



Single-cell analysis of diversity in human stem cell-derived neurons

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Abstract

Neural stem and progenitor cells produce one of the most remarkable organs in nature, the human brain. Among neural stem cell progeny, post-mitotic neurons are likewise remarkably diverse. Single-cell transcriptomic approaches are now cataloging a long-sought-after molecular taxonomy of neuronal diversity in the brain. Contemporary single-cell omic classifications of neuronal diversity build from electrophysiological approaches that for decades have measured and cataloged diverse biophysical properties of single neurons. With the widespread application of human pluripotent stem cell-based models of neurogenesis to investigate disease pathology and to develop new drugs, a high-resolution understanding of neuronal diversity *in vivo* is essential to benchmark the state of *in vitro* models of human neurological disease.

Keywords Neural development · Neuronal diversity · Single-cell analysis · Neural stem cells · Pluripotent stem cells

Introduction

Neural stem and progenitor cells (NSCs, NPCs) give rise to diverse neuronal and glial progeny during brain development (Lodato and Arlotta 2015; Masland 2004). While Golgi and Cajal first glimpsed the extraordinary morphological diversity of neurons in the cerebral cortex over a century ago (Cajal 1890, 1891; Golgi 1886), careful accounting of neural diversity at the molecular level continues today. A complete census of neural subtypes and circuit composition will further our understanding of how complex behaviors and neurological diseases arise from neural circuits.

Tissue-wide measurement of any brain region obscures the underlying molecular diversity, so rare cell types go unmeasured and diversity among common cell types becomes population-averaged (Fig. 1). Thus, measurement of single cells is essential to define neuronal diversity (Eberwine and Kim 2015). The ongoing development of *in vitro* human pluripotent stem cell (hPSC)-based models of neurological disease adds urgency to the need for a high-resolution understanding of diversity among primary neurons (Brennan

et al. 2015a, b). The limitations of current hPSC-based neurogenesis approaches are best evaluated by comparison to primary human neurons; innovative *in vitro* approaches can then focus on clear obstacles with well-defined measures of success.

Single-cell analysis has been fundamental to neuroscience since the patch-clamp technique allowed one to record the electrical activity of single neurons (Neher and Sakmann 1976). Patch-clamp has been used to functionally classify neurons based on their response to synaptic inputs, ion channel composition, and intrinsic firing properties (Contreras 2004; Migliore and Shepherd 2005). Following from these studies, the maturation of hPSC-derived neurons is typically validated based on the presence of sodium and potassium currents that underlie action potential generation. More careful characterization of hPSC-derived neuronal subtypes is assessed based on the extent to which their electrophysiological response matches the target cell type in slice recordings.

Today, single-cell transcriptome measurement is routine (Macaulay and Voet 2014). Efforts to map neuronal diversity in mouse and human cortex have produced several thousand single-cell transcriptomic profiles which are sorted into known cell types based on the expression of one or a few marker genes (Lake et al. 2016; Linnarsson 2013; Tasic et al. 2016). As expected, further analysis of gene expression revealed sub-types within the known types, and ongoing analysis of these data provides evidence of additional diversity. Current approaches to generate hPSC-derived neurons produce neurons that express known marker genes, but neuronal

