

# Human Models Are Needed for Studying Human Neurodevelopmental Disorders

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The analysis of animal models of neurological disease has been instrumental in furthering our understanding of neurodevelopment and brain diseases. However, animal models are limited in revealing some of the most fundamental aspects of development, genetics, pathology, and disease mechanisms that are unique to humans. These shortcomings are exaggerated in disorders that affect the brain, where the most significant differences between humans and animal models exist, and could underscore failures in targeted therapeutic interventions in affected individuals. Human pluripotent stem cells have emerged as a much-needed model system for investigating human-specific biology and disease mechanisms. However, questions remain regarding whether these cell-culture-based models are sufficient or even necessary. In this review, we summarize human-specific features of neurodevelopment and the most common neurodevelopmental disorders, present discrepancies between animal models and human diseases, demonstrate how human stem cell models can provide meaningful information, and discuss the challenges that exist in our pursuit to understand distinctively human aspects of neurodevelopment and brain disease. This information argues for a more thoughtful approach to disease modeling through consideration of the valuable features and limitations of each model system, be they human or animal, to mimic disease characteristics.

## Introduction

Animal models, particularly rodent models, are essential for determining the molecular mechanisms of gene regulation and have been instrumental in our understanding of neurodevelopment and disorders. However, the advancement of imaging, genomic, and genetic technologies has increasingly revealed human-specific aspects of neural development, genetics, pathology, and disease mechanisms. Fundamental differences between humans and animal models raise questions about the value of information gained primarily from animal models, particularly widely used rodents. In addition, the failure of clinical trials that have been based on animal studies has further highlighted the limitations of animal-model-focused human disease research.

Analysis of human-specific characteristics of the brain is hindered by the difficulty in acquiring developing and diseased human brain tissue. Human pluripotent human stem cells (hPSCs) provide an important alternative for studying the development and function of brain cells. To facilitate brain disease modeling, human embryonic stem cells (hESCs) and human induced pluripotent stem cells (hiPSCs) provide paradigms for defining human-specific biology and identifying disease mechanisms. However, questions remain about the utility and value of these cell-culture-based models and how best to move the technology forward. In this review, we summarize human-specific features of neurodevelopment and neurodevelopmental diseases, present the gaps between animal models and human diseases, demonstrate how human stem cell models can bridge some of these gaps, and discuss the challenges for further improvement.

## Unique Characteristics of Human Brain Development

### *Conserved Characteristics among Humans and Animal Models*

The neocortex in mammals is involved in higher cognitive functions, such as sensory perception, generation of motor commands, spatial reasoning, conscious thought, and in humans, language. The higher-order functions that distinguish humans from rodents are accomplished through complex and evolutionarily emergent differences in cortical expansion, arealization, and connectivity. Yet, specific fundamental aspects of gross neuroanatomy, neuronal function, and principles of initial neocortical development are similar in all mammals, and thus our understanding of these aspects of brain development come largely from studying animal models.

The neuroepithelium of the neural tube is the origin of the entire central nervous system. Cell genesis during cortical development follows an intrinsic time sequence, which begins with the development of neurons, followed by astrocytes and then oligodendrocytes. The cortex forms in an “inside-out” manner whereby deep layers (layers 4–6) form before upper layers (layers 1–3).<sup>1–3</sup> This sequence of events is largely conserved in animals.<sup>4</sup>

Two major classes of neurons populate the mammalian cortex: excitatory and inhibitory neurons. Complex neuronal information processing depends on precise spatial and temporal coordination of the activity of principal excitatory neurons that constitute the majority of neurons in the brain. Such coordination is provided by network oscillations that synchronize activity within local cortical circuits. Inhibitory interneurons, although constituting less than 20% of cortical neurons, form extensive local connections, such that each interneuron innervates thousands of principal neurons, enabling them to exert

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decisive forms of control over large neuronal assemblies. The interactions between excitatory and inhibitory neurons are critical in generating and regulating network oscillations, synchronizing the activity of principal neurons, and setting time windows for synaptic integration. Thus, these interactions drive circuit activity<sup>5,6</sup> and switch networks between different states of activities.<sup>7,8</sup> The balance between the actions of excitatory and inhibitory neurons is critical in all mammals in that it enables information on the mechanisms of neuronal function gained from rodents to be applied to humans.

Proper brain functioning relies on the correct number and placement of excitatory and inhibitory neurons. This is a remarkably complex process because each type of neuron has a different developmental origin. Excitatory glutamatergic projection neurons arise from progenitor cells in the *dorsal* ventricular zone; these migrate to their proper cortical layer destinations via radial migration.<sup>2,9–12</sup> In contrast, inhibitory GABAergic interneurons develop from progenitors in the ganglionic eminences of the *ventral* forebrain and migrate first radially in the ventricular zone and then tangentially to the cortex.<sup>13–27</sup> Correct formation, and thus function, of the cortex relies on synchronization of these two developmental programs. Comparison of human fetal brain tissue with information gleaned from animal models suggests that basic steps in excitatory and inhibitory neuron development are similar in mammals.<sup>28–40</sup> These results imply that mechanisms of cortical neuron development in rodents are relevant to humans.

These specific shared aspects of neocortical development in humans and mammals suggest that knowledge gained through animal modeling can be attributed to human development. Yet, emerging evidence makes it clear that the development of the human neocortex includes additional mechanisms to form the highly complex structure that can carry out higher-order functions, and it is in these higher-order functions from which disorders of intellectual disability often arise.

#### **Human-Specific Characteristics of Brain Development**

Despite the similarity between humans and rodents, there are a number of human-specific characteristics of neocortical development, including cortical expansion, protracted time of development, and genetics. A consequence of these differences between humans and rodent models is that not all aspects of human neurological diseases can be recapitulated in animal models, leading to misinterpretation of data and potentially failed clinical trials.

**Cortical Expansion.** The most obvious difference between rodent and human brains is the much larger size of the human cortex. The evolutionary expansion of the cerebral cortex is reliant on increased cell numbers, which is in turn dependent on an increase in the numbers of neural progenitors.<sup>41–46</sup> Specific cellular and molecular characteristics of cortical neural progenitors that are unique to primates drive this expansion. Neural progenitor cells,

specifically radial glia that give rise to neurons, undergo more symmetric divisions in primates, enabling the generation of more neural progenitors. Further, these neurogenic radial glia (oRGs) translocate to an additional outer subventricular zone (oSVZ), where they undergo mitosis to provide vastly more proliferative neuronal progenitors that, in turn, differentiate into more neurons.<sup>26,27,47–49</sup> Although oRGs have been found in rodents, they are rare in number, and their contribution to rodent brain development remains unclear.

Molecular mechanisms that regulate the expansion of the human cortex have been gleaned primarily from global transcriptomic analyses of the developing human brain. A human-specific gene, *ARHGAP11B* (MIM: 616310), encoding Rho GTPase activating protein 11B, promotes basal progenitor amplification and neocortex expansion and might have contributed to the evolutionary expansion of the human neocortex.<sup>50,51</sup> Other suggested molecular regulators include non-coding RNAs, such as microRNAs (miRNAs) and long non-coding RNAs (lncRNAs), that have undergone dynamic evolutionary changes.<sup>52–55</sup> Further, DNA methylation is dynamic during human brain development.<sup>56</sup> Thus, the ability to accurately model the expansion of the human cortex requires recapitulation of these critical molecular regulators, and so accurate modeling of cortical expansion is not possible in rodents.

