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## Human induced pluripotent stem cells for modeling neurodevelopmental disorders

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### Abstract

Currently, we have a poor understanding of the pathogenesis of neurodevelopmental disorders, owing to the fact that post-mortem and imaging studies are only capable of measuring the postnatal status quo and offer little insight into the processes that give rise to the observed outcomes. Human induced pluripotent stem cells (hiPSCs) in principle should prove powerful in elucidating the pathways that give rise to neurodevelopmental disorders. HiPSCs are embryonic stem cell-like cells that can be derived from somatic cells. They retain the unique genetic signature of the individual from whom they were derived from, and thus allow researchers to recapitulate that individual's idiosyncratic neural development in a dish. In the case of diseased individuals, we can reenact the disease-altered trajectory of brain development and examine how and why phenotypic and molecular abnormalities arise in these diseased brains. In this paper, we review various aspects of hiPSC biology and experimental design as well as discuss existing hiPSC models of neurodevelopmental disorders. As already shown by some studies discussed in this review, our hope is that iPSCs will illuminate the pathophysiology of developmental disorders of the CNS and lead to therapeutic avenues for the millions that today suffer from neurodevelopmental disorders.

### Keywords

neural stem cells; progenitor; human; development; genome; epigenome; mosaicism; brain; cerebral cortex; transcriptome; iPSC; organoid; monolayer; glutamate; GABA; synapse

### 1. Introduction

Normal development of neuronal networks requires a delicate balance of proliferation and differentiation of specific neuronal lineages. Furthermore, it requires the proper migration and integration of these specific neuronal subtypes into the appropriate neuronal circuits.

The development of this neuronal circuitry and of the human brain as a whole has intrigued scientists for generations, yet we have not been able to explore the development of human-specific neural networks because of the limitations of current methodologies for investigating the human brain and disorders that affect it. A substantial portion of our understanding of the processes underlying normal brain development comes from our study of neurodevelopmental disorders, in which such critical developmental pathways are disrupted. However, the emerging use of human induced pluripotent stem cells (hiPSCs) can reproduce the disease-altered trajectory of early brain development while retaining a patient's unique genetic signature, enabling us to disentangle the contribution of genetic and non-genetic factors to the construction of normal and diseased neuronal circuitry.

Here, we discuss biological and experimental challenges associated with hiPSC derivation and review applications of hiPSC derived neuronal cells to model neurodevelopmental diseases. We begin by discussing the current state of our understanding of neurodevelopmental disorders as derived from neuroimaging, genetic, and pathological studies. We then discuss the shortcomings of such studies and the power hiPSCs possess to overcome them and advance our understanding. Following this we discuss the specifics and mechanics behind building hiPSC model systems and end with a comprehensive overview of existing hiPSC models of neurodevelopmental disorders.

## 2. Neurodevelopmental disorders — the potential of hiPSCs

Neurodevelopmental disorders are a broad group of disorders in which development of the CNS is altered so that the way sensory, motor and cognitive information is acquired and processed in postnatal development is disturbed. In turn, these disturbances affect how neural networks are modified by ongoing neuronal activity, resulting in a wide spectrum of emotional, cognitive, and motor deficits – including, for example, impaired language and/or non-verbal communication, impaired memory and learning, and motor dysfunction.

Neurodevelopmental disorders, and more generally CNS development, are extraordinarily difficult to study for several reasons. Rodent models of brain development have proved insightful, but are limited by their lack of complexity and sophistication compared to the human brain, especially the human cortex. Mice and rats are estimated to have 3–4 orders of magnitude fewer neurons than humans<sup>1</sup>, and, owing to non-linear scaling of neuronal density and connections with brain size, the development and structure of the human brain differs considerably from that of a rodent's. The development of rodent brains is also much faster than that of humans. As a result, the prefrontal and temporal cortices, along with other interconnected association areas, are highly-developed in humans compared with rodents<sup>2</sup>; these areas are often associated with higher cognitive functions such as language, planning, logical thought, expression of personality and emotions and are regions that are most relevant to neurodevelopmental disorders. The lack of efficacious CNS drugs discovered via rodent models supports the idea that preclinical animal models of neurodevelopmental disorders have limited validity<sup>3–6</sup>.

In studying human brain development, post-mortem tissue offers only a single endpoint reading that provides little insight into the altered trajectory of early brain development in

neurodevelopment disorders. Indeed, over two thirds of human genes are expressed in the developing brain itself<sup>7</sup>. Therefore, even if symptoms develop postnatally, all genetic developmental disorders are likely to involve prenatal brain structural and/or functional alterations.

Genetic studies have helped to confirm the high heritability and within family recurrence risk of some neurodevelopmental disorders, such as autism and schizophrenia<sup>8–11</sup>. Improvements in genome sequencing have enabled numerous genome-wide association studies, candidate gene discoveries and exome-sequencing studies<sup>12–21</sup>. However, these genomic studies can identify loci that contribute to disease risk, but do not assess the functional consequences of genetic variants. Transcriptome analyses are able to assess these functional consequences to some degree, but are subject to environmental influence and other confounding variables. Furthermore, brain transcriptome studies cannot distinguish between primary causes and secondary consequences of disease. Ideally, genetic studies should, therefore, involve simultaneous transcriptome and genome analyses in a disease-relevant tissue, and should not be limited by the availability of disease-relevant tissue in the form of autopsy specimens from patients with neurodevelopmental disorders<sup>22</sup>.

The ideal experimental model for studying human early brain development is represented by embryonic stem cell (ESC), but several ethical issues prevent their use in this way.

Furthermore, genetically affected embryos are accessible to only small number of laboratories, and the derivation of affected ESC lines requires connection to a PGD performing center. The discovery of human induced pluripotent stem cells (hiPSCs) has provided an alternative model, by allowing the derivation of ESC-like cells *in vitro* from virtually any type of somatic cell. Additionally, hiPSCs, as opposed to ESCs, can be generated from patients with defined clinical phenotypes, thus allowing to link *in vitro* phenotypes to the clinical presentation *in vivo*. The hiPSC model system shows great promise in overcoming many of the problems with the approaches discussed above and elucidating the pathogenesis of neurodevelopmental disorders.

In contrast to postmortem human brains, hiPSC-derived model systems are actively developing and express dynamic genetic programs that regulate the process of cell proliferation, differentiation into neural precursors and subsequently into mature neurons and glial cells. These systems consequently enable the study of genetic programs that are active in the prenatal brain, as gene expression changes dramatically at the time of birth<sup>23</sup>. As noted above, postmortem brain tissue is also often distorted by other disease processes, making it hard to distinguish causes from consequences and experimental artifacts. In principle, hiPSCs can recapitulate the progression of brain development from embryonic day zero to various stages of maturity. One drawback is that hiPSC-derived brain cells are not as complex as those in the brain, and technical reasons currently limit our ability to grow these cells long enough *in vitro* to recapitulate the perinatal and adult brain. Nevertheless, hiPSC-derived models can allow us to examine and understand how the aberrations in brain structure, composition and connectivity we observe in postmortem and imaging studies develop, and to derive quantifiable measures of neuronal morphology, function,

electrophysiology, connectivity, and gene expression from multiple timepoints during embryonic brain development (Figure 1).

Similarly, implementation of genome-scale deep sequencing technologies with hiPSC model systems has increased the potential of these systems. These techniques can reveal the consequences of gene mutations on the entire cellular transcriptome, and, in turn, how changes in transcriptomics translate into cellular phenotypes. Genome engineering technologies should also help to determine which of the myriad developmental alterations are crucial for a given cellular and molecular phenotype. Cellular and molecular consequences of mutations can be explored in animal models and cultured human cell lines, but hiPSC-derived modeling provides information that is immediately applicable to humans because hiPSCs have a specific human genetic background and, given sufficient sample size, can reveal how inter-individual genetic variations influence phenotypes.

In summary, hiPSCs allow us to replicate the disease-altered trajectory of early brain development and examine when phenotypic and molecular abnormalities arise in these diseased brains. Furthermore, hiPSCs retain the patient's unique genetic signature and thus can recapitulate the patient's idiosyncratic neural development. In future, hiPSC-based studies, imaging studies, and perhaps other patient-based observational studies could be integrated in such a way that various technologies can inform each other<sup>22,24–26</sup>.

### 3. Generation of hiPSC models

HiPSC model generation is a two-step process. The process begins by taking a somatic cell (any cell that isn't a sex cell) and reversing it (known as reverse differentiating and/or reprogramming) back to its embryonic stem cell-like state, known as the hiPSC state. The hiPSC then allows the experimenter to generate, through the differentiation process, the somatic cells required to model the disorder of interest (in the context of neurodevelopmental disorders this would likely be some brain region, neuronal network, or neuronal subtype).