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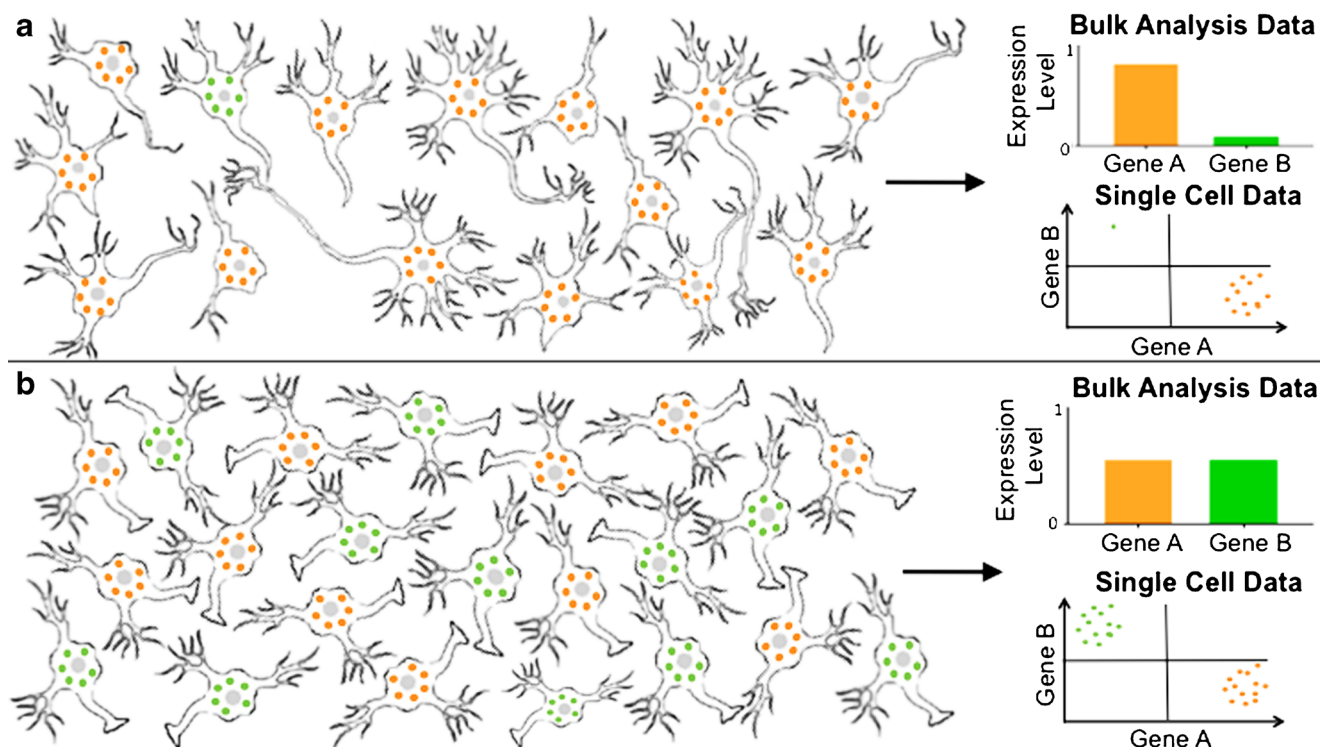


Fig. 1 Single cell approaches are essential to measure neuronal diversity. **a** In a morphologically diverse population of neurons (*left*), transcriptomic diversity present only in a rare population of neurons is lost in bulk measurement. Schematized gene expression measurements from bulk tissue and from single cells (*right*) are used for illustration. Bulk analysis indicates that all neurons express high levels of gene A

(*orange*), and low levels of gene B (*green*); whereas single-cell measurement identifies neuronal diversity. **b** Diversity among morphologically similar neurons (*left*) is also obscured by bulk measurement. As in (**a**, *right*), bulk measurement indicates that all neurons express equal levels of genes A and B, whereas single cell measurement can reveal distinct subpopulations

diversity *in vitro* is still limited. Here, we review single-cell approaches to define neuronal diversity in primary tissue and assess current hPSC-based models of neurogenesis.

Single-neuron morphology and physiology

Microscopy and differential staining methods revealed exquisitely distinct morphological diversity among neurons; however, measurements of the fundamental electrical properties of single neurons required the patch-clamp technique. Patch-clamp approaches yield information about the resting membrane potential, membrane resistance, and intrinsic excitability of single neurons, as well as synaptic function. In turn, these differences reflect electrical diversity among NSC progeny.