**Protracted Time of Development.** The development of the human brain occurs over a prolonged time course in comparison with that of animal models.<sup>4</sup> The gestational period is 40 weeks in humans and 3 weeks in rodents. The bulk of human brain formation occurs prenatally. The neural tube is completely formed by embryonic day 27 (gestational age of 6 weeks) in humans and embryonic day 10 in mice. Human brains thus have 34 weeks, versus 11 days in mice, to develop before birth. The major period of generation and migration of cortical neurons in humans occurs from 10 to 25 weeks of gestation.<sup>2,43,55</sup> Cortical neurons generated in the ventricular zone, primarily in the oSVZ, reach their destination in the cortical plate by both radial and non-radial migration.<sup>25,57–59</sup> Newly born neurons are added to the cortical plate in an “inside-out” fashion and differentiate to form the cell-specific layers of the cortex.<sup>60,61</sup> Although most neuroblasts have been generated by the 16<sup>th</sup> gestational week, many neurons have not yet reached their final destination.<sup>2</sup> Furthermore, medium and small neurons, as well as glia, can continue to be produced after 16 weeks of gestation,<sup>2</sup> and neurogenesis continues at low levels after birth into old age.<sup>62</sup> Glial genesis continues throughout infancy and early childhood.<sup>55,63,64</sup> Infancy is also characterized by the formation of synaptic connections, including outgrowth of dendrites and axons. Structural and molecular reorganization of cells and synapses continues into early adolescence.<sup>43,55</sup> This long-drawn-out timing of neurodevelopment provides opportunities for mechanisms to increase complexity in the human brain, and this is difficult to model in rodents.

*Increased Complexity.* Although the functions of excitatory and inhibitory cortical neurons are conserved, interneurons in humans have distinct features. Recent evidence indicates that the human cortex contains more diverse types of interneurons and that humans have greater reliance on interneurons than other mammals.<sup>32</sup> Interneuron progenitors arise from the ventral forebrain in the ganglionic eminences (GEs), although there is dispute about whether interneurons in humans also arise from the dorsal forebrain.<sup>65–71</sup> The medial GE is the primary source of interneurons in mice, and its development is well characterized. The medial GE gives rise to 70% of the total cortical interneurons in mice, but recent data suggest a greater contribution from caudal GE progenitors in primates.<sup>72,73</sup> Human cortical neuron development integrates unique mechanisms, including prolonged neurogenesis and migration,<sup>74,75</sup> to generate interneurons.<sup>27,32,47,76–78</sup> Thus, developmental mechanisms that regulate interneuron development in primates are more complex and could lead to the generation of different classes and/or proportions of interneuron subtypes than in rodents.<sup>79</sup> In fact, a new human interneuron subtype was recently identified with features never described in rodents.<sup>80</sup>

*Unique Genetics.* Comparisons of DNA between human and animal models reveal only subtle differences in protein-coding regions. However, transcribed non-protein-coding sequences, including many species of non-coding RNA, are significantly less conserved than protein-coding mRNAs.<sup>44,63,81–83</sup> Thus, differences in regulatory elements, non-coding genes, and limited human-specific genes, as well as human-specific gene removal, can contribute significantly to phenotypic differences between human and animal brains.

An increase in comparative genomic analyses between the human or primate brain and the rodent brain in the last 10 years has led to reports of different expression patterns of the same genes in humans and mice.<sup>44,55,81,82,84,85</sup> Some of these differences can be accounted for by chromosome-level regulation, given the divergent chromosomal organization of human and mouse genes.<sup>86</sup> Differences in chromosomal architecture can impede the ability to genetically model disorders that are due to large changes in chromosomes, including aneuploidies (e.g., trisomy 21, also known as down syndrome [DS (MIM: 190685)]) and duplication syndromes (e.g., chromosome 15q11–q13 duplication syndrome [MIM: 608636]). Several examples of divergent regulation of the same genes have recently emerged.<sup>87,88</sup> A number of studies have identified human accelerated regions (HARs): short, evolutionarily conserved DNA sequences that have acquired significantly more DNA substitutions than expected in the human lineage since our divergence from chimpanzees.<sup>89,90</sup> Many of the HARs, such as *GLI2* (GLI family zinc finger 2 [MIM: 165230]) and *NPAS3* (neuronal PAS domain protein 3 [MIM: 609430]), are enhancers near genes and have important functions in development. For example, Prabhakar et al. identified *cis*-regulatory elements that have

undergone human-specific accelerated evolution, and one of them, human-accelerated conserved non-coding sequence 1 (*HACNS1*), leads to enhanced gene expression in limbs and potentially affects digit development and dexterity.<sup>91,92</sup> The impact of HARs in human neural development is currently unknown. In addition, recent evidence has revealed uniquely human enhancers that might drive human cortical neurogenesis.<sup>93,94</sup> Johnson et al. surveyed the transcriptome of 13 brain regions, including prefrontal cortical regions, of mid-gestation human brains and discovered genes differentially expressed and differentially spliced within subregions of the neocortex.<sup>44</sup> Interestingly, a significant number of differentially expressed genes, including forkhead box P2 (*FOXP2* [MIM: 605317]), are involved in speech and language and have undergone human-specific accelerated evolution.<sup>44</sup> Besides genomic regulatory elements, differences in untranslated regions (UTRs) of mRNAs can lead to differential gene regulation. In the developing human cortex, nitric oxide synthase 1 (*NOS1* [MIM: 163731]) mRNA is bound by fragile X mental retardation protein (FMRP), and its protein translation is regulated by FMRP. However, *NOS1* mRNAs in rodent and other non-primate mammals lack a G-quartet motif for FMRP binding, and so such regulation is absent.<sup>44,84,95</sup> The authors hypothesize that such differential post-transcriptional regulation could be responsible for reduced amounts of *NOS1* in individuals with human fragile X syndrome (FXS [MIM: 300624]); such differential regulation is not found in FXS mouse models. Relevant to neuron function and neural disorders, Qiu et al. compared activity-dependent gene expression in neurons differentiated from either human or mouse ESCs and found that basal neuronal gene expression is less divergent than depolarization-triggered gene expression.<sup>88</sup> For example, human ETS proto-oncogene 2 (*ETS2* [MIM:164740]) is induced more strongly and rapidly than mouse *Ets2*. This difference could be due to the presence of a human-specific AP-1 transcription factor binding site in the human *ETS2* promoter but not in the mouse *Ets2* promoter. The differential regulation and expression of this gene is particularly relevant to DS because *ETS2* is encoded on human chromosome 21, and so its expression and downstream consequences could be very different between humans and mouse models. Thus, an increasing number of examples of divergent gene expression between rodents and humans could lead to differences in function and relevance to disease.

Genome-wide approaches have identified a small number of human-specific genes that are likely to be important for neurodevelopment.<sup>50</sup> Some of these human-specific genes result from alternative splicing that is highly enriched in developing human brains.<sup>44</sup> For example, Kallikrein-related peptidase 8 (*KLK8*, also known as neuropsin [MIM: 605644]) encodes a secreted-type serine protease that is involved in synaptogenesis, neurite outgrowth, and plasticity in the hippocampus and neocortex. *KLK8* is involved in learning and memory and has a long spliced

form that is expressed only in humans (not in non-human primates) and produces human-specific type II KLK8.<sup>96</sup> Other human-specific genes could be products of human-specific duplications or variants of otherwise evolutionarily conserved genes. *ARHGAP11B* is a human-specific duplication of *ARHGAP11A* (MIM: 610589), is highly expressed in radial glia, and promotes cell proliferation and cortical expansion.<sup>50</sup> SLIT-ROBO Rho GTPase activating protein 2 (*SRGAP2* [MIM: 606524]) has two human-specific duplications, *SRGAP2B* and *SRGAP2C*. It has been shown that *SRGAP2C* inhibits *SRGAP2*, leading to increased dendritic spine density.<sup>97</sup> In addition to duplications, human-specific gene removal (deletion) also occurs. One example is an *Alu*-mediated frameshift mutation of the gene encoding the enzyme cytidine monophospho-N-acetylneuraminic acid (CMP-Neu5Ac) hydroxylase (*CMAH*, named *CMAHP* for the pseudogene in humans [MIM: 603209]). Whereas other mammals, including our closest relative (chimpanzee), express functional *CMAH* to convert Neu5Ac to Neu5Gc, humans do not, leading to an accumulation of amino acid Neu5Ac in the human brain.<sup>98</sup> The functional impact of such gene loss is unclear, and at least one study has shown that a lack of Neu5Gc prevents malaria parasite infection.<sup>99</sup> Other examples of gene loss, although not human specific, could also contribute to differences between humans and animal models. For example, primates (including humans) and guinea pigs have gained mutations in the gene encoding gulonolactone oxidase (*GULOP*), which produces ascorbic acid (vitamin C).<sup>100</sup> Humans and some primates have lost the enzymatic activity of urate oxidase (*UOX*), which catalyzes the oxidation of uric acid to allantoin during primate evolution, thus predisposing us to conditions including gouty arthritis and renal stones.<sup>101</sup> How human-specific gene addition or removal contributes to the differences observed between rodent and human neurodevelopment remains unclear. These studies further bolster the idea that studying animal models alone will yield incomplete information about human neural development and function.