#### a. Reprogramming

The reprogramming process involves the re-activation of key genes in the somatic cell, that are important in normal embryonic stem cells to maintain their characteristic pluripotent state. This is a highly specific, inefficient, and intricate processes triggered by introduction of a set of key transcription factors required to dedifferentiate a somatic cell into an hiPSC, a process which leads to a unique "open" chromatin profile (Box 1)<sup>27–31</sup>. The efficiency of this process is influenced by many other factors including bioenergetic factors, cell cycle regulators and microRNAs<sup>32</sup> as well as dosage of reprogramming factors<sup>33</sup>. Far less is understood about establishing and maintaining a pluripotency circuitry than is understood about the initial reprogramming step. What is known is that several new genes are activated and consequently several developmental transcription factor cascades turned on. We refer the reader to an excellent review by Buganim et al. for a more in-depth discussion of the subject<sup>34</sup>. This process is far from perfect and hiPSCs may still retain some of their originator somatic cell's gene expression patterns and chromatin modification patterns, biasing their differentiation potential towards their originator cell lineage. However, when

human hiPSCs and ESCs were compared controlling for genetic background, clonality and sex, no consistent differential gene expression or epigenetic signature could be found, suggesting that hESCs and hiPSCs are molecularly and functionally equivalent<sup>35</sup>.

## b. Differentiation

Any neuronal differentiation process consists of an initial neural induction of hiPSCs, mediated by appropriate concentrations and gradients of several morphogenetic factors that are normally expressed in the developing brain. In the case of autism, schizophrenia, intellectual disabilities, and in many other disorders primarily affecting higher cognitive functions, several *in vitro* protocols have been developed with the ultimate goal of mimicking the development of the cerebral cortex. The mature cerebral cortex is a six-layered structure composed of two main classes of neurons: glutamatergic excitatory neurons and GABAergic inhibitory interneurons. Moreover, different subtypes of neurons are located in different cortical layers. The fate specification and differentiation of the different subtypes of cortical neurons is determined by the expression of specific combinations of transcription factors in each subpopulation, responsible for their functional diversity. The hiPSC neuronal differentiation strategy should be chosen to mimic the differentiation process with the aim of understanding its derailment in developmental disorders.

## c. Monolayers versus organoids

The two main neuronal differentiation models of hiPSCs are monolayer cultures or 3D organoid cultures (Figure 1). For each of these models, several protocols have been developed and are continually being improved.

In general, monolayer neuronal cultures are derived from dividing neuronal precursor cells that can be dissociated from each other, expanded under growth factor stimulation, and frozen, providing a useful source for later analyses. The advantages of these types of cultures are that a large number of neuronal progenitor cells can be obtained and differentiated into mature neurons, and analyses of cellular morphology (such as neuronal branching and spine quantification) are easier to perform, owing to the 2D nature of these *in vitro* systems. One of the most commonly used monolayer protocols in the ES and hiPSC fields is the dual SMAD inhibition method<sup>36</sup>; the original version of this resulted in conversion of ES and hiPSCs to neuronal cells with an efficiency >80%, and an adaptation of this protocol has successfully been used to generate dopaminergic neurons to study Parkinson disease<sup>37,38</sup> and to generate cortical interneurons<sup>39</sup>. An alternative protocol, that combined dual SMAD inhibition with retinoid signaling led to a 95% efficiency of neuronal differentiation<sup>40</sup> and has been used to model Alzheimer disease and schizophrenia<sup>41–43</sup>. In addition, many more variations of 2D protocols exist, which have been developed and successfully used to generate specific neuronal subtypes, such as GABAergic inhibitory neurons<sup>44</sup>, glutamatergic neurons<sup>44</sup>, and hippocampal granule neurons<sup>45</sup> (see table 1 for more information on different monolayer protocols).

The main disadvantage of monolayer methods is the lack of spatial organization that is found in the brain. Moreover, monolayer approaches confer highly unphysiological

mechanical and adhesive properties to differentiating neurons, which alter neuronal morphology and gene expression. As a result, the quantity and types of neurons produced are highly dependent upon subtle variations in cell-to-cell contacts and interactions, which are in turn influenced by the initial plating density of the neuronal progenitors, the adhesive substrate, and the exogenous factors added to the preparation.

Organoid culture protocols have been developed in the past few years to overcome the drawbacks of monolayer cultures and better mimic the 3D structure, organization, composition, and connectivity of the human brain. The main difference with the monolayer culture is that cells are in direct contact with each other for much of their membrane surface. Thus, organoid cultures allow the recapitulation of a greater range and complexity of cellular interactions and morphogen gradients under minimally perturbed conditions and the preservation of cell-to-cell contacts allows the self-organization of progenitors and neurons in layers that resemble the early stages of mammalian forebrain development.

The advent of 3D cultures started with the pioneering work of Y. Sasai and colleagues in 2008, in which they derived 3D-organized aggregates of neuronal cells from mouse and human ES cells<sup>46</sup>. In this study the authors also showed that the regional identity of specific cortical neurons could be specified and manipulated by the addition of patterning factors into the medium. Similar organoid protocols were refined and optimized for the 3D differentiation of human iPS cells<sup>47,48</sup>. More recently Lancaster and colleagues developed an organoid method designed to mimic the organization of the entire brain and used this method to model intrinsic neuronal differentiation defects in individuals with microcephaly<sup>49</sup>. Organoids obtained with this method are heterogeneous in the sense that they contain several different brain regions (such as cerebral cortex, brainstem, retina and choroid plexus) within each organoid. We have used 3D organoid cultures to study of ASD<sup>50</sup>, and used a method that differed in the initial enrichment of neural progenitor cells through the manual selection of neural rosettes, which allows a more homogeneous differentiation of each organoid in dorsal and ventral neurons mainly representing the telencephalon.

#### 4. Experimental design in disease modeling with hiPSCs

Here we will only mention some experimental design either accessible only via hiPSCs based models, or where hiPSCs based models offer a distinct advantage. Matched pair design is a widely used and powerful experimental design in which cases are matched to controls based on the value of one or more factors, allowing to control for confounding variables, e.g. sex, age and genetic background, in the design stage as opposed to the analysis stage. It has been successfully applied to post-mortem tissue samples<sup>51-53</sup>, as well as to hiPSC studies<sup>35,54</sup>. Matching is more efficient than controlling for confounding in the analysis when the sample size is small, if cost of matching is lower than cost of increasing the sample size. This is certainly true in the case of hiPSCs. On the downside, it is not always possible to find the required match, and this is especially true in the case of post-mortem tissue samples, due to the limited availability of covariates-matched tissue samples. The family design can be considered a subclass of the matched pair design, where the family (genetic) background is the controlled factor. It has been extensively used in genomic studies to investigate genetic transmission within families; for example, to discover de novo



mutations. It is a powerful approach that was successfully implemented in a recent hiPSCs based autism study<sup>50</sup>. This design is not really accessible using post-mortem tissue. The advantage of a family design in the context of hiPSC studies is that it is better suited to a low number of samples in that it allows better control of genetic background effects, which have been shown to drive a large portion of variability across hiPSC lines. A potential drawback may be the intrinsic age difference between probands and controls, when children used as probands are opposed to their parents. Various groups have shown that the “age signature” of the cell donor appears to be reset by the reprogramming process<sup>55,56</sup>, while this reversal does not appear to occur when the somatic cell is directly converted into neurons<sup>57</sup>. In particular, hiPSCs derived from older donors do not seem to present significant transcriptional differences with respect to younger donors. Thus, age difference between probands and controls is less of concern when hiPSC-derived samples are compared. Another potential problem of the family design is that it cannot be used to study the influence on the phenotype of genetic risk factors that are shared within each family. To mitigate this, the design should incorporate “diseased” and “non-diseased” families, as commonly done in genomic studies. Indeed, a distinct advantage of a family design is that since it is commonly used in genomics, it may simplify correlations between the genetic variation and aspects of the phenotype that are investigated via hiPSC modeling.

Finally, hiPSC studies are increasingly using the isogenic design, usually implemented to analyze the functional effect of some (epi-)genetic perturbation on the same cell line (discussed earlier on in this review). This is an extremely powerful approach as it allows, for instance, the functional characterization of a genetic mutation by correcting it in the cells from the same patient carrying the mutation (e.g. genome engineering by CRISPR technology).

## 5. Induced-pluripotent stem cell models of neurodevelopmental disease

Many studies have used hiPSCs for modeling neurodevelopmental disorders (Tables 1–5). In many cases, the models have confirmed or expanded upon our existing understanding of the pathology gained from neuroimaging, postmortem and rodent model studies. However, hiPSC models have allowed us to understand, for the first time, the cellular and molecular mechanisms that give rise to these disorders and drive its progression throughout human development.

The engineering of specific disease-relevant penetrant mutations in hiPSCs is particularly powerful in studying disorders in which such a mutation exists (e.g., FMR1 in Fragile X syndrome or TSC1/2 in tuberous sclerosis). However, hiPSCs can be also useful for studying complex genetic disorders, such as schizophrenia and autism. In these disorders, given sufficient sample size, the derivation of hiPSCs from populations of diseased individuals with appropriate controls can be equally powerful.

Below, we will discuss existing hiPSC-based models of neurodevelopmental disorders, both those that involve a single disease-penetrant mutation and complex disorders in which hundreds of genes have been implicated. We define neurodevelopmental disorders as including any disorders that alter the course of normal human brain development and for

which etiological factor(s) are active in prenatal or early postnatal life. Such disorders include Rett Syndrome, Timothy Syndrome, Fragile X, Autism, Tourette syndrome, and Schizophrenia. While there is some evidence that Bipolar disorder has developmental origins [see review <sup>58</sup> and a few prominent hiPSC studies <sup>59–61</sup> have been carried out] overall the evidence is not conclusively pointing to a definite developmental etiology. It is important to note that because of the high resource and time requirements of hiPSC studies, some of the published works on the subject are only able to conduct n=1 i.e., one control vs. one affected hiPSC without any genetic risk variant assessment. It is unlikely that these papers can add any conclusive evidence on disease pathology and so we purposely tried to exclude them from our analysis unless there is something especially novel about the experiment and/or its design.