In the neocortex, neurons are broadly defined as excitatory or inhibitory based on which neurotransmitters are released from the cell upon activation. However, apart from a cell's synaptic output, neurons also differ in their response to activation, and neural circuits are composed of cells with varying electrophysiological properties and firing patterns (Connors and Gutnick 1990; Mountcastle et al. 1969). The firing properties of neocortical neurons can be broadly divided into five categories: regular spiking, intrinsically bursting, fast spiking,

fast repetitive bursting, and cells with low threshold spikes (Connors and Gutnick 1990; Contreras 2004), though a cell's firing class is flexible and may change based on changes in ion conductance in response to surrounding activity (Steriade 2004). Firing patterns are highly related to neuronal morphology (Connors and Gutnick 1990; Mason and Larkman 1990), particularly the number and branching of dendritic spines (Mainen and Sejnowski 1996). Additionally, patching cells followed by single-cell sequencing has led to the discovery that electrophysiological subtypes are often correlated with aspects of the cells' transcriptomic profiles (Cadwell et al. 2016; Foldy et al. 2016; Fuzik et al. 2016).

The question arises whether cortical neuron classification is entirely driven by NPC fate decisions, or whether electrophysiological subtypes are determined as the cells form into circuits. There is some evidence that the electrical properties of neurons are in part pre-determined while they are intermediate progenitors (Tyler et al. 2015); however, it has been shown that transplanted neural stem cells can also integrate into an already established circuit (Avaliani et al. 2014; Espuny-Camacho et al. 2013), indicating that circuit activity surrounding the cell is at least partially responsible for cell fate. Additionally, morphological constraints during development can affect a neuron's firing properties, again showing that the

local environment contributes to the development of electrical diversity (Zhu et al. 2016).

Fluorescent biomarkers and live or time-lapse imaging have been extensively used in animal models to track the fate of stem cells during development, including neural stem cells both during development and the role of neurogenesis in the adult brain (Gobel et al. 2007; Mizrahi 2007; Schroeder 2011; Yokota et al. 2007). Although it is not usually feasible to perform imaging of this extent in humans because there is usually a requirement for pre-loading stem cells with a dye or label, proton magnetic resonance spectroscopy has been used to visualize levels of neurogenesis in the human hippocampus at different ages (Manganas et al. 2007). Some morphological features of cortical neuron diversity are accurately reflected in hPSC-based neurogenesis. For example, neurons expressing superficial versus deep cortical layer markers can be found in cultures (Handel et al. 2016; Mariani et al. 2012). In 3D cultures, imaging has shown that newly differentiating neurons undergo a process similar to interkinetic nuclear migration (Pasca et al. 2015). The cortical spheroids in this study also show a clear lamination pattern, with deep layer markers concentrated towards the center of the spheroid and upper-layer markers towards the outside, mimicking *in vivo* cortical lamination. While it seems that cortical diversity is being represented in this way, functional characterization distinguishing cells from different regions has been limited.

Different morphological subtypes are observed in *in vitro* cultures; however, the extent to which this correlates with *in vivo* data is unclear. In a study on Dravet syndrome patient-derived neurons, Liu et al. showed that bipolar-shaped cells and pyramidal-shaped cells show very limited differences in basal membrane properties, despite a slightly decreased membrane resistance in pyramidal cells (Liu et al. 2013). However, other studies in hPSC-derived neuron cultures have shown a correlation between properties such as dendritic branching with more mature neurons in hPSCs (Bardy et al. 2016).

Physiological diversity *in vitro*

Although there is evidence that hPSC-derived neuron cultures may mimic diversity found in primary cortex, technical variation is a large source of heterogeneity in hPSC-derived neuron cultures. There is a wide discrepancy in the literature in reported electrophysiological properties of hPSC-derived neurons. Some of this diversity can be explained by differing culture methods. Factors such as astrocyte co-culture (Kuijlaars et al. 2016; Rushton et al. 2013; Tang et al. 2013) and altered cell culture medium (Bardy et al. 2015; Kemp et al. 2016) have been shown to alter the time scale of neuron maturation. However, even among neurons of the same origin cultured the same way, there is a range of neuron function, including neurons that do not exhibit firing behavior at all

alongside neurons with robust spiking activity. This makes it difficult to analyze spiking using traditional measures, such as a plot of current injection versus frequency, since the high rate of non-responders skews the data. To overcome this, many studies have categorized neurons into firing ‘types,’ and analyzed spiking metrics within those categories (Bardy et al. 2015; Belinsky et al. 2014; Kemp et al. 2016; Lam et al. 2017; Song et al. 2013).