Even evolutionarily conserved genes can have different functions in humans and animal models. One example is *FOXP2*, the only gene that has been firmly linked to speech and language development in humans through gene identification of individuals with language disorders (MIM: 602081).<sup>102,103</sup> *FOXP2* has gone through accelerated evolution in humans. *FOXP2* encodes a transcription factor and is among the top 5% of highly conserved genes among mammals, such that only three amino acids differ between the human and mouse proteins, and only two amino acids differ between the human protein and those of other primates.<sup>104</sup> In mice, *FOXP2* has been found to regulate lung development, and *Foxp2*-null mice die before adulthood as a result of severe motor and respiratory defects.<sup>105</sup> However, mice with *FOXP2* variants that cause language disorders in humans do not exhibit apparent vocalization deficits, suggesting differential functions of

*FOXP2* between humans and mice.<sup>106–109</sup> In fact, chromatin immunoprecipitation analysis demonstrates that human *FOXP2* has distinct transcriptional targets compared with those of its counterparts in rodents and chimpanzees.<sup>110</sup> Therefore, the two amino acid substitutions in *FOXP2* could affect its specificity for transcriptional targets that are uniquely important for human language development. Recapitulation of specific human gene mutations does not, therefore, predictably recapitulate gene function in animal models.

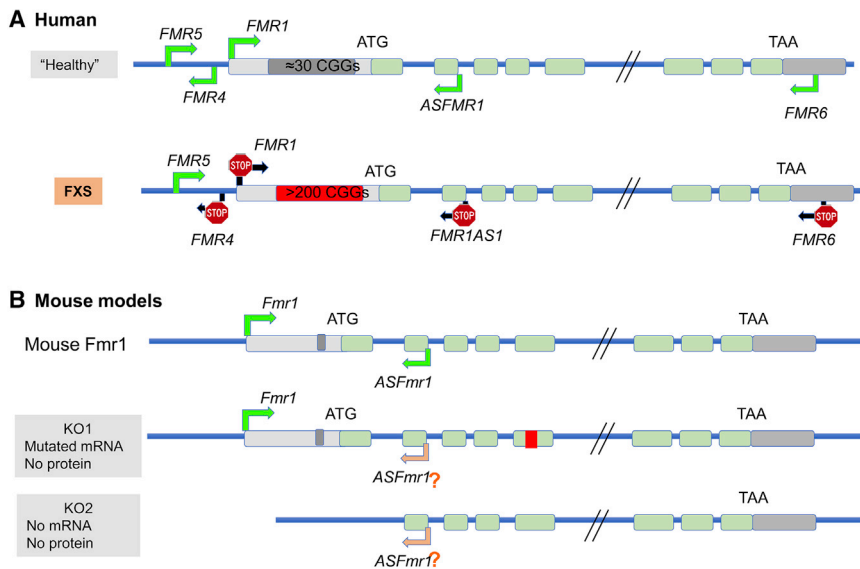
A large portion of both human and mouse genomes is transcribed into non-protein-coding RNAs. A comparison of orthologous human and mouse gene pairs has revealed that the sequence, number, and length of exons are highly conserved, but both the sequence and the length of introns vary significantly between species (e.g., human genes have larger introns).<sup>111</sup> The functional significance of this divergence in introns remains unclear, but non-coding RNA genes are sometimes embedded within introns.<sup>112</sup> In fact, both the human and mouse genomes encode a large number of non-coding RNAs that include both lncRNAs that are over 200 bases long<sup>112,113</sup> and small RNAs that are 20–30 nucleotides in length.<sup>114</sup> Unlike protein-coding genes, lncRNAs are less conserved. Approximately one-third of lncRNAs are primate specific.<sup>115</sup> Also, a number of conserved miRNAs seem to play similar functions in both humans and rodents. For example, both *MIR9-1* (MIM: 611186) and *MIR124-1* (MIM: 609327) are enriched in neurons and promote neural stem cell (NSC) differentiation into neurons in multiple species.<sup>116</sup> On the other hand, human-specific miR-941 is highly expressed in hPSCs, and its expression decreases upon differentiation.<sup>117</sup> The functional importance of this miRNA in human-specific traits remains unclear, but the fact that it targets hedgehog- and insulin-signaling-pathway genes that are important for brain development suggests that it could have a critical function in neural development. As with protein-coding genes, the impact of human-specific RNA species on neural development and function remains unclear but expands the known differences between humans and mice.

Together, expansion and complexity of the human cortex, at both the cellular and genomic levels, most likely lead to human-specific aspects of disease when they go awry. Thus, some aspects of human neurological diseases cannot be recapitulated in animal models.

### Human-Specific Genetic Characteristics of Neurodevelopmental Disorders

Mistakes in any of the unique aspects of human brain development summarized above lead to neurodevelopmental disorders that generally result in intellectual impairment. Two of the most prevalent genetic causes of intellectual disability in humans are FXS and DS. We will use these two disorders as examples to discuss human-specific traits of neurodevelopmental disorders and the ability to investigate their causes through model systems.





**Figure 1. The Genetics of Human FXS versus Mouse Models of FXS**

(A) The human *FMR1* locus contains several primate-specific lncRNAs, *FMR4*, *FMR5*, and *FMR6*. *ASFMR1* exists in both humans and mice. In FXS-affected individuals with a full mutation, most of these lncRNAs are also silenced.

(B) Mouse models of FXS have been created through the insertion of a neomycin cassette into exon 5 (KO1) or the deletion of the promoter and exon 1 (KO2), leading to a lack of protein production in both KO1 and KO2. *ASFmr1* expression is unknown in mouse models.

### Unique Genetics

Although both FXS and DS are categorized as genetic neurodevelopmental disorders, they represent two ends of the spectrum in terms of genetic mechanisms: single-gene mutation (Figure 1) versus whole-chromosome duplication (Figure 2).

The causal mutation in FXS affects a single gene, *FMR1* (MIM: 309550; Xq27.3), whose protein-coding sequence is highly conserved among species: the murine homolog is 97% homologous to the human amino acid sequence.<sup>121,122</sup> However, human *FMR1* has a large CGG repeat region that is absent in mouse *Fmr1*. The expansion of this CGG repeat in the 5' UTR of human *FMR1* leads to a chromosomal fragile site on the X chromosome<sup>123</sup> and epigenetic silencing of *FMR1*, resulting in FXS. The number of CGG repeats in human *FMR1* is highly polymorphic (the mode peak is 30 or 31), and more than 90% of the human population has fewer than 40 CGG repeats.<sup>123,124</sup> When the CGG repeat number exceeds 200, the CGGs become methylated and *FMR1* is transcriptionally silenced. Interestingly, when CGG repeats are expanded to 55–200, *FMR1* transcription is enhanced and leads to a pathological condition called fragile X pre-mutation. Individuals with pre-mutation have an increased risk of developing fragile-X-related primary ovarian insufficiency (FXPOI) and fragile-X-associated tremor and ataxia syndrome (FXTAS) in older age.<sup>125</sup> In addition, the CGG repeats in individuals with pre-mutation are unstable and tend to expand further during meiosis in the germline, leading to increased risk of FXS as a result of repeat expansion in the next generation. Despite extensive efforts, it remains unclear how the variable CGG-repeat length in *FMR1* affects *FMR1* transcription and why having more than 200 repeats leads to DNA methylation and gene shutdown.

