative potential.

## DATA AVAILABILITY STATEMENT

The original contributions presented in the study are included in the article/supplementary material, further inquiries can be directed to the corresponding author/s.

## AUTHOR CONTRIBUTIONS

EK and LMJ contributed to the conceptualization, writing, and editing of this manuscript. All authors contributed to the article and approved the submitted version.

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