### a. Penetrant mutations

The most extensive work in hiPSC modeling of neurodevelopmental disorders has been done in the context of the monogenic disorder, Rett Syndrome (RTT). However, Fragile X syndrome (FXS) and Timothy syndrome (TS) are other monogenic neurodevelopmental disorders that have been studied with hiPSC models; the symptoms of both of these disorders include symptoms of autism spectrum disorder (ASD).

**Rett syndrome**—Rett Syndrome (RTT) is part of the family of Autism Spectrum Disorders. Classical RTT is a neurodevelopmental disorder clinically characterized by the initial appearance of normal early growth and development followed by a period of regression followed again by recovery or stabilization. Diagnosis is usually considered when deceleration of head growth is observed postnatally, though this may not be true in all cases. RTT patients often have partial or complete loss of acquired purposeful hand skills and/or acquired spoken language. It is also often accompanied by the advent of gait abnormalities and stereotypic hand movements <sup>62</sup>. RTT almost exclusively affects females and is most often caused by X-linked mutations in the MECP2 gene, a transcription factor crucial to normal brain development. Rett syndrome is also associated with mutations in CDKL5 or FOXP1 though to a far lesser extent.

Pathological studies of RTT brains have found evidence of decreased neuronal and brain size, defects in synaptogenesis, and reduced dendritic arborizations and spines. Some studies have also identified altered (mostly reduced) neurotransmitter levels <sup>63–65</sup>. All of these are indicators of less mature and less complex neurons. Defects in synaptogenesis may lead to alterations in neuronal processes <sup>66</sup> such as learning, memory, and information retrieval. Reduced dendritic arborizations can be considered as sign of a less complex neuron. Similarly, spine density on excitatory glutamatergic neurons is a strong indicator of these neuron's maturity and ability to potentiate synaptic connectivity, as dendrites with reduced number of spines can receive less inputs. Furthermore, spine formation, plasticity, and maintenance depend on contact mediated and long range signals that are in part genetically encoded and in part regulated by synaptic activity and can be modulated by one's sensory experiences <sup>67–69</sup>. Since the synaptic activity and sensory experiences are likely abnormal for RTT patients, this implies at some level a vicious cycle if RTT patients do in fact have



reduced spines as pathological studies so far suggest. Gene expression profiling of post mortem RTT brains also hinted at specific deficits in expression of presynaptic markers <sup>70</sup>.

While the first derivation of Rett syndrome hiPSC lines with known mutations in MECP2 was performed by Hotta et al. in 2009 <sup>71</sup>, to our knowledge, the first of these models accompanied by extensive phenotypic characterization and later corroborated by other groups was created by Marchetto et al. who in 2010 derived hiPSCs from the fibroblasts of RTT patients with MECP2 mutations. They found that RTT patient-derived neurons had fewer synapses, reduced spine density, and smaller cell body compared to wild type (WT) controls. Furthermore cultures had a reduced density of the glutamate transporter VGLUT1 on glutamatergic neurons. Knockdown of MECP2, that is the reduction of the expression of the MECP2 gene so as to mimic RTT patient-derived neurons, in WT hiPSCs resulted in a similar glutamate transporter phenotype. Overexpression of MECP2 in WT and RTT patient-derived neurons resulted in increased density of VGLUT1, suggesting a dose-dependent relationship between the MECP2 gene and glutamate transport. Interestingly, overexpression of MECP2 is also pathogenic, and recently, a primate model of MECP2 overexpression showing autism-like social deficit has been generated<sup>72</sup>. A human hiPSC model for MECP duplication syndrome (MECP2dup), established by Nageshappa et al. showed that cortical neurons derived from affected hiPSC lines have increased synaptogenesis and dendritic complexity as well as altered neuronal network synchronization <sup>73</sup>.

Similar to the RTT patient-derived neurons and RTT pathology previously described, WT neurons with MECP2 knockdown showed decreased spine density and soma size. RTT patient-derived neurons also exhibited functional alterations at the neural network level – a decrease in intracellular calcium oscillations and a decrease in frequency and amplitude of spontaneous post-synaptic excitatory currents compared to WT neurons <sup>74,75</sup>. Calcium has wide-ranging effects in neurons ranging from microsecond level control of neurotransmitter secretion on the pre-synaptic side of the neurons to gene regulation in the neuron's nucleus (which can last for hours or days). Calcium is also required for activity-dependent synaptic plasticity, a process crucial to learning and memory <sup>76–79</sup>. The decrease of frequency and amplitude of spontaneous post-synaptic excitatory currents is consistent with the decrease in intracellular calcium transients and also indicates a decreased likelihood of action potential firing in the RTT patient-derived neurons. Note how these observations in RTT patient hiPSC-derived neurons would have been impossible to make in patients. From a translational perspective the Marchetto et al. study is also particularly promising as the group also showed rescue of certain neuron cell defects such as reduced VGLUT1 density with the administration of IGF-1 and/or gentamicin <sup>74,75</sup>.

Several other studies <sup>80–86</sup> have also studied the morphology of hiPSC-derived neuronal progenitors and/or neurons from RTT patients with MECP2 mutations. Many of these have largely corroborated the results of Marchetto et al., showing small cell body size, deficits in neuronal maturation, and decreased dendritic complexity. Djuric et al <sup>86</sup>, went on to also show that RTT neurons had decreased cell capacitance, dysfunction in action potential generation, dysfunction in voltage-gated sodium currents, and dysfunction in miniature excitatory synaptic current frequency and amplitude. Two studies demonstrated particularly well the power of hiPSCs by showing that RTT neurons had a delayed

functional switch in GABA from excitatory to inhibitory<sup>84</sup> and neuronal progenitors from RTT patients had an increased frequency of L1 retrotranspositions<sup>83</sup>, conclusions almost impossible to derive from any other experimental medium. Tang et al. went on further to show that IGF1 treatment rescued GABA functional deficits<sup>84</sup>. In addition, Rett gain of function by gene duplication (MECP2dup) has also been modeled in hiPSC lines, showing a phenotype in many respect opposite to that of Rett loss of function, namely increased synaptogenesis and dendritic complexity along with altered neuronal network synchrony<sup>73</sup>.

HiPSC models from RTT patients with rarer mutations have also been created. Groups have derived hiPSCs and neurons from RTT patients with CDKL5 and FOXP1 mutations showing reduced number of synaptic contacts, aberrant spine structures, increased tendency to differentiate into inhibitory synapses, and reduced excitatory synaptic markers<sup>85,87–89</sup>. Other studies modeling Rett Syndrome<sup>90,91</sup> have differentiated hiPSCs into astrocytes, supporting cells that form and maintain the environment and microstructure for neurons, and suggested that mutant RTT astrocytes have adverse effects on the morphology and function of WT neurons, which were partially rescuable with IGF1 treatment. Globally, the evidence suggests that Rett is a non-cell autonomous disorder (disorder in which genotypically mutant cells causes other non-genotypically mutant cells to exhibit a mutant phenotype) and that the glial effect on neuronal morphology is separate from the intrinsic neuronal deficit in RTT mutant neurons. Delepine et al. found that microtubule-dependent vesicle transport in mutant astrocytes was altered and they were able to reverse this with Epothilone D<sup>90</sup>.

Though Rett syndrome is a monogenic disorder and should theoretically be easier to model, being an X-linked disorder introduces unique challenges due to the phenomenon of somatic X chromosome inactivation (Box 2) Nevertheless, this same phenomenon creates the unique opportunity of an isogenic control (with the same genetic background) since hiPSC lines derived from one RTT patient could either express the mutant or WT allele. Cheung et al. exploited this opportunity and derived isogenic control hiPSC lines (from RTT patient fibroblasts) expressing only the WT or the mutant MECP2 allele<sup>92</sup>. This pattern of X-chromosome inactivation was maintained through neuronal differentiation, and similar to Marchetto et al<sup>74</sup>, Djuric et al<sup>86</sup>, and Ananiev et al.<sup>80</sup> (who also had an isogenic control), these studies found that RTT patient derived neurons with the mutant MECP2 allele had a reduced soma size compared to the isogenic controls patient derived neurons expressing WT MECP2.

**Fragile X Syndrome**—FXS is one of the most common causes of syndromic autism, and often also causes moderate to severe intellectual disability, speech delays, growth and motor abnormalities, hyperactivity, and anxiety<sup>93,94</sup>. FXS is thought to be the result of decreased expression of the FMR1 gene, which encodes FMRP, an RNA-binding protein that inhibits mRNA translation. Regulation of mRNA translation is thought to be important for synaptic plasticity and neuronal maturation, thus making this gene crucial for normal brain development. More specifically, expansion of CGG repeats in the 5' UTR of the FMR1 gene results in hyper-methylation and consequent silencing of the gene in an X-linked dominant manner. Healthy individuals have ~6–40 CGG repeats in their FMR1 gene whereas affected individuals have >200–230 CGG repeats<sup>95</sup>. Elevated protein synthesis at baseline as a consequence of FMR1 reduced expression leads to an absence in synaptic

activity-dependent protein synthesis, which in turns disrupts several higher cognitive processes<sup>96–98</sup>. Without synaptic activation-dependent protein synthesis, processes that are involved in the physical encoding of information storage in the brain circuitry cannot function fully.