In a study by Bardy et al. 2016, neurons were separated into five ‘functional states’ based on firing activity, which was subsequently correlated with transcriptome data from the single cells (Bardy et al. 2016). While there was a general increase in the more mature functional states with increased culture duration, immature cells were present at all time points. Similar to findings in *in vivo* studies, a cell’s firing activity was shown to be correlated with the cell’s transcriptome; in this case, the expression of certain genes involved in higher-level neuron function, such as synaptic plasticity. This indicates that functional variability is in fact reflecting differences in maturity as assessed by transcriptome analysis. Thus, different electrophysiological subtypes found in these cultures are more likely to reflect neurons in various stages of development, and unlikely to reflect the kind of variability that is present within an adult cortex. Because of this, hPSC-derived neurons are currently not an ideal method to study functional subtypes in the adult cortex.

While a difference in maturity may be the root cause of variability in neuron function within a single culture, this does not necessarily negate the notion that stem cell-derived neurons accurately reflect some aspects of *in vivo* diversity. The field standard for functional maturity in these cultures is that of an immature neuron *in vivo* (Randall 2016). One indicator of this is the membrane resistance, which is on the order of 1–4 G Ω in hPSC-derived neurons as opposed to less than 1 G Ω in an average adult neuron in animal studies (Livesey et al. 2016). However, this aligns with membrane resistance values from fetal cortex tissue, which similarly show values up to 5 G Ω , and also show immature, abortive action potentials similar to those seen in stem cell-derived cultures (Moore et al. 2009). With this in mind, it is possible that the heterogeneity of neuron cultures is accurately mimicking *in vivo* developmental timelines, as all neurons do not reach functional maturity at the same rate.

While having such wide variability can make using hPSC-derived neurons as a valid model of mature neural circuits difficult, it also offers an opportunity to study factors that contribute to electrophysiological development, including why some neurons seem to mature faster than others and what relevance this may have to later function. It is also important to note that hPSC-derived neurons accurately reflect properties of immature cortical neurons, and, as techniques improve to culture these neurons long-term or accelerate maturation, it is possible that circuitry more reminiscent of a mature cortex

will develop. This will be a valuable tool for investigating what factors contribute to circuitry formation and how electrophysiological diversity is established.

Single neuron transcriptome analysis

Transcriptomic analysis is routinely employed to validate pluripotency and lineage potential in hPSC lines (Muller et al. 2011; Tsankov et al. 2015). Likewise, transcriptomic analysis has become a central tool for phenotyping patient-derived hPSC lines. Several groups have taken this approach to study hPSCs derived from patients with neuropsychiatric disorders such as schizophrenia (Brennan et al. 2011, 2015a, b). Studies looking at the transcriptome of hPSC-derived neurons are typically performed on large cell populations, which necessarily obscure neuron-to-neuron diversity. With the development of single-cell transcriptomic approaches, fine-grained maps of neuronal diversity are rapidly emerging.

Transcriptome diversity in vivo

Various single-cell approaches have been used to better understand cellular diversity within known subpopulations of primary NPCs and neurons. Specific neural subtypes are purified from bulk tissue using fluorescence-activated cell sorting (FACS). Single-cell transcriptomes can then be measured on targeted collections of hundreds of specific genes using multiplexed qPCR and microfluidic devices (e.g., Fluidigm Biomark) or comprehensively on RNA libraries by next generation sequencing. Two examples of these approaches are highlighted from human cerebral cortex; however, several similar studies have also examined other populations of NPCs and neurons (Dulken et al. 2017; Liu et al. 2016; Tasic et al. 2016).

Johnson et al. (2015) isolated two cortical progenitor cell populations: apical and outer (i.e., non-apical) radial glia from fetal human brain based on expression of known proteins (i.e., GLAST and LexA) and differential abundance of the apical marker prominin. Diversity in these human cortical progenitor cell types is of particular interest because the development of large, complex germinal zones is associated with evolutionary expansion of the human neocortex. Multiplexed qPCR was performed on 546 single progenitor cells to query the expression levels of dozens of genes. In contrast to previous bulk analysis of microdissected germinal zones, this single-cell study identified new markers of outer radial glia and found diverse transcriptional states involving co-expression of both classic radial glia markers and pro-neural transcription factors in both apical and non-apical cell types.