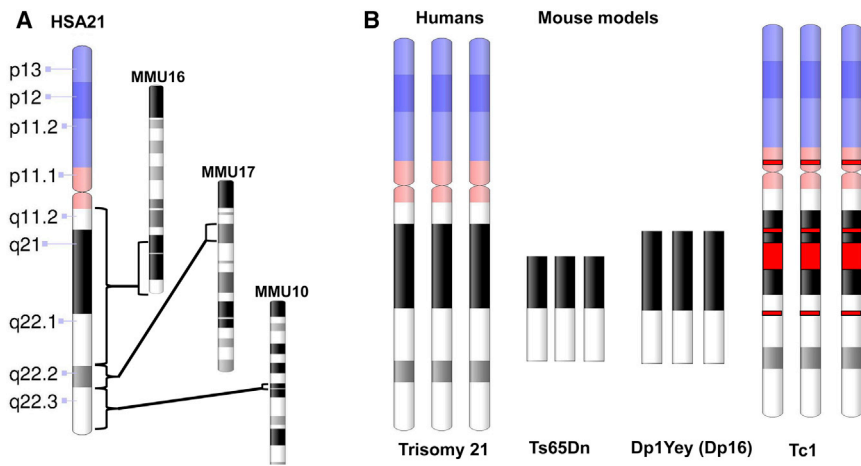
Mouse *Fmr1* has very few (about six) CGG repeats at the 5' UTR, and no CGG expansion has been observed in

any experimental mouse lines, thus limiting the utility of the mouse for studying the mechanisms of *FMR1* shutdown. Several mouse models of fragile X pre-mutation have been

created through the insertion of human CGG repeat (~100) sequences into the 5' regions of mouse *Fmr1*. Although these mice exhibit some deficits found in human individuals with pre-mutation, such as reduced cognitive ability, increased oxidative stress, RNA-induced toxicity, and instability of repeats, the CGG repeats do not expand into full mutation in the germline as they do in humans.<sup>126</sup> Therefore, mouse FXS models rely on *Fmr1* deletion, a completely different mechanism than the human mutation.

The protein product of *FMR1* and *Fmr1*, FMRP, is an RNA-binding protein that binds to specific mRNAs to control their stability, localization, and protein translation. The function of FMRP in human neuronal development and how a lack of FMRP causes the characteristics in FXS-affected individuals are largely unknown. FMRP's targets and function might differ between humans and rodents, leading to different phenotypes when the protein is not expressed. An example has been demonstrated by FMRP binding and translational regulation of human *NOS1* mRNA,<sup>95</sup> but not of mouse *Nos1* mRNA. However, a comprehensive identification of FMRP targets in human neurons has not yet been realized, defining a large gap in our knowledge of the similarities and differences of FMRP function between mice and humans.

Importantly, the human *FMR1* locus also produces several human-specific lncRNAs. Among them, *FMR4* (MIM: unavailable), *FMR1AS1* (MIM: 300805), and *FMR6*, but not *FMR5*, are silenced in FXS.<sup>127</sup> The functions of these lncRNAs are largely unclear, although *FMR4* has been shown to promote neural progenitor proliferation.<sup>128</sup> Therefore, it is currently unknown whether human FXS pathology is a result of the deletion of FMRP alone or the silencing of several coding and non-coding genes. Answering these questions will require human experimental models that have the full repertoire of human *FMR1* components.



**Figure 2. Comparison of Human Chromosome 21 and Mouse Models**

(A) Genes in human chromosome 21 have orthologous regions on three mouse chromosomes.

(B) Schematic of chromosome 21 genes that are represented in three mouse models: Ts65Dn (the most widely studied mouse model), Dp16, and Tc1 (a transchromosomal mouse line).<sup>118–120</sup>

generated<sup>118,119,141–146</sup> (Figure 2). Trisomy 16 mice were originally the most complete HSA21 trisomy model,<sup>147–149</sup> but their utility is limited because fetuses do not survive

DS (also known as trisomy 21) is a complex multigenic disorder and the most common viable human aneuploidy.<sup>129</sup> Neurological consequences include intellectual disability and early-onset Alzheimer disease (AD). The complexity of an additional chromosome provides a daunting problem for deciphering the underlying mechanisms, especially genotype-phenotype investigations. Further, the variability of characteristics among DS-affected individuals suggests that genetic, environmental, and stochastic influences are likely to play a role in defining the characteristics of DS.<sup>130–132</sup>

Chromosome 21 (HSA21), comprising an estimated 225–400 genes, is the smallest human chromosome,<sup>133,134</sup> which could explain why trisomy of this chromosome and not others (e.g., 13 and 18) is compatible with life and results in only mild to moderate intellectual disability. The short arm (p) of HSA21 is very small and highly repetitive, and its loss has only rarely been reported to result in significant disease phenotypes. Well-studied genes on the long arm (q) of HSA21 include genes that encode cell-adhesion molecules (e.g., DS cell-adhesion molecule [*DSCAM* (MIM: 602523)]), signaling molecules and kinases (e.g., dual-specificity tyrosine phosphorylation-regulated kinase 1A [*DYRK1A* (MIM: 600855)]), and transcription factors (e.g., *ETS2* [MIM: 164740]). Several genes on chromosome 21 have been implicated in DS and other disorders: familial AD (amyloid beta precursor protein [*APP* (MIM: 104760)]), familial amyotrophic lateral sclerosis (superoxide dismutase 1 [*SOD1* (MIM: 147450)]), and a predisposition for leukemia (runt-related transcription factor 1 [*RUNX1* (MIM: 151385)] and GATA binding protein 1 [*GATA1* (MIM: 305371)]). Using rare partial trisomies, human studies have tried to pinpoint the critical or smallest HSA21 region whose triplication might confer specific attributes of DS.<sup>135–140</sup> However, some phenotypic characteristics might map outside the minimum critical region, and it is likely that the combination of genes and their downstream effects on other genes result in the characteristics of DS.

Genes on HSA21 are encoded across three mouse chromosomes, and mouse DS models that contain various combinations of orthologous HSA21 genes have been

as live-born animals, and so analysis is limited to prenatal development. In the most widely used mouse model, Ts65Dn,<sup>150</sup> a large trisomic segment of mouse chromosome 16 contains most regions orthologous to HSA21. Mouse models Ts1Cje and Ts2Cje contain smaller regions.<sup>143,144</sup> The mouse strain Dp1Yey (also known as Dp16) contains the largest number of orthologous chromosome 16 genes. In addition, a “transchromosomal” mouse model that contains most of HSA21 has been developed.<sup>120</sup> Although these varied mouse models of DS contain substantial portions of HSA21 gene content, these genes lack the same regulation as human genes. As discussed above, the regulation of genes and their expression can vary in a human context. Thus, none of the mouse models are trisomic for all HSA21 orthologs, some have additional triplicated mouse genes, and none have human-specific gene regulation, so the full impact of human trisomy 21 has not been realized in mouse models. Although mouse models have provided a wealth of information about DS, it is crucial to examine the effects of the full trisomy 21 in the context of human cells, where human-relevant genetics and development come into play.

#### Success and Limitations of Animal Models for Neurodevelopmental Disorders

FXS- and DS-affected individuals exhibit distinct yet overlapping phenotypes. FXS-affected individuals have mild to severe cognitive impairment, increased incidence of childhood seizures, reduced motor coordination, and heightened anxiety. DS-affected individuals also have cognitive impairment (although it is often milder and includes deficits in different domains) and motor dysfunction characterized by hypotonia and a small seizure risk. Language, a human trait, is affected in both syndromes. These characteristics of both FXS and DS are due to affected higher-order functions that are largely mediated by the cerebral cortex and are unique to humans. Thus, models of these disorders must account for the varied human-specific aspects of cortical development, cortical expansion, human genetics, and genetic regulation.