HiPSC modeling of Fragile-X syndrome is complicated in a similar way as is modeling of Rett syndrome. Eiges et al. showed that in ESCs derived from embryos carrying the FMR1 mutation (determined through preimplantation genetic diagnosis), the FMR1 gene is expressed in ESCs but undergoes transcriptional silencing following differentiation<sup>99</sup>. The same group later showed that FXS patient-derived hiPSCs, following reprogramming of FXS-patient-derived somatic fibroblasts, had epigenetic inactivation of the FMR1 gene<sup>100</sup> (Box 1). The same group also tested in their FXS hiPSC several chromatin remodeling drugs to assess the reactivation of FMR1 gene expression. To our knowledge, only two groups<sup>101,102</sup> created hiPSC models of FXS and studied the phenotype of FXS neurons. Both reported aberrant neuronal differentiation of these hiPSCs and showed that FXS neurons had reduced neurite outgrowth and fewer and shorter processes, echoing reports on FMR1 knockout mouse models<sup>103,104</sup> and post-mortem brain tissue<sup>105,101,102</sup>. Halevy et al. have also assessed and partially reversed the abnormal transcriptional signatures that may underlie the abnormalities<sup>106</sup>. Interestingly these findings imply early neurodevelopmental alterations, prior to synaptogenesis, although more studies are needed to understand in depth the pathophysiology of these defects. Four groups have used FXS<sup>107–109,110</sup> patient-derived hiPSCs to create proof of concept high-throughput drug discovery systems, though unfortunately the sensitivity and robustness of these screens is still poor and will require significant work. Promisingly though, Park et al.<sup>111</sup> used a genomic engineering technique known as CRISPR/Cas (Box 3) to ablate CGG repeats in FXS patient-derived hiPSCs and thus restore expression of FMR1 mRNA and consequently FMRP protein. Unfortunately, they did not assess for any phenotypic reversal in this study.

**Timothy Syndrome**—Timothy Syndrome is much rarer than FXS but also often results in syndromic ASD. It is caused by a mutation in the CACNA1C gene, which encodes an L-type voltage gated calcium channel. The mutation leads to decreased calcium- and voltage-dependent inactivation of the channel. Calcium entry into excitable cells is a crucial cellular signal and calcium- and voltage-dependent inactivation, also known as CDI and VDI, are forms of negative feedback regulation following membrane depolarization. Calcium channels play an important role in neuronal development because they control dendritic growth and arborization and excessive calcium entry due to the loss of feed-back can lead to detrimental effects on brain development and growth as seen in Timothy Syndrome. Pasca et al. showed that hiPSC-derived Timothy Syndrome neurons showed impaired calcium signaling and electrophysiology, and had defects in activity-dependent transcription compared to WT<sup>112</sup>. Interestingly, neurons also showed a high expression of tyrosine hydroxylase (an enzyme that converts the amino acid tyrosine to the dopamine precursor) and consequently had increased production of norepinephrine and dopamine. HiPSCs derived from these patients had abnormal differentiation tendencies and differentiated neurons had reduced expression of genes marking lower cortical layers and callosal projection neurons. The same group later used a bioinformatics approach, integrating co-

expression network analysis and transcription factor binding analysis, to provide mechanisms by which altered calcium signaling generates altered level of calcium-dependent transcriptional regulators leading to the transcriptional network changes observed in TS patient-derived neurons<sup>113</sup>. Another group reported that hiPSC-derived neurons from individuals with Timothy syndrome have activity-dependent dendrite retraction<sup>114</sup>. Both groups promisingly were able to partially reverse some of the deficits in TS patient-derived neurons.

### b. Complex genetic disorders

HiPSCs have proved quite helpful in further validating findings from previous pathological and rodent model studies of monogenic disorders, especially when coupled with a comparison between isogenic lines. Since most of the variation between hiPSC lines is due to genetic background effects<sup>35</sup>, comparison between isogenic lines when investigating the phenotypic effect of single genes can be quite powerful. Yet, isogenic pairs do not completely resolve the issue of modeling disorders. One reason for this is that even in the so-called monogenic disorders, penetrance can vary substantially depending upon the genetic background. This requires the investigation of single genes's mutations in multiple genetic backgrounds.

HiPSCs have proved to be useful in modeling of monogenic disorders, but the real power of these models lies in their ability to model complex genetic disorders, where multiple genes and multiple genetic backgrounds are typically at work in disease pathogenesis and for which it is not informative to engineer a single penetrant mutation into an ESC line or rodent. The approach for modeling complex genetic disorders with hiPSCs has been to compare phenotypes and gene expression in a sufficient number of patient-derived and control lines in order to get mechanistic insights into the neurobiological bases of the disorder (Table 1). In these studies, the choice of patients and controls, the overall design of the study and the clinical phenotyping are key.

**Schizophrenia**—Schizophrenia is a highly heterogeneous and complex disorder. The disorder has classically been thought of us as being caused by the interaction of predisposing genes and detrimental environmental factors. However, heritability has been estimated to be as high as 80% with a sibling recurrence risk ratio of 8.6<sup>16</sup>. As a result, we primarily focus in this review on the genetic component and briefly discuss modeling environmental factors in a later section.

Schizophrenia comes in two phenomenological forms – Type 1 which is often accompanied by hallucinations, delusions and disorganized thinking and Type 2 which is often accompanied by cognitive deficits and disturbances in social and affective functions<sup>115</sup>. The disorder is considered a developmental disorder because several perturbations during pregnancy are known to increase risk, and furthermore schizophrenia-associated gene and epigenetic factors point to early cortical development<sup>116,117</sup>. In particular, schizophrenia-associated DNA methylation is closely related to cellular and transcriptome changes occurring during the transition between pre and postnatal life<sup>118</sup>. Neuropathological studies of Schizophrenia have revealed reduced brain volume, cell size, and spine density as well as

altered neuronal distribution in the prefrontal cortex and hippocampus compared to controls<sup>119–123</sup>. Furthermore, pharmacological studies have shown that NMDA receptor antagonists, such as ketamine and phencyclidine (PCP), can cause psychotic and cognitive abnormalities reminiscent of schizophrenia, indicating some detrimental effect caused by reduced glutamate in SCZD<sup>124–126</sup>.

In a pioneering study, Brennand et al.<sup>127</sup> constructed the first hiPSC model of Schizophrenia (SCZD). The Brennand et al. study interestingly echoed many of the neuropathological and pharmacological results showing diminished neuronal connectivity, decreased neurite number, decreased PSD95-protein levels, and decreased glutamate receptor expression. However they were also able to build on post-mortem studies in that they revealed further information about the cellular pathway underpinnings of SCZD – namely, their gene expression profiles of SCZD patient derived neurons had abnormal expression of components of the cyclic AMP and WNT signaling pathways<sup>127</sup>. The same group later showed that SCZD patient-derived neuronal progenitor cells (NPCs) had aberrant migration, increased oxidative stress, and perturbed responses to environmental stresses<sup>128</sup>. A separate study by this group suggests increased protein synthesis due to increased levels of ribosomal and translation initiation and elongation factor proteins in SCZD patient-derived cultures, hinting at yet another possible mechanism of disease<sup>54</sup>.

Other hiPSC models of SCZD have gone even further in demonstrating cellular and molecular elements of the disorder's pathogenesis previously undetectable through post-mortem, genetic, or neuroimaging studies. Some have focused on abnormal miRNA expression profiles<sup>129</sup>, some on cellular level deficits in adherens junction and apical polarity<sup>43</sup>, and synaptic vesicle release deficits have also been noted<sup>130</sup>. Furthermore, Robicsek et al. studied patient-derived hiPSCs differentiated into dopaminergic and glutamatergic neurons, in contrast to the more frequently used general forebrain differentiation program, showing perturbations in neural differentiation and mitochondrial function<sup>131</sup>.

Interestingly, some of the results from SCZD patient-derived hiPSCs and neurons are very similar to those of hiPSC model studies in other disorders. For example, SCZD hiPSC-derived neurons also show increased L1 retrotransposition (similar to Rett syndrome)<sup>132</sup>, increased tyrosine hydroxylase, and consequent increased catecholamines, dopamine, norepinephrine and epinephrine (similar to Timothy syndrome)<sup>133</sup>, potentially hinting at similar pathologies between these neurodevelopmental disorders.

**Autism**—Our group recently reported on using hiPSC-derived organoids to model an idiopathic neurodevelopmental disorder – Autism Spectrum Disorder (ASD). This is, to our knowledge, the first published model of non-syndromic, idiopathic autism i.e., independent of any other disorder such as Rett Syndrome, Fragile X, that makes a phenotypic assessment.