A second single-cell study by the Zhang and Chun laboratories used NeuN expression to separate neuronal nuclei from non-neuronal nuclei and investigated cortical neuron diversity

in 6 discrete regions of the human cerebral cortex (Lake et al. 2016). Notably, they found that sequencing of nuclear RNA (Grindberg et al. 2013; Lacar et al. 2016) was superior to laser microdissection of intact neurons. A total of 3227 neuronal datasets were classified based on 10-fold differentially expressed genes using an iterative clustering approach, then excitatory and inhibitory populations were identified based on expression of known marker genes. Neuronal diversity was classified into 8 excitatory and 8 inhibitory subtypes with additional diversity associated with regional identities. However, the authors note that even within one region there is still heterogeneity in gene expression among single cells that remains to be explored.

An unbiased alternative to these FACS-based approaches is to perform RNAseq analysis on all single cells in a defined brain region. Zeisel et al. (2015) performed RNAseq on 3005 single cells from mouse somatosensory cortex and hippocampal CA1 region. Cluster-based analysis identified pyramidal neurons and interneurons, as well as 6 non-neuronal subtypes, namely oligodendrocytes, astrocytes, microglia, vascular endothelial cells, mural cells, and ependymal cells. Distinct subclasses of cells were identified retrospectively based on expression of cell marker genes. Sixteen subclasses of interneurons were discovered and the authors noted that interneurons within the same region had the most similar transcriptomic profiles.

Transcriptome diversity in vitro

Single-cell transcriptome analysis of primary neurons provides an essential benchmark for hPSC-derived neurons. Handel et al. (2016) conducted a critical study assessing the similarities in transcriptomes of primary tissue and hPSC-derived cortical neurons. They discovered that hPSC-derived neurons had mixed layer identities based on the expression of specific upper and deep layer markers. However, this heterogeneity in cell layer marker expression was also present in primary fetal and adult neurons, suggesting that there is neuronal diversity throughout neurogenesis and that hPSC-derived cortical neurons may provide us with a reliable model to examine how that diversity is formed. Indirectly, this study also shows why transcriptional profiling will be a key method to identify cell subtypes moving forward. In both primary tissue and hPSC-derived cultures, there were mixed layer identity cells; thus, transcriptional signatures may for the foreseeable future be the best tool to disambiguate those cortical layer identities.

The direct comparison between in vivo and in vitro single-cell transcriptomic analysis has also been examined in mice. Dulken et al. (2017) specifically looked at transcriptional dynamics in adult neural stem cell lineage through single-cell RNA-seq of the adult mouse subventricular zone in transgenic GFAP-GFP mice. They observed four cell populations:

astrocytes, quiescent neural stem cells (qNSCs), activated neural stem cells (aNSCs), and NPCs. Through PCA analysis, they found a continuum of quiescence, activation, and differentiation in aNSCs. Further investigation of this subpopulation revealed differential gene expression depending on the state of the NSC. Earlier aNSCs expressed cell cycle genes while later aNSCs expressed neuronal genes. When the authors compared the *in vivo* NSCs to *in vitro*, they found a greater enrichment for neuronal genes *in vivo* while the *in vitro* NSCs had enrichment for astrocyte genes (Dulken et al. 2017). This study highlights one of the key issues in the study of *in vitro* neurogenesis: determining real versus artificial heterogeneity. The *in vitro* condition showed more heterogeneity than the *in vivo* condition in that there was a greater spectrum of neural stem cells in various stages of activation. The study did not follow the NSCs far into the differentiation process, however, it is still unclear if the heterogeneity in the NSCs translated into more diverse hPSC-derived neurons compared to primary tissue.