### Modeling Neurological Symptoms and Behavioral Deficits

Neurodevelopmental disorders encompass a broad spectrum of phenotypes with potential contributions from a large number of genes.<sup>151–153</sup> Although mice and humans are 80 million years apart in evolution, most protein-coding genes are highly conserved; therefore, mice have been used extensively for modeling human diseases. Behavioral tests of rodents have been used for modeling human deficits with both successes and limitations.<sup>142,154–157</sup> Symptoms of social impairment can be evaluated through interactions, preference, and recognition of other mice. The communication between mice can be assessed by their responses to vocalizations or olfactory cues and their interactions. Repetitive behavior and resistance to change in routine can be observed in stereotypical behavior or in reversal learning tasks. Cognition is standardly evaluated in rodent models of neurological disorders via the assessment of memory and learning through novel-object recognition, the Morris water maze, fear conditioning, and passive avoidance tests. These tests can define memory differences due to specific disease-mutation modeling but still cannot completely model human-specific aspects of cognition, including language and conscience. The hope is that these measurements can be used for assessing specific gene contributions to neurodevelopmental disorders and evaluating potential drug candidates in a pre-clinical model system.

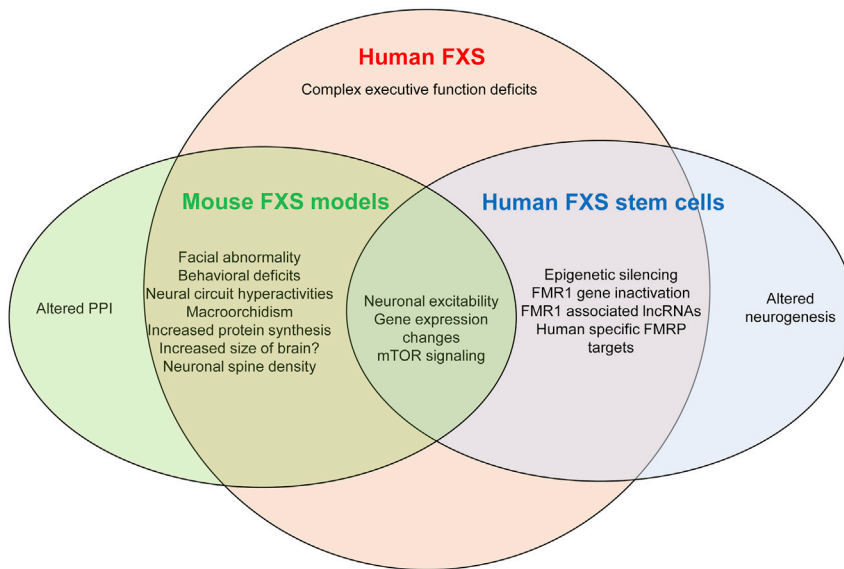
**Fragile X Syndrome.** Animal models of FXS have been developed in *Drosophila*, zebrafish, mice, and rats. Extensive behavioral modeling has been done primarily with mouse models of FXS.<sup>158</sup> In the most widely used mouse model (*Fmr1* knockout [KO]), an insertion in exon 5 of *Fmr1* produces a mutant *Fmr1* mRNA but not FMRP<sup>159</sup> (Figure 1). Another *Fmr1*-mutant mouse line (*Fmr1* KO2) has been generated by germline deletion of the floxed promoter and the first exon of *Fmr1*, and neither mRNA nor protein is produced.<sup>160</sup> Ever since its creation, the *Fmr1*-KO mouse model has been extensively tested with the hope that it will faithfully recapitulate human FXS phenotypes. Human male FXS-affected individuals have macroorchidism, an elongated face, prominent ears, and flexible joints. *Fmr1*-KO mice indeed have enlarged testes; however, joint flexibility has not been effectively assessed in mouse models.<sup>161</sup> Dense-surface modeling techniques have been used for assessing the facial features of human FXS and *Fmr1* KO.<sup>162</sup> *Fmr1*-KO mice do not show an increased anterior-posterior length of the skull nor increased length of the frontal bone. However, *Fmr1*-KO mice exhibit an increased vertical dimension of the mandible skull, increased width of the premaxilla, and nasal bone differences, which could resemble the more oval face and wide nose of FXS-affected individuals. Seizure is prevalent in neurodevelopmental disorders. About 10%–20% of FXS-affected individuals have childhood seizures, but these seizures are generally infrequent and can be controlled by medication. *Fmr1*-KO mice exhibit susceptibility to audiogenic seizures triggered by loud sounds.

The mouse model exhibits transient and mild seizure phenotypes that could be similar to human FXS, but the seizures occur only transiently in young mice and only in certain mouse strains.

The core behavioral deficits of human FXS-affected individuals include cognitive deficits, increased anxiety, hyperactivity, social phobia, and repetitive behaviors. Although FXS is primarily a single-gene disorder, FXS-affected individuals display variable degrees of severity. Because *FMR1* is an X-linked gene, the differences in clinical phenotypes between males and females are expected, and in fact, FXS-affected females tend to have less severe disability as a result of compensation by the second X chromosome. However, even males with the full mutation exhibit heterogeneous characteristics. Both the size of the CGG expansion and the extent of CGG methylation vary significantly among males with the full mutation. Furthermore, mosaic CGG methylation and partial *FMR1* inactivation have been detected in somatic cells of some individuals.<sup>163</sup> Therefore, genetic differences lead to different levels and patterns of FMRP expression and are responsible for additional variability in clinical presentations and severity of this single-gene disorder. This heterogeneity is somewhat recapitulated in FXS mouse models that are developed in different genetic backgrounds.<sup>164,165</sup>

*Fmr1*-KO mice exhibit several human FXS-related behavioral deficits, but not others (for a comprehensive review of behavioral studies of FXS mice, please see Kazdoba et al.<sup>157</sup>). FXS-affected individuals have impaired working memory, short-term visual memory, visual-spatial abilities, sequential information processing, executive function, and attention. Several mouse behavioral tests have been used for assessing these complex cognitive functions in *Fmr1*-KO mice. Although some studies have shown that *Fmr1*-KO mice are impaired in passive avoidance, contextual fear conditioning, delayed trace conditioning, the Morris water maze, novel-object recognition, and novel-location tests, other laboratories have found no change or even opposite results (Figure 3 and Table 1).

FXS-affected individuals exhibit significant anxiety despite the genetic background. Anxiety can be assessed in rodents with the open-field activity test, dark-light exploration, and the elevated plus maze. These tests are non-invasive and are based on the natural tendency of mice to seek dark and protected locations. However, the mouse models display increased, decreased, or no change in anxiety depending on the genetic background, age, methods, and labs tested.<sup>155,157</sup> Compulsive and repetitive behaviors are frequently seen in FXS-affected individuals. *Fmr1*-KO mice have shown altered reverse learning. The marble-burying test has been used to show compulsive burying of strange objects in mice.<sup>155</sup> *Fmr1*-KO mice in some backgrounds have been shown to bury more marbles.<sup>166</sup> Impairment of social behavior in FXS is largely due to social anxiety. The social-interaction results of mouse models are mixed—some studies have shown decreased anxiety, whereas others have found no



**Figure 3. Human FXS Neurological and Behavioral Deficits that Are Represented in Mouse Models and/or Human Stem Cell Models**

Although FXS-affected individuals also exhibit reduced pre-pulse inhibition (PPI), mouse models show either increased or no change in PPI.

difference in social interaction in *Fmr1*-KO mice. A recent study has shown that *Fmr1*-KO mice might have trouble recognizing strangers in a social-novelty test.<sup>167,168</sup> Whether this is a result of learning or social ability remains to be validated. Thus, the behavioral phenotypes that are relevant to FXS-affected individuals are not robustly recapitulated in FXS mouse models.