Studies of rare exonic mutations have suggested that genes expressed during early fetal cortical development are etiologically implicated in ASD<sup>134,135</sup>. In an effort to directly model early cortical development of patients with idiopathic autism, we produced hiPSC-

derived telencephalic organoids from the fibroblasts of ASD patients with macrocephaly. The telencephalon is the embryonic structure that gives rise to the cerebral cortex, hippocampus, basal ganglia, and olfactory bulb. The ventral telencephalon gives rise to the basal ganglia, the principal source of cortical inhibitory interneurons, while the dorsal telencephalon gives rise to the excitatory projection neurons of the cerebral cortex. Telencephalic organoids contain both glutamatergic (excitatory) cortical neurons and GABAergic (inhibitory) cortical neurons. Macrocephaly refers to an increased head circumference due to increased brain size, and is one of the most consistently replicated phenotypes in ASD<sup>136–138</sup>. Furthermore, ASD patients who present with macrocephaly usually have more severe symptoms and poorer outcomes. In this study, organoids were used to reflect early to mid-fetal telencephalic development in humans.

Gene network analyses of transcriptomes obtained from these organoids revealed upregulation of genes involved in cell proliferation, neuronal differentiation, and synaptic assembly in ASD. Interestingly, GABAergic genes were strongly upregulated. Immunocytochemical analyses confirmed that, indeed, ASD patient-derived organoids exhibited an accelerated cell cycle and an overproduction of GABAergic inhibitory neurons compared to control organoids derived from the patients' unaffected parents. This study further showed, using RNA interference, that increased expression of the master regulatory transcription factor FOXG1 in ASD patient-derived organoids is the cause for the increased production of GABAergic neurons. The data are intriguing, because the model clearly mimics a very early stage of cortical development, suggesting that what is modeled through hiPSC is predisposition to disease. Yet, the altered expression of gene network modules positively correlated with symptom severity, albeit in a small patient sample. It is possible that increased production of GABA is responsible for aberrantly increased synaptogenesis, as GABA functions as an excitatory neurotransmitter promoting activity-dependent synapse formation in prenatal development<sup>139</sup>. The increase in synapse-related transcripts detected in hiPSC-derived organoids echoes a neuropathological study where a morphological increase in synaptic connections was identified in unselected patients with idiopathic autism<sup>140</sup>. Increased density of cortical neuron minicolumns<sup>141</sup> and areas of aberrant cortical neuron layering<sup>142,143</sup> have been shown to occur in autism, but there are as yet no neuropathological studies with sufficient number of cases to demonstrate an imbalance of inhibitory over excitatory neurons in the cortex. Remarkably, the increase in FOXG1 expression in idiopathic ASD with macrocephaly has been replicated in an independent set of cases using a non-organoid neuronal differentiation protocol, along with the increase in GABAergic neuronal progenitors<sup>144</sup>, consolidating the idea of an involvement of FOXG1-mediated GABAergic disturbance in ASD and suggesting that hiPSC modeling is robust and can yield reproducible data across hiPSC lines, patients, and laboratories. The above mentioned study<sup>144</sup> has also hypothesized that the Wnt signalling pathway, which when elevated causes excessive proliferation of NPCs<sup>145,146</sup>, is paradoxically reduced in ASD with macrocephaly, and somehow this is related to the aberrantly increased proliferative activity, as agents that increased the canonical b-catenin/BRN2 cascade normalized cell proliferation. Further support for an involvement of this signaling pathway in ASD is provided by the observation that ASD patients have mutation in CHD8, a negative regulator of canonical Wnt signalling pathway<sup>17,147–149</sup>.



Other studies have engineered hiPSCs with specific mutations associated with rare cases of autism with variable penetrance. Griesi-Oliveira et al.<sup>150</sup> derived hiPSCs and consequently neurons from an individual with non-syndromic autism with a de novo balanced translocation disruption of TRPC6, a cation channel that plays a fundamental role in calcium homeostasis. The group showed that these TRPC6 mutant hiPSC-derived neurons had shorter and less arborized neurites, reduced density of dendritic spines, significantly lower density of VGLUT1 puncta, and, predictably, impaired sodium and calcium currents. Promisingly, restoration of TRPC6 expression or treatment with IGF1 or hyperforin (a TRPC6-specific agonist) partially corrected these abnormal neuronal phenotypes. In contrast to the majority of hiPSC models, Wang et al. used CRISPR/Cas technology to engineer in hiPSCs a heterozygous loss of function mutation for CDH8, an ATP-dependent chromatin remodeler strongly associated with rare cases of autism. The mutation produced differential expression of thousands of genes enriched in GO functions such as  $\beta$ -catenin/Wnt signaling in mutated neurons, demonstrating the combined power of genome engineering and hiPSCs<sup>151</sup>. This literature is still emerging, and it will be interesting to see whether a few common pathophysiological mechanisms underlying autism will emerge from these studies. In this respect, it will be essential to take into consideration technical and experimental variables that may influence the results.

Among these, one of the most important is the quality of the hiPSC lines and the potential presence of epigenetic abnormalities and somatic mutations<sup>152–155</sup>. Poor quality hiPSC lines are more likely to fail or give variable outcomes using any differentiation method, complicating the interpretation of the results. A good practice is to differentiate between 2 and 3 different hiPSC lines for each individual in each experiment. The second most important variable is the method of differentiation. Variables that are often associated with different differentiation protocols include initial enrichment and selection of NPCs with different techniques such as manual isolation, FACS-sorting or bead-isolation, and whether NPCs are cultured in a monolayer or in a 3-D environment. For example, monolayer protocols typically involve a lower cell density, decreased cell-to-cell and increased cell-to-matrix signaling; these variables, along with matrix rigidity and stiffness, have all been shown to influence cell fate.

Regardless of the differentiation method used, it is imperative that the regional/cellular specificity of a neuronal differentiation protocol is properly analyzed. Few cellular markers are generally not sufficient for this purpose, and a better way to address this question is to perform global transcriptome analysis exploiting rich and well curated databases of developmental transcriptome of the human brain, like Brainspan<sup>156</sup>, by classifying samples against it. A very simple classification algorithm, based on correlation analysis, showed that the organoid's transcriptome resembled best human brain development at 8 to 10 post-conceptional weeks, with weaker correspondence to later stages of fetal development<sup>50</sup> suggesting that the in-vitro developmental timeline mimics early in-vivo brain development. A more sophisticated machine learning based algorithm, CoNTEXT<sup>157</sup>, was later developed, which identified strong conservation of transcriptomics network signatures between primary human neural progenitor cells and developing human fetal brain, but highlighted differences between these primary human neural progenitor cells and hiPSC-derived neural progenitors from multiple laboratories. However, CoNTEXT had not yet been applied to transcriptome

data derived from organoids, which may be different from neural progenitors dissociated and grown in 2D culture.

Several groups have identified CNVs in the context of ASD patients<sup>158–162</sup>, although only a few have yet been modeled in hiPSCs. In particular it is interesting that de novo duplications of the 7q11.23 region is associated with ASD, whereas deletion of the same region causes Williams-Beuren syndrome, characterized increased sociability<sup>163</sup>. Williams syndrome has been modeled in hiPSC and it has been shown that Williams syndrome progenitors have increased doubling time and apoptosis, while neurons show increased dendritic spines and synapses compared with typically developing neural cells<sup>164</sup> as well as prolonged repolarization times and a deficit in voltage-activated K<sup>+</sup> currents<sup>165</sup>.

### c. Environmental factors

The models described above exploit the ability of hiPSCs to be differentiated into neurons while retaining the unique genetic signature of the individual from whom they were derived, thereby replicating the developmental trajectory of an individual's brain development on the basis of their genetic code. So-called microenvironmental effects can also be studied in hiPSC-based organoid systems; these effects include cell-to-cell interactions, the effects of age and the effects of diffusible substances generated by developing cells; it is well known that the transcriptome and epigenome are dynamically altered by the cellular micro-environment. However, hiPSCs by themselves offer little to no insight into the macro-environmental component of the pathogenesis of neurodevelopmental disorders, such as organism-level effects and interactions (for example, hormones and sensory experiences). HiPSC organoid systems could, therefore, be exposed to toxic chemicals or immune challenges that mimic detrimental environmental effects to study the consequences on development.

One very recent example of modeling neurodevelopmental disorders primarily driven by environmental factors is the modeling of Zika infection with brain organoids. Modeling infection in early embryonic stages, as would be the case when modeling with hiPSCs, can be useful in predicting some problems later on in life. hiPSCs have allowed these researchers to investigate areas of the disease' biology that were previously intractable – such as what the Zika virus actually targets in embryonic stages that results in the various physical birth anomalies (e.g., microcephaly) and later issues in brain development. In the small period of time since when Zika became a critical public health concern, researchers have been able to isolate exactly why babies born from Zika virus infected mothers had microcephaly. Tang et al. showed that the virus readily infects forebrain-specific cortical neural progenitors, the building blocks of the human cortex<sup>166</sup>. The same study went further to show that at the molecular level the infection resulted in downregulation of cell-cycle genes and upregulation of apoptosis genes in NPCs. This was further evidenced by abnormal progression of cell cycle, increased cell death/apoptosis, and disrupted neurogenesis by the same study as well as many others<sup>167–173</sup>. For a more in-depth overview of the subject see review by Ming et al.<sup>174</sup>.