Single-neuron genome analysis

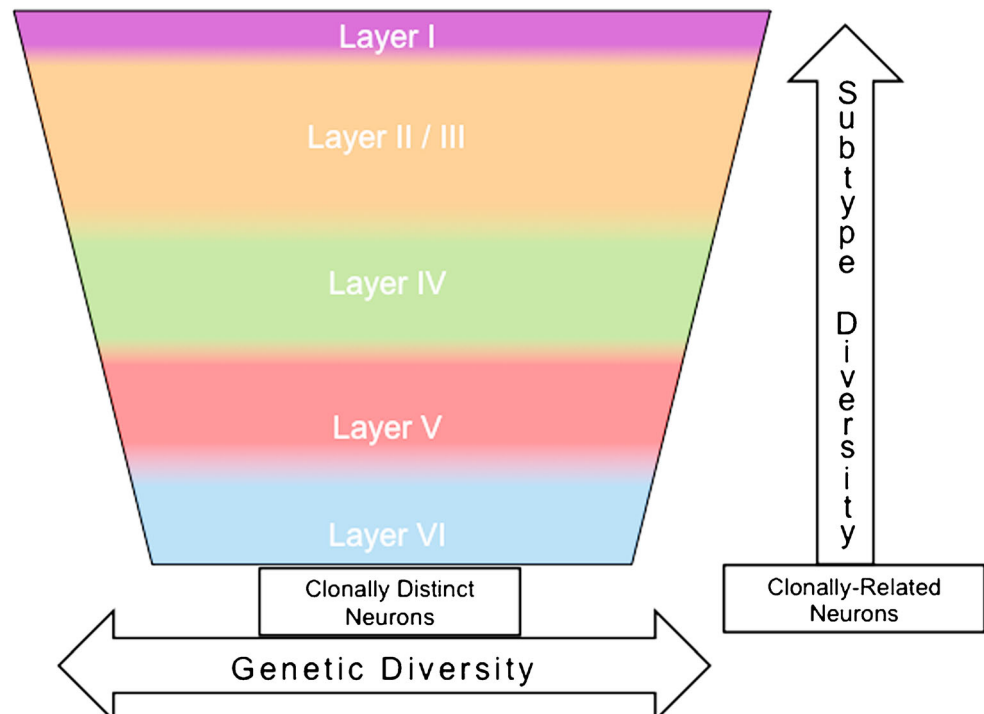
Single-cell analysis has also revealed surprising diversity in neuronal genomes (McConnell et al. 2017). Brain somatic mosaicism is brought about by single nucleotide variants (SNVs), small insertion/deletion mutations (Hazen et al. 2016; Lodato et al. 2015), structural variants (e.g., copy number variants; CNVs) (Cai et al. 2014; Knouse et al. 2016; McConnell et al. 2013), and mobile genetic element insertions

(Baillie et al. 2011; Erwin et al. 2016; Evrony et al. 2012; Upton et al. 2015). Collectively, the variety and abundance of somatic mutations indicates that every neuron in a circuit may be expected to have a different genome.

Cell proliferation during development is a prominent source of somatic mutations. Given about 20 billion neurons in human cortex, and more than 50% cell death during neurodevelopment, cortical NPCs must undergo at least 40 billion cell divisions (Lui et al. 2011). Errors in DNA replication and repair are estimated to lead to 1–3 mutations per cell division (Lynch 2010); thus, estimates of greater than 1000 somatic SNVs per neuronal genome (Lodato et al. 2015) are quite reasonable. However, it is interesting to consider that the distribution of diverse genomes in the cortex is expected to be orthogonal to the arrangement of diverse cell types.

Clonally-related neurons of different cell types are born through a series of asymmetric cell divisions by an individual NPC (Namba and Huttnner 2017). Surviving progeny then reside in functionally connected radial columns (Yu et al. 2009) with early-born neurons in deep cortical layers and subsequently-born neurons migrating to increasingly superficial layers. Neuronal cell-type diversity is often described in the context of layer VI neurons expressing different genes than layer V neurons, and so forth. Likewise, neurons in different cortical layers have different morphological and physiological properties. Somatic mutations in NPCs, as a consequence of iterative cell fate decisions, will be shared among many of that NPC progeny, while cortical columns that arise from other NPCs could have distinctly different genomes (Fig. 2).