Many FXS-affected individuals are hyperactive and have impaired sustained attention. They have difficulty performing difficult tasks requiring switching and inhibiting responses. *Fmr1*-KO mice have not shown impaired attention in the five-choice serial-reaction-time task. However, analyses of open-field activity have shown increased locomotor activity in some studies, but not others.<sup>157</sup> Sensorimotor gating is impaired in many neurodevelopmental disorders, including autism and FXS, which can be assessed with pre-pulse inhibition (PPI) tests for both rodents and humans. FXS-affected individuals are more sensitive to pure tones, as are *Fmr1*-KO mice. The severity of PPI deficits correlates with the severity of impaired attention and cognition in FXS. However, the PPI results from mice have been confusing because most studies have found that PPI is enhanced in *Fmr1*-KO mice, contradictory to the reduced PPI observed in FXS-affected individuals.

The assessment of language impairment is difficult in rodents. Ultrasonic vocalization emitted by pups separated from their mother has been used for assessing language in rodents; however, the findings are inconsistent. Importantly, it is not clear how relevant rodent vocalization is to human language.

Altogether, it is not clear whether the variable results observed in FXS mouse models reflect variability in the disease phenotypes that are being modeled or whether these mouse models simply fail to recapitulate human disease phenotypes.

**Down Syndrome.** Non-neural DS features, including hallmark craniofacial characteristics, heart malformations, and

leukemia, have been modeled in some of the DS animal models.<sup>169–174</sup> DS is characterized by learning, memory, and language abnormalities that result in mild to moderate intellectual disability.<sup>175–182</sup> Additional neurological hallmarks of DS include motor dysfunction and hypotonia<sup>178,183–185</sup> and predisposition to AD pathology.<sup>178,186–189</sup> In recent years, research in DS has been increasingly focused on

the degeneration and dementia hallmarks of AD in DS. DS and neurodegeneration are reviewed in detail in other recent reviews.<sup>190–192</sup> Here, we focus on neurodevelopment, although the two are probably linked.

Cognitive disability in DS-affected individuals suggests that hippocampus-dependent mechanisms are affected but that other cortical regions and cerebellum contribute. Hippocampus-dependent learning and motor learning are impaired in DS mouse models, as shown in behavioral tests and electrophysiology (Figure 4 and Table 2).<sup>193–208</sup> Yet, thorough analyses of all the different trisomic mouse models are still underway, and results are not consistent among models.<sup>142,145,196,204,209</sup> Importantly, the different mouse models have differing or no phenotypes that might link to DS disease characteristics.<sup>210</sup>

Although the behavioral phenotypes of some of the DS mouse models could be reminiscent of cognitive characteristics of DS-affected individuals, structural and molecular mechanisms that underlie the phenotypes are different.

#### **Modeling Structural and Cellular Deficits**

**Fragile X Syndrome.** Whether FXS-affected individuals have an enlarged brain is unclear because larger brain size has been found in some affected individuals but not others.<sup>211</sup> Compared with those of wild-type mice, the brains of *Fmr1*-KO mice show no significant changes in size, weight, or overall number of cells.<sup>159</sup> A number of studies have assessed the size of the brain and major brain structures in FXS-affected individuals by using magnetic resonance imaging (MRI) and revealed abnormalities including reduced cerebellar vermis, increased hippocampal volume, larger volume of the caudate nucleus, and enlarged ventricles, although these findings are not consistent across studies.<sup>212,213</sup> *Fmr1*-KO mice have also been analyzed by MRI. Although an early analysis found no significant change in gross structure,<sup>214</sup> two later studies found reduced volumes in the deep cerebellar nuclei<sup>215</sup>



**Table 1. Comparison of Characteristics of FXS-Affected Individuals and Model Systems**

	<b>FXS-Affected Individuals</b>	<b>Rodent Models</b>	<b>Human Stem Cell Models</b>
Physical symptoms	facial abnormality (long face, prominent ears)	some mild facial abnormality	ND
	flexible joints	ND	ND
	gastrointestinal issues	ND	ND
	macroorchidism <sup>a</sup>	macroorchidism <sup>a</sup>	ND
	facial abnormality (long face, prominent ears)	some mild facial abnormality	ND
Behavioral symptoms	reduced PPI	increased, decreased, or no change in PPI	ND
	severe cognitive deficits (working memory, short-term memory, executive functioning, attention)	some mild cognitive deficits and variable results <sup>b</sup>	ND
	motor deficits	ND	ND
	hyperactivity	variable results <sup>b</sup>	ND
	significant anxiety, social anxiety	variable results <sup>b</sup>	ND
	compulsive behaviors <sup>a</sup>	impaired reversal learning, marble burying <sup>a</sup>	ND
	childhood seizures <sup>a</sup>	audiogenic seizure in juvenile mice <sup>a</sup>	ND
Brain and neuronal deficits	increased brain size	both embryonic and adult NPCs have increased proliferation	ND
	ND	embryonic NSCs have increased neuronal differentiation; adult NSCs have reduced neuronal differentiation	reduced neuronal differentiation, no difference, variable results
	ND	reduced dendritic complexity <sup>a</sup>	reduced dendritic complexity <sup>a</sup>
	increased spine density <sup>a,c</sup>	increased spine density <sup>a,c</sup>	ND
Neuronal functional deficits	neural circuit hyperactivity <sup>a</sup>	neural circuit hyperactivity <sup>a</sup>	ND
	neuronal hyperexcitability <sup>d</sup>	neuronal hyperexcitability <sup>d</sup>	neuronal hyperexcitability <sup>d</sup>
	ND	reduced synaptic pruning	ND
	ND	impaired LTD	ND
	ND	impaired LTP	ND
	ND	impaired development of GABAergic inhibitory neurons	ND
Genetic basis	<i>FMR1</i> CGG expansion and gene silencing <sup>a</sup>	<i>Fmr1</i> deletion in exon 5 (KO1 produces mutant mRNA and no protein) or exon 1 (KO2 produces no mRNA or protein) <sup>e</sup>	<i>FMR1</i> CGG expansion and gene silencing <sup>a</sup>
	mosaic gene silencing <sup>a</sup>	ND	mosaic gene silencing <sup>a</sup>
	human lncRNAs <i>FMR4</i> and <i>FMR6</i> inactivated <sup>a</sup>	ND	human lncRNAs within <i>FMR1</i> locus <sup>a</sup>
	<i>FMRIAS1</i> inactivated <sup>a</sup>	ND	<i>FMRIAS1</i> inactivated <sup>a</sup>
Molecular deficits	increased protein synthesis <sup>a</sup>	increased protein synthesis <sup>a</sup>	ND
	altered mTOR signaling <sup>d</sup>	altered mTOR signaling <sup>d</sup>	altered mTOR signaling <sup>d</sup>
	elevated PI3K activities <sup>a</sup>	elevated PI3K activities <sup>a</sup>	ND
	ND	signaling pathways (e.g., APP, ERK, IGF, and P53)	ND

(Continued on next page)

**Table 1. Continued**

	<b>FXS-Affected Individuals</b>	<b>Rodent Models</b>	<b>Human Stem Cell Models</b>
Molecular targets	gene expression changes <sup>c</sup>	gene expression changes <sup>c</sup>	gene expression changes <sup>c</sup>
	some FMRP targets (e.g., <i>Map1b</i> ) <sup>d</sup>	some FMRP targets (e.g., <i>Map1b</i> ) <sup>d</sup>	some FMRP targets (e.g., <i>Map1b</i> ) <sup>d</sup>
	human-specific FMRP targets (e.g., <i>NOS1</i> ) <sup>a</sup>	ND	human-specific FMRP targets (e.g., <i>NOS1</i> ) <sup>a</sup>

The following abbreviations are used: LTD, long-term depression; LTP, long-term potentiation; ND, no data.

<sup>a</sup>Consistent between FXS-affected individuals and only one of the experimental models.

<sup>b</sup>Large variability in behavioral deficits was observed. See Kazdoba et al.<sup>157</sup> for a review.

<sup>c</sup>Spine density has only been shown in specific brain regions and in very few human cases.

<sup>d</sup>Consistent among FXS-affected individuals and both experimental models.

<sup>e</sup>Not consistent between experimental models and FXS-affected individuals.

and increased white-matter volume.<sup>216</sup> In these studies, no change in brain volume was found. Because *Fmr1*-KO mice exhibit mild facial structural changes,<sup>162</sup> changes in regional brain structures and cell number could be too subtle for MRI detection.

Neurons have complex morphological development that is critical for their function. A highly cited finding is that neurons in post-mortem brain tissue from humans with FXS have long, thin dendritic spines. This observation was first reported in apical dendrites of pyramidal neurons of the isocortex in one FXS-affected male and was later confirmed in neocortical neurons of three more individuals.<sup>217–219</sup> A subsequent quantitative analysis comparing the layer V pyramidal neurons of temporal visual cortices of three FXS-affected individuals with that of three control individuals corroborated the spine phenotypes.<sup>220</sup> However, the limited analyses, in terms of both types of neurons and numbers of subjects, limit the potential generalizability of this finding to phenotypical modeling in animal models. Nevertheless, because this dendritic-spine abnormality is one of the only neuronal deficits identified in human neurons, spine analysis has been widely carried out to identify “human FXS-individual-relevant phenotypes” in *Fmr1*-mutant mouse models<sup>221</sup> despite the fact that the spine phenotype has been inconsistent in animal models.<sup>222</sup> More analysis of human neurons and the identification of additional neuronal deficits in human FXS neurons are critically needed to guide animal studies and to expand our understanding of the impact of FXS on the brain.

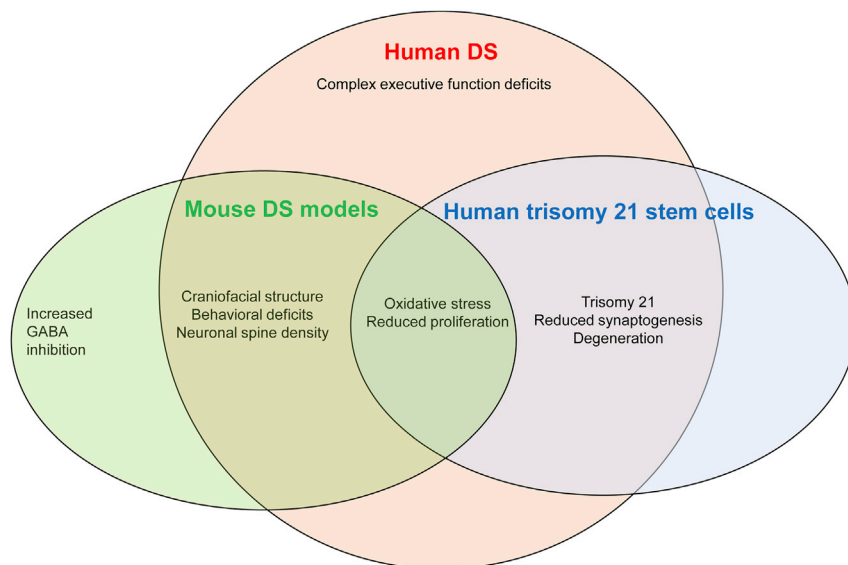
Electroencephalography (EEG) analysis has revealed that FXS-affected individuals exhibit hyperexcitability in the neocortex and altered functional connectivity, suggesting impaired excitatory and inhibitory balance.<sup>223–226</sup> The *Fmr1*-KO mouse model enables cellular physiology analysis that is impossible in humans. Altered EEG has also been found in *Fmr1*-KO mice, and it has been used as a readout for pre-clinical assessment.<sup>227</sup> These studies have shown that *Fmr1*-KO mice exhibit altered synaptic plasticity and a disrupted critical period for neuronal maturation (see the review by Contractor et al.<sup>228</sup>). Validation of these morphological and functional neuronal deficits in human models is needed.

**Down Syndrome.** Post-mortem observations and volumetric MRI studies have indicated that people with DS, in contrast to those with FXS, have reduced brain volumes.<sup>229–234</sup> Fewer neurons in the developing cerebral cortex of DS-affected individuals have been consistently reported over the last 70 years.<sup>229–233,235–239</sup> Cortical neuron deficiency appears near the end of cortical neurogenesis (>23 weeks of gestation)<sup>238,240</sup> and, according to limited histopathological analyses, is area, cell-type, and age specific. Data suggest that primarily small, granular, presumably GABAergic neurons in layers II and IV of the cortex are affected.<sup>239</sup> Additional hypocellularity has been reported in other brain regions, including the hippocampus and cerebellum,<sup>241–243</sup> during development, suggesting a general defect in neurogenesis in DS.<sup>244</sup>

Impaired neurogenesis has been reported in DS mouse models<sup>241,245–247</sup> but does not consistently decrease the number of neurons in the mouse brain to recapitulate the reduced number of neurons in the brains of DS-affected individuals. For example, increased interneuron progenitor proliferation and increased production of interneurons is found in T65Dn,<sup>248</sup> in contrast to observations of fewer interneurons present in human DS cortex. Further, at least one mouse model, Dp16, that contains the largest number of triplicated orthologous HSA21 genes has no detectable neurodevelopmental defects.<sup>210</sup> Neuronal structure is also altered in the brains of DS-affected individuals, such that neurons show fewer and shorter dendritic spines.<sup>229,240,249–255</sup> A reduced number of spines has been reported in DS mouse models, although the morphology varies among models.<sup>209,256–258</sup> These results highlight the inability of DS mouse models to recapitulate the most established neuroanatomical characteristics of DS-affected individuals.

### **Modeling Molecular Mechanisms**

**Fragile X Syndrome.** Extensive studies using animal models have revealed several significant molecular functions of FMRP. The most prominent function of FMRP is regulating protein synthesis and acting as a brake for metabotropic glutamate-receptor-dependent protein syntheses. In the absence of FMRP, exaggerated mGluR5 signaling leads to enhanced protein synthesis and changes in synaptic



**Figure 4. Human DS Neurological and Behavioral Deficits that Are Represented in Mouse Models and/or Human Stem Cell Models**

Increased GABAergic inhibition has not yet been shown in human DS.

plasticity. According to this mGluR5 theory,<sup>259</sup> a number of pharmacological preclinical studies targeting mGluR5 have been performed and shown remarkable effects in correcting *Fmr1*-KO mice. Subsequent large human clinical trials were carried out and were able to successfully alleviate disease symptoms.<sup>158</sup> Another group of favorite drug targets for FXS is GABA receptors and drugs to enhance GABAergic signaling. Some of these drugs were found to correct behaviors in *Fmr1*-KO mice, and hence clinical trials were moved forward, but they did not show a successful response in FXS-affected individuals.<sup>161,260</sup> Although the failure of these trials could be due to many reasons, including differences in drug bioavailability, drug metabolism, and methods of delivery and outcome assessment, there was a clear lack of validation in human experimental systems before the trials. In fact, only one study assessed mGluR5 in humans and found only mild increases with marginal significance ( $p = 0.058$ ) in the density and amount of mGluR in post-mortem prefrontal cortex.<sup>261</sup> Importantly, the activity of mGluR5 has not been assessed in human FXS neurons, and so it remains unclear whether pathways targeted for therapy in FXS mouse models are the correct targets for human disease.

Several FMRP-regulated downstream pathways have been discovered, and the most well-known mechanistic target is rapamycin (mTOR) signaling.<sup>262,263</sup> However, a critical role of mTOR signaling has not been validated in human FXS. Many signaling pathways, including mitogen-activated protein kinase 1 (MAP2K1) signaling and Wingless-type MMTV integration site family (WNT) signaling, have been shown to alter mTOR signaling in *Fmr1*-KO mice. Targeting these pathways has had remarkable rescue effects in mice, but none of these changes have been validated in human neurons.<sup>158</sup> Therefore, it is unclear whether any of these pathways are important mechanisms underlying FXS in humans.

*Down Syndrome.* Most lines of research to identify molecular mechanisms underlying DS have focused on the premise that specific genes located on chromosome 21 are responsible for DS symptoms and that these genes might exert direct effects that lead to manifestations of DS or have secondary effects on other genes. Thus, intense focus on the contribution of a few individual HSA21 genes to regulating aspects of DS pathology has

been carried out with DS mouse models. In particular, DYRK1A, a kinase that plays a role in many signaling pathways (including those involved in neural development), has been linked to behavioral deficits in mouse models and has been targeted as a potential therapeutic candidate.<sup>264–266</sup> More recently, the role of DYRK1A has been implicated in the proliferation and differentiation of human neural progenitors.<sup>267</sup> Regulator of calcineurin 1 (RCAN1) regulates calcineurin—so its overexpression might affect neural development—and it has been linked to aspects of DS.<sup>268–271</sup> One chromosome 21 gene, *USP16*, has been shown to be a controller of stem cells in both animals and humans and can affect the proliferation and differentiation of many cell types that are affected in DS.<sup>272</sup> Most of these studies do not analyze the role of genes in the context of trisomy 21 but instead provide good data for each chromosome 21 gene product's roles in neural development.

The predominant candidate mechanism that has emerged from mouse model studies as underlying intellectual disability in DS is an imbalance between excitation and inhibition in the cortex, specifically over-inhibition. Excitation-inhibition imbalance is a mechanism commonly proposed in disorders characterized by intellectual disability and cortical dysfunction. The establishment of this phenotype in mouse models has led to the targeting of over-inhibition in the cortex by various drugs, including GABA agonists and epigallocatechin-3-gallate.<sup>273–277</sup> It is important to note that in the Ts65Dn mouse model, the one on which therapeutic targets and clinical trials have been based, 25% of trisomic genes are not human chromosome 21 orthologs and 45% of human chromosome 21 orthologs are not trisomic.<sup>118,141</sup> Importantly, the over-inhibition phenotype has not been validated in human DS brain tissue or neurons, and the fact that the human DS brain has fewer interneurons suggests that over-inhibition might not underlie cortical dysfunction in DS-affected individuals.

**Table 2. Comparison of Characteristics of DS-Affected Individuals and Model Systems**

	<b>DS-Affected Individuals</b>	<b>Rodent Models</b>	<b>Human Stem Cell Models</b>
Physical symptoms	altered craniofacial structure <sup>a</sup>	altered craniofacial structure <sup>a</sup>	ND
	short stature	ND	ND
Behavioral symptoms	severe cognitive deficits (learning and memory, executive function)	some cognitive deficits and variable results <sup>b</sup>	ND
Brain and neuronal deficits	decreased brain size	ND	reduced neuronal differentiation, no difference, variable results
	reduced excitatory and inhibitory neurons	increased inhibitory neurons <sup>c</sup>	reduced inhibitory neurons
	dendritic spine defects	dendritic spine defects	ND
	Alzheimer disease pathology <sup>d</sup>	increased amyloid beta protein <sup>d</sup>	increased amyloid beta protein <sup>d</sup>
Neuronal functional deficits	oxidative stress <sup>d</sup>	oxidative stress <sup>d</sup>	oxidative stress <sup>d</sup>
	decreased neurogenesis and proliferation	variable increased and decreased neurogenesis <sup>b</sup>	decreased neurogenesis and proliferation
	ND	increased inhibition	ND
	decreased myelination <sup>a</sup>	decreased myelination <sup>a</sup>	ND
Genetic basis	trisomy 21 <sup>a</sup>	each animal model has different combinations of orthologous HSA21 genes; some have triplicated non-HSA21 genes <sup>c</sup>	trisomy 21 <sup>a</sup>
Molecular deficits and targets	upregulation of some HSA21 genes; downstream consequences on whole genome <sup>a</sup>	different sets of genes; potentially different downstream consequences <sup>c</sup>	upregulation of some HSA21 genes; downstream consequences on whole genome <sup>a</sup>

The following abbreviation is used: ND, no data.

<sup>a</sup>Consistent between DS-affected individuals and only one of the experimental models.

<sup>b</sup>Large variability in behavioral deficits was observed. See Herault et al.<sup>142</sup> for a review.

<sup>c</sup>Not consistent between experimental models and DS-affected individuals.

<sup>d</sup>Consistent among DS-affected individuals and both experimental models.

The examples of data from mouse models of the two most common genetic causes of intellectual disability highlight important knowledge gained but also reflect differences, inconsistencies, and limited (sometimes contradictory) associations with disease characteristics. Many biochemical or structural differences identified in animal models have not been validated in humans and therefore might not be of physiological relevance to human disease. Therapeutic targets that have not been validated in affected individuals are being pursued. So, the impact of these therapeutic interventions could be limited to treatments that reverse mouse phenotypes rather than disease characteristics. Thus, human models are critical to identifying therapeutic targets that will be more likely to benefit affected persons. To do so, we need to better understand foundations of human development and developmental disorders through the use of human tissue, cells, and models.

### The Advantages of Human Stem Cell Models

#### *Human Experimental Models for Neurodevelopmental Disorders*

Various peripheral cell types, including lymphocytes, amniocytes, and fibroblasts, have been used for studying molecular aspects of neurological disease. The primary

advantage of these cells is their accessibility in humans. These peripheral cells can also serve as critical platforms for identifying biomarkers for early diagnosis if characteristics of these cells are shared with neural cells or, more likely, if strong correlations between neural function and specific peripheral cell phenotypes are defined. However, in the context of understanding the causes of neurodevelopmental disorders, cells of the developing cortex are dynamic and have cell-type-specific characteristics that cannot be represented by these surrogate cells. Thus, studying human neural cells is critical to providing insight into the foundations of neurodevelopmental disorders.

*Post-mortem Brain Tissues.* Post-mortem brain tissues have long been a major experimental source for analyses to inform our understanding of neurological diseases. The fact that the bulk of the formation of the human brain occurs prenatally necessitates study of the fetal period to understand human cortical development. The use of human post-mortem fetal tissue is limited by access, ethical and sometimes political controversy, and static time points. Yet, primary fetal tissue provides a critical opportunity to view human brain development and thus remains the gold standard by which to compare other model systems. In fact, fetal tissue samples were essential for recent transcriptomic analysis of normal human brain



development and identifying differences in cortical development in DS.<sup>64</sup>

Some of the technical, but not societal, limitations of post-mortem human fetal tissue can be overcome to some extent by forcing expansion of precursor cells within the tissue. Human neural progenitor cells (hNPCs) provide a relatively pure population of neural cells that can be used for studying early brain formation. Exposure of primary fetal cortical tissue to mitogens causes the formation of aggregates of progenitor cells, termed “neurospheres.”<sup>278</sup> Neurospheres contain uncommitted and restricted neural progenitor cells. Progenitors within the neurospheres divide when cultured in the presence of growth factors for extended periods of time and differentiate into neurons and glia in the absence of growth factors.<sup>279–282</sup> Yet, there are significant limitations of using hNPCs to study neural development. First, the isolation of cells from tissue, as in any primary-tissue *in vitro* paradigm, removes them from their developmental niche and therefore provides information about the potential of hNPCs but not necessarily what the cells would do *in vivo*. hNPCs differentiate into limited neuronal subtypes because they are derived from fetal tissue at a single time point, approximately 20 gestational weeks. hNPCs are not very valuable for studying synaptic development because synaptically active cortical neurons have not been successfully generated from hNPCs. Therefore, the utility of hNPCs for studying human neural development and neurodevelopmental disorders is limited.

Despite these limitations, critical information about neurodevelopmental disorders, particularly DS, has been gleaned from studies of hNPCs. The ability to diagnose DS prenatally and to terminate a pregnancy has allowed for access to DS fetal tissue. Although hNPCs are derived from a specific developmental time point, this particular time point has proved to be useful for identifying differences in DS cortical development, such as reduced neuron generation<sup>283–285</sup> and increased astrocyte generation,<sup>286,287</sup> suggesting a possible change in developmental timing during cortical development in DS.

hPSCs overcome many limitations of primary brain tissue and are an important tool for studying the development of specific cell types, including neural cells.<sup>288–294</sup> hPSCs, including hESCs