## 6. Conclusions

To date, hiPSC model systems have not only confirmed previous findings about the pathogenesis of neurodevelopmental disorders, but provided further detail and nuances, and shed light on the mechanisms underlying the associated manifestations. Altered synaptic connectivity and density, excitation inhibition imbalance, and altered neuronal activity had all been proposed as having a role as a result of postmortem, neuroimaging and genetic studies, and hiPSCs have been able to establish a level of causality for these mechanisms, because they provide the means for experimentally manipulating the system. Promisingly, these causal relationships have been used to successfully reverse observed abnormal phenotypes. Furthermore, hiPSCs have demonstrated when these phenotypic alterations first occur, the events leading up to their first occurrence, and how they progress, whereas postmortem studies were only able to examine a single time point, often remote from the genesis of the disorder. In some cases the phenotypic observations, such as neuron size and detailed spine morphology, made in patient-derived neurons are entirely novel and were previously unobservable. Still, we think there is much left to be discovered especially in using hiPSCs to generate non-neuronal brain cells, or glial cells, which in fact make up at least half of brain cells<sup>175</sup>. Additionally, investigations must be made into reducing the resource and time requirements of conducting hiPSC studies so more labs can contribute to the field with high n studies. One potential avenue through which this can be achieved is direct conversion to neurons. A challenge for this approach is, however, that the factors that are required for the determination and maintenance of defined neuronal types have not been completely elucidated, and there is the risk of creating “hybrid” or otherwise non-physiological neurons. There are still challenges in the iPSC field that will need to be addressed in the future years, to allow greater translational potential. An obvious difficulty is to reproduce the cellular and regional complexities of the human brain. Another is the fact that iPSC-derived neurons are often “timed” to a prenatal stage. It is important to recognize these limitations, and consider the complexity of these problems when trying to apply knowledge derived from iPSC studies to patients.

Our long-term goal should be to make hiPSC-based drug discovery systems a reality. The modern field of tissue engineering has advanced considerably and, although the human brain is far more difficult to model than mechanical tissue, keeping this as our long-term goal will likely help us develop more mature and sophisticated human neuron differentiation protocols. Such hiPSC-based drug discovery systems are also already well on their way. In some cases, abnormal neurobiological phenotypes have already been reversed with existing drugs<sup>74,176,177</sup>. It is likely that the predictive validity of this system will only increase as the neurons used become more sophisticated and more accurately representative of human neurons. Groups have exploited neurobiological phenotypes they found in patient-derived neurons to conduct high-throughput genetic screens for compensatory mutations and potential drug targets<sup>176–180</sup>. In contrast, the majority of current subtype characterization for clinical trials is done almost entirely through notoriously imprecise clinical diagnostic criteria. Gene network and neurobiological analyses on hiPSC-derived neurodevelopmental models could be used instead to identify subtypes based on specific genes/pathways that are altered. Finally, another therapeutic avenue may be through transplantation. While this is

already in clinical trial for some adult neurological disorders such as Parkinson's disorder, we believe it is challenging for disorders of childhood, especially for neurodevelopmental disorders where, as demonstrated by this review, there isn't much evidence of neuronal loss or neural tissue damage that is in need of being regenerated. One recent clinical trial, not directly regarding neurodevelopmental disorders but in the area of CNS disorders, is the use of hiPSC-derived retinal pigment epithelial cells for the treatment of macular degeneration<sup>181</sup>. Broadly, the idea of editing mutations in patient-derived hiPSCs and then transplanting them back into the patient is exciting. This is mainly because transplantation normally is accompanied by host rejection risk, however this would not be the case with hiPSCs as the cells are derived from the patient themselves.

All of these areas of growth will require the collaboration of multiple scientific and engineering disciplines. As we move from studying single genes and single mutations to studying networks that integrate the two, we will require collaboration among neurobiologists, bioinformaticians, statisticians, and computer scientists. And as we move to push hiPSCs into the translational space, we will require not only the expertise but the support of clinicians.

## **Glossary 1: Neurobiology Glossary 2: Stem Cells**

### **Neurons**

Core component cells of the brain. The function of these electrically excitable cells is to receive, conduct, and transmit signals. Neurons typically have three parts – the soma, dendrites, and an axon.

### **Cortical neurons**

Neurons of the cerebral cortex

### **Glial cells**

Non-neuronal brain cells that provide support and protection to neuronal cells

### **Soma**

Cell body – contains the cell nucleus

### **Axon**

Conducts signals from the cell body to other target neurons

### **Dendrites**

Extend and branch out from the cell body like antennae providing an enlarged surface area through which neurons receive signals from other neurons

### **Synapse/Synaptic connection**

Neuronal structure that allows a neuron to pass an electrical or chemical signal to another neuron. Neurons are connected to each other through what are known as synaptic connections.

### **Neuronal network**

A groups of neurons and their synaptic connections is referred to as a neuronal network. It is classically thought that neurons form these networks in order to execute some function or process or store information.

**Connectivity**

The number of synaptic connections between a group of neurons or in a neuronal network

**Synaptogenesis**

The generation of new synapses

**Dendritic arborization**

Branching of dendrites, increased branching results in increased surface area through which neurons can receive signals

**Spines**

Protrusions from dendrites that receive and help transmit to the cell body, signals from single synapses. Spines are hypothesized to serve as the storage site for memories and be crucial to learning processes. Spine plasticity is hypothesized to mediate synaptic plasticity.

**Spine plasticity**

The process by which neuronal activity affects the size, shape, and density of dendritic spines. These changes in dendritic spines can be short or long term. This process is hypothesized to play a crucial role and learning and memory processes.

**Synaptic plasticity**

The process by which neuronal activity affects the strength of synaptic connections. This change can arise through changes in the quantity or sensitivity of neurotransmitter receptors or through the quantity of neurotransmitter release.

**Neurite**

Can refer to any projection from the soma i.e., axon or dendrite. This term is frequently used when describing neurons in development as it sometimes hard to distinguish axon vs. dendrites

**Glutamate/Glutamatergic**

Excitatory neuron

**GABA/GABAergic**

Inhibitory neuron

**PSD95**

Post-synaptic density protein

**Monogenic disorders**

Disorders that are the result of a single defective gene

**Complex disorders**

Disorders that are the result of >1 defective genes (more commonly the result of 1000s of genes interacting with each other through complex and dynamic pathways and networks)

**Proliferation**

Reproduction of cells, results in an increased number of cells

**Differentiation**

The process by which a stem cell or less specialized cell becomes a more specialized cell. This occurs through regulation of gene expression as different genes are turned on and off at different levels in different cells.

**Embryonic stem cells**

These pluripotent cells are derived from the inner cell mass of the blastocyst

**Epigenetic**

Changes in gene expression or regulation that do not involve alterations in primary sequence or copy number of DNA

**Pluripotent**

Able to differentiate into any adult cell type

**Induced Pluripotent stem cell**

Pluripotent stem cells, resembles embryonic stem cell, and can be derived via reprogramming from virtually any somatic cell type

**Somatic cell**

Any cell that is not a sex cell (i.e., not sperm or zygote)

**Neural/Neuronal progenitor cell**

These cells are somewhere in-between a stem cell and neuron in terms of differentiation and specialization. They have less replication capacity than stem cells but are more primed to differentiate into neurons.

**Organoids**

Tridimensional aggregates generated from pluripotent ES or iPSC cells, which differentiate over time in a self-directed and organized fashion into a particular tissue or organ, under appropriate conditions *in vitro*.

In the case of the brain, these 3D cultures mimic the brain's cell type composition and layer/tissue cytoarchitecture.

**Reprogramming**

The process by which a somatic cell is dedifferentiated into a iPSC. The dedifferentiation step involves erasing a somatic cell's epigenetic signatures, switching the cell's epigenetic state from closed to open, and establishing pluripotency networks.

**Differentiation**



Follows reprogramming and is the conversion of an iPSC into the desired type of somatic cell. The differentiation step involves establishing the epigenetic signatures and transcription factor networks associated with the desired somatic cell type.

#### **Direct differentiation/direct lineage reprogramming**

The process of a converting a somatic cell into another desired somatic cell type, but skipping the pluripotent stage

#### **Embryoid bodies**

Tridimensional aggregates that can be generated from pluripotent ES and iPSC cells, with the potential to differentiate in the three embryonic germ layers under appropriate stimulations *in vitro*.

#### **Neural rosettes**

Radially self-organized neuroepithelial structures composed of early neural progenitor cells, resembling the developing neural tube.

#### **High throughput screening**

A process through which a large number of compounds/molecules can be assessed systematically for their biological activity (or more commonly for their ability to reverse or partially reversed a targeted abnormal phenotype)

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**Box 1****Epigenetics and hiPSCs**

The potential of hiPSCs to mitotically divide indefinitely *in vitro* and to differentiate into any cell type (including placental, see <sup>182</sup>), derives from their unique epigenetic state characterized mainly by open chromatin structure, similar to that of embryonic stem cells <sup>31,35,183,184</sup>. Open chromatin structure, or euchromatin, refers to a loose packing of the DNA around histone proteins in nucleosomes and easier access of transcription factors and other regulatory factors to the DNA template. The result is usually active transcription.

Reprogramming of a somatic cell into an hiPSC is a three-step process from an epigenetic perspective. In the first step epigenetic and other transcription factors must work together to erase the somatic cell's current epigenetic state. Following this, they must establish a pluripotent epigenetic state in the cell. Finally, they must maintain this pluripotency as well as immortality, i.e. ability for indefinite *in vitro* mitotic division. Lineage-committed somatic cells often have a relatively "closed" epigenetic state with many highly condensed heterochromatin foci <sup>31,185–187</sup>. The tight packing of the DNA around histone proteins in nucleosomes restricts access of transcription factors to the DNA template and reduces overall transcriptional activity. As mentioned earlier, the polar opposite holds for chromatin in hiPSCs, which maintain an "open" epigenetic state with more accessible chromatin domains and less heterochromatin foci, resulting in more active transcription.

**Box 2****X chromosome inactivation**

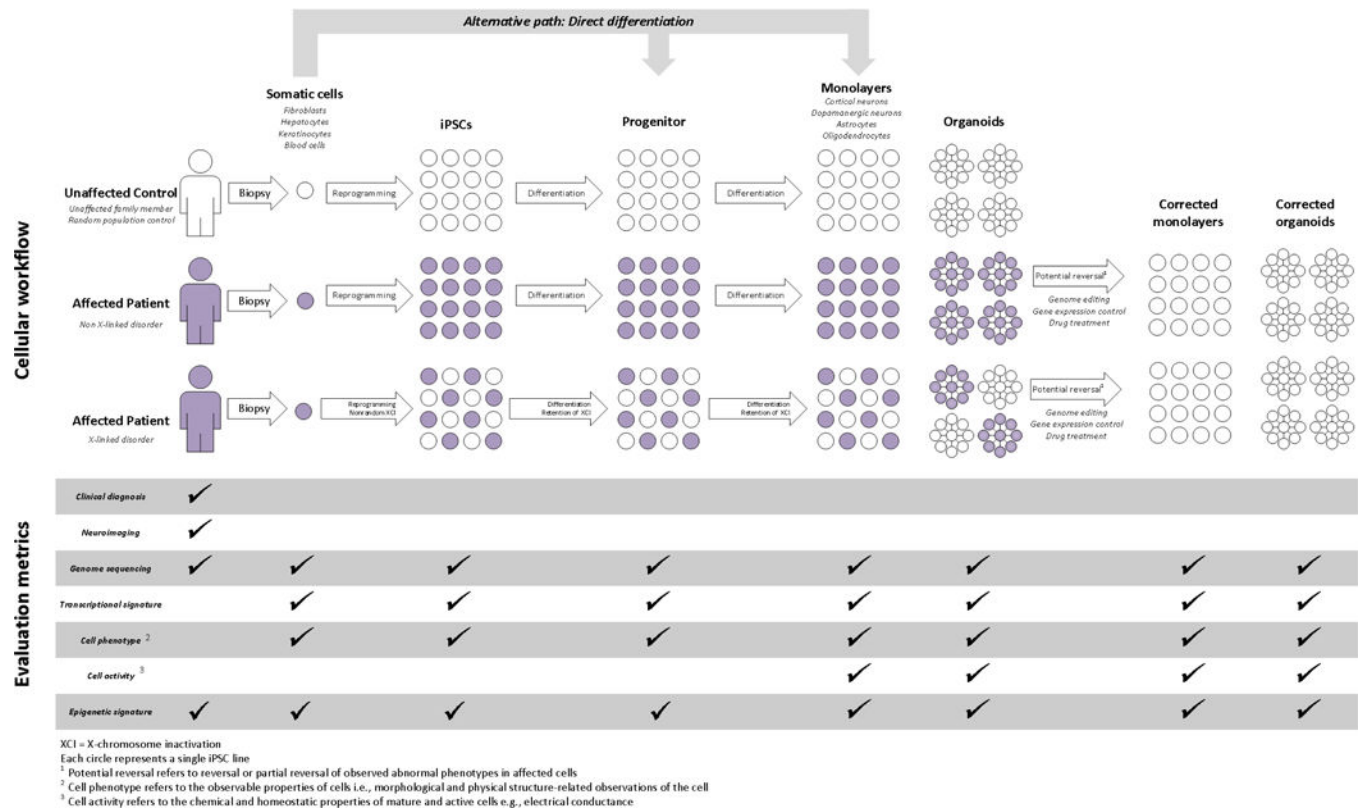
Females are the homogametic sex – that is, they have two of the same chromosome (XX). Males on the other hand are the heterogametic sex and are XY. In order for females not to receive double the dosage of genes on the X chromosome, reduction of gene expression via transcriptional silencing on one of the X chromosomes is required. This sex chromosome dosage compensation effect is commonly known as X chromosome inactivation (XCI) and is thought to be required for normal embryonic development. The process is mediated largely by the large noncoding RNA Xist, which coats the X chromosome in *cis*, mediating transcriptional silencing of X-linked genes and inducing repressive chromatin character along the entire chromosome<sup>188</sup>. However, which X chromosome gets coated and consequently inactivated is random in the sense that the maternal X chromosome and paternal X chromosome each have equal 50% likelihood of being silenced<sup>189</sup>. Interestingly, it was recently shown that X chromosome inactivation in female hiPSCs actually exhibits a non-random pattern<sup>190</sup>. X-chromosome inactivation, which is retained in hiPSCs, presents a powerful addition to the potential of hiPSCs to model X-linked neurodevelopmental disorder such as Rett syndrome (Figure 1). For example, one can then derive hiPSCs from a heterozygous female patient with a mutation in an X-linked gene such that different hiPSC lines either express either the wild-type or the mutant form of that gene. In this way, both the mutant and wild-type form have the same genetic background, making the wild-type form an isogenic control within the experiment. To date the only neurodevelopmental disorder this has been done with is Lesch-Nyan<sup>191</sup> and Rett syndrome as discussed in the text. However, such an experimental design may not be as powerful as it initially seems. While matched genetic backgrounds give more statistical power, family studies are more informative, as they allow comparisons of functional effects of mutated alleles in different genetic backgrounds. Additionally, caution should be exerted though since female hiPSC undergo progressive “erosion” of the pattern of X chromosome inactivation over passage, with associated transcriptional derepression of genes on the inactive X<sup>191</sup>.



**Box 3****CRISPR/Cas**

Genome engineering techniques allow researchers to edit sequences of DNA in their endogenous context, thus allowing for modulation of expression of the gene that that sequence of DNA encodes. The latest of genome engineering technologies, known as CRISPR/Cas, was discovered through the study of the prokaryotic immune system. Bacteria and archaea have a unique adaptive immune system in that they use RNA-guided enzymes to cleave and destroy foreign DNA. Prokaryotes are able to integrate pieces of foreign DNA (usually from invading viruses) into their own DNA into what are known as spacer regions. These spacer regions are regularly interspaced throughout the prokaryote's DNA and serve as a memory of previous invaders' DNA, thus allowing for acquired immunity. Spacer regions are preceded in the prokaryote's DNA by short palindromic sequences. Segments of prokaryotic DNA containing these short palindromic sequences followed by spacer DNA are known as Clustered regularly interspaced short palindromic repeats, or CRISPR. CRISPR sequences serve as the RNA-guide for the DNA endonucleases known as CRISPR associated proteins, or Cas. These enzymes are the ones that actually destroy foreign pieces of DNA, which they recognize via the spacer regions of CRISPRs. Researchers today utilize known Cas proteins and engineer their own CRISPR sequences to act as RNA guides in order to target for endonuclease cleavage specific regions of cellular DNA. Such cleaved DNA can be repaired via endogenous non-homologous end joining which creates small insertions/deletion that typically lead to alteration of the DNA reading frame, generating gene knockouts. Alternatively, Cas-cleaved DNA can be repaired by homologous recombination, where the complementary strand is experimentally introduced in the cell, allowing for precise editing of the DNA sequence. CRISPR/Cas allows researchers the ability to knockdown/edit multiple genes simultaneously in a highly scalable fashion <sup>192,193</sup>

### Experimental workflow for iPSC models of neurodevelopmental disorders



**Figure 1.** Experimental workflow for hiPSC models of neurodevelopmental disorders. Different experimental options are shown with regards to type of controls (cross-sectional, matched pair or family control), choice of reprogrammed cell type, type of differentiation protocol, and outcome metrics. For patients with X-linked disorders, different colored cells represent cells with either the wild type or the mutated X allele. Corrected cells represent the same patient-derived cells after genome editing or drug treatment.

## hiPSC models of Rett Disorder

Table 1

Mutated Gene	Differentiated cell type	Phenotype (compared to controls)	Phenotypic reversal or rescue with drug?	Isogenic control?	Organoid or monolayer?	Reference
MECP2	Astrocytes	Mutant RTT astrocytes and their conditioned media have detrimental effects on the morphology and function of WT neurons; Glial effect on neuronal morphology is independent of the intrinsic deficits in mutant neurons.	IGF-1 is able to partially rescue the neuronal deficits caused by mutant RTT astrocytes	Yes	Monolayer	91
MECP2	Neurons	Smaller neuron nuclear size	NA	Yes	Monolayer	80
MECP2	Neurons	RTT hiPSCs displayed defects in neuronal maturation	NA	No	Monolayer	81
CDKL5	Neurons	NA	NA	No	Monolayer	87
MECP2	Neurons	Reduced glutamatergic synapse number and neurite spine number, smaller soma size, altered calcium signaling and decreased frequency of spontaneous postsynaptic currents	Overexpression of MECP2, addition of IGF1 or addition of geniamicin partially reversed RTT neuron phenotype and increased the glutamatergic synapse number	No	Monolayer	74
MECP2	Neurons	Reduced soma size	NA	Yes	Monolayer	82
MECP2	Neuronal progenitor cells	Increased frequency of L1 retrotransposition	NA	No	Monolayer	83
CDKL5	Neurons	Significantly reduced number of synaptic contacts; Aberrant dendritic spine structures	NA	No	Monolayer	89
MECP2	Neurons	Significant deficit in KCC2 (potassium chloride cotransporter) expression and consequently a delayed GABA functional switch from excitation to inhibition (GABA, prenatally is excitatory and switches to being inhibitory postnatally)	Overexpression of MECP2 or IGF1 treatment rescued GABA functional deficits	No	Monolayer	84
FOXP1	Neurons	Increased inhibitory synaptic markers and GluDI (preferentially induces GABAergic differentiation); reduced excitatory synaptic markers	NA	No	Monolayer	88
MECP2 and CDKL5	Neurons	Both MECP2 and CDKL5 mutated neurons had increased GRID1 (encodes for GluDI). GluDI controls synaptic differentiation and shifts the balance toward inhibitory synapses (over excitatory synapses). Mutant hiPSCs had reduced GRID1.	NA	No	Monolayer	85
MECP2	Astrocytes	Microtubule -dependent vesicle transport is altered	Epothilone D, a brain-penetrant microtubule stabilizing natural product,	No	Monolayer	90

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Mutated Gene	Differentiated cell type	Phenotype (compared to controls)	Phenotypic reversal or rescue with drug?	Isogenic control?	Organoid or monolayer?	Reference
MECP2	Neurons	Deficits in neuronal maturation – decreased soma size, dendritic complexity, and cell capacitance; Dysfunction in action potential generation, voltage-gated sodium currents, and miniature excitatory synaptic currents’ frequency and amplitude	restored proper microtubule dynamics Overexpression of appropriate MECP2 isoform resulted in reversion of reduction in soma size	No	Monolayer	86
MECP2	hiPSC	This was the first hiPSC model of Rett Syndrome with known mutation in MECP2	NA	NA	NA	71

Table 2

## hiPSC models of Fragile X Syndrome

Mutated Gene	Differentiated cell type	Phenotype (compared to controls)	Phenotypic reversal or rescue with drug?	Isogenic control?	Organoid or monolayer?	Reference
FMR1	Neurons and glia	Aberrant neuronal differentiation; Glial differentiation was variable (some looked like controls in morphology and number others didn't); FXS neurons had fewer and shorter processes	NA	No	Monolayer	102
FMR1	Neurons	NA	NA	No	Monolayer	100
FMR1	Neurons	FXS neurons had decreased expression of neuronal differentiation and axon guidance genes; REST, a transcription factor which acts to suppress neural genes in non-neuronal tissues (and thus its expression levels must be downregulated as neural differentiation progresses) fails to undergo downregulation and silencing as differentiation progresses in FXS-derived neurons; REST is regulated by microRNA has-mir-382 which has lower levels in FXS-derived neurons	Introduction of a miR-382 mimic, repressed REST and upregulated its target axon guidance genes	No	Monolayer	106
FMR1	Neurons	NA	Tested several chromatin remodeling drugs to assess re-activation of FMR1 gene expression; proof of concept for hiPSC-based drug screen model	No	Monolayer	110
FMR1	Forebrain neurons	Reduced neurite outgrowth; Significantly fewer and shorter processes	NA	No	Monolayer	101
FMR1	Neurons	NA	FXS hiPSCs CGG repeats were completely ablated using CRISPR/Cas9 resulting in expression of FMR1 mRNA. FMR1 promoter showed near complete demethylation and differentiated mature neurons showed reactivation of FMRP	No	Monolayer	111
FMR1	Neural stem cells	NA	Proof of concept high throughput drug discovery screen	No	Monolayer	108
FMR1	Neuronal progenitor	NA	Proof of concept high throughput drug discovery screen	No	Monolayer	107
FMR1	Neural progenitor cells	NA	Proof of concept high throughput drug discovery screen	No	Monolayer	109

**Table 3**

hiPSC models of Timothy Syndrome

Mutated Gene	Differentiated cell type	Phenotype (compared to controls)	Phenotypic reversal or rescue with drug?	Isogenic control?	Organoid or monolayer?	Reference
CACNA1	Neurons	Defects in calcium signaling and activity-dependent gene expression, abnormalities in differentiation (decreased expression of lower cortical layer and callosal projection neurons), and abnormal expression of tyrosine hydroxylase and consequent increased in production of norepinephrine and dopamine	Roscovitine, a cyclin-dependent kinase inhibitor and atypical L-type-channel blocker, reversed the phenotype	No	Monolayer	112
CACNA1	Neurons	Altered calcium signaling in patient-derived neurons resulted in an alteration in calcium-dependent transcriptional regulators which led to molecular dysregulation and transcriptional changes in TS patient derived neurons	NA	No	Monolayer	113
CACNA1	Neurons	Activity-dependent dendrite retraction	Dendrite retraction was inhibited by overexpression of the channel-associated GTPase Gem	No	Monolayer	114

**Table 4**

hiPSC models of Autism

Mutated Gene	Differentiated cell type	Phenotype (compared to controls)	Phenotypic reversal or rescue with drug?	Isogenic control?	Organoid or monolayer?	Reference
TRPC6	Neurons	Shorter and less arborized neurites, density of dendritic spines was reduced, significantly lower density of VGLUT1 puncta, impaired sodium and calcium currents	Restoration of TRPC6 expression or treatment with IGF1 or hyperforin (a TRPC6-specific agonist) corrected abnormal neuronal phenotypes	No	Monolayer	150
Idiopathic	Cortical neurons	Decreased cell cycle length of undifferentiated hiPSCs, overproduction of synapses, and imbalanced overproduction of GABAergic inhibitory neurons, increased expression of FOXG1 and other GABA neurons transcription factors	Dowregulation of FOXG1 overactivity by shRNA was able to revert the overproduction of GABAergic inhibitory neurons to physiological levels	Yes	Organoid	50
CHD8	Neurons	Differential expression of genes enriched in GO functions: neural development, $\beta$ -catenin/Wnt signaling, extracellular matrix, and skeletal system development	NA	No	Monolayer	151
Idiopathic	GABAergic neurons	First derivation of hiPSC (and subsequent differentiation into GABAergic neurons) from peripheral blood mononuclear cells derived from the whole blood of autistic children	NA	No	Monolayer	44
Idiopathic	Cortical neurons	Increased proliferation of progenitors, increased number of GABA neuron progenitors, decreased neurite arborization, decreased beta-catenin-Brm2 canonical pathway, increased expression of FOXG1 and other GABA neurons transcription factors	Partial correction of deficit in network activity with insulin growth factor 1 (IGF-1)	No	Monolayer	144



Table 5

## hiPSC models of Schizophrenia

Mutated Gene	Differentiated cell type	Phenotype (compared to controls)	Phenotypic reversal or rescue with drug?	Isogenic control?	Organoid or monolayer?	Reference
22q11.2 Del	Neurons	Differential expression of several microRNAs	NA	No	Monolayer	129
15q11.2 microdeletion	Neural progenitors	Defects in adherens junctions and apical polarity	NA	No	Monolayer	43
Idiopathic	Neurons	Increased catecholamines and elevated secretion of dopamine, norepinephrine and epinephrine; Tyrosine hydroxylase, a catalyst for catecholamine biosynthesis, positive neurons was also increased.	NA	No	Monolayer	133
One had a 22q11.2del the other two idiopathic	Neurons	Delay in reduction of pluripotency marks during neuronal differentiation	NA	No	Monolayer	194
Idiopathic	Neurons	Decreased neuronal connectivity, neurite number, PSD-95 protein levels, and glutamate receptor expression; Altered expression of cyclic AMP and WNT signaling pathway genes	Partial reversal following treatment with loxapine	No	Monolayer	127
Idiopathic	Neuronal progenitor cells	Abnormal expression of genes and proteins related to cytoskeletal remodeling and oxidative stress; Aberrant migration and increased oxidative stress	NA	No	Monolayer	128
Idiopathic	Dopaminergic and glutamatergic neurons	Affected dopaminergic cells showed severely impaired ability to differentiate; Affected glutamatergic cells were unable to mature; Affected cells showed perturbation in mitochondrial structure and function;	NA	No	Monolayer	131
DISC1	Cortical neurons	Synaptic vesicle release deficits and dysregulation of synapse-related genes	NA	No	Monolayer	130
22q11 deletions	Neurons	Increased L1 retrotransposition	NA	No	Monolayer	132
Idiopathic	Hippocampal dentate gyrus (DG) granule neurons	Reduced neuronal activity, spontaneous neurotransmitter release, hippocampal dentate gyrus granule neurons generation	NA	No	Monolayer	45
Idiopathic	NPCs	Perturbed responses (i.e., high variance in levels of heat shock factor 1 activation) to environmental stressors in SZ neurons	NA	No	Monolayer	195