Fig. 2 Genomic diversity may be greater within single neuronal subtypes, than among clonally-related neurons. The molecular taxonomy of excitatory cortical neurons is based on laminar residence. Morphological, physiological, and transcriptomic diversity is cataloged by comparing layer VI neurons, to layer V neurons, for example. However, genomic diversity is expected to expand in an orthogonal direction. Radial cortical columns contain neurons of each subtype that were born from the same NPC, these clonally related progeny are expected to share somatic mutations. Tangentially distributed columns, on the other hand, arise from different NPCs and are expected to accumulate distinct mutational profiles from other columns



A central interest is whether somatic mutations are more prevalent in the brain than in other tissues. Although somatic mosaicism occurs in probably all organs, the long lifespan and direct influence on the behavior of neural circuits suggest a direct consequence of somatic mosaicism in both neurotypical and diseased brains. In vitro models of human neurogenesis offer some insight into this question.

Single-cell genomic analysis showed that NPCs derived from hPSCs exhibit few CNVs, whereas CNVs are abundant in 8-week-, differentiated neurons (McConnell et al. 2013). Using an L1 reporter system, L1 retrotransposon activity is likewise absent in hPSC-derived NPCs, but then observed during the first week of neuronal differentiation (Coufal et al. 2009). Additional study of the L1 promoter shows transcriptional regulation similar to that of the master neuronal transcription factor, NeuroD. Hybrid Sox2/TCF-LEF motifs MeCP2-deficient phenotypes (Coufal et al. 2009) support a model wherein Sox2-binding and MeCP2 activity suppress L1 mobility in NPCs, and Sox2 de-repression coupled with Wnt signaling promote L1 transcription during neurogenesis (Kuwabara et al. 2009). If hPSC-based neurogenesis is faithfully recapitulating the mechanisms that lead to primary brain somatic mosaicism, it will prove to be a valuable model system in defining the intersection between cell-type diversity and genomic diversity in human neurons.

Future directions

In addition to the single-cell approaches highlighted here, other advances in single-cell epigenomic (Farlik et al. 2015; Gravina et al. 2016) and proteomic approaches (Budnik and Slavov 2017), as well as multi-omic approaches (Bock et al. 2016; Macaulay et al. 2017), a high-resolution molecular taxonomy of neuronal diversity is rapidly taking shape. Multiplex protein measurement approaches such as flow cytometry provide additional quantification of diverse neural types in specific brain regions. Pruszek et al. used fluorescence-activated cell sorting to analyze heterogeneous hESC derived neural stem cells at various stages of differentiation and showed that specific surface antigens corresponded to the development or maintenance of neural precursor cells or neurons (Pruszek et al. 2007). Mass cytometry extends multiplex capability to at least 50 separate analytes on single cells by conjugating antibodies with heavy metals rather than fluorophores (Bandura et al. 2009; Bendall et al. 2014). Together with novel computational approaches, Zunder et al. used mass cytometry to track the dynamic process of iPSC reprogramming by looking at markers of pluripotency, differentiation, cell cycle status, and cell signaling (Zunder et al. 2015).

Development of improved approaches for hPSC-based neurogenesis is likewise proceeding with great pace. Three-

dimensional culture approaches such as cerebral organoid technology (Lancaster et al. 2013) show improved tissue patterning over standard two-dimensional approaches; however, additional advances are required to fully recapitulate neuronal diversity in primary tissue. For example, neural vasculature is known to provide an important NPC niche, yet existing organoids are avascular. Without such a niche, limited diversity is not unexpected.

Very large single-cell datasets from many individuals will ultimately be needed to establish a complete molecular taxonomy of neuronal diversity. While current single-cell datasets number in the thousands, droplet-based approaches permit simultaneous capture, amplification, and barcoding of tens of thousands of single cells. Notably, the first application of DropSeq was to explore neural diversity in the retina using a dataset of more than 10,000 cells (Macosko et al. 2015). Large datasets provide the statistical power not only to define a reference taxonomy but also to begin to determine how neural diversity may vary among neurotypical individuals and in disease states. Moreover, a fine-grained map of primary brain tissue such as this will further inform our appreciation of the successes and limitations of hPSC-based models of neurogenesis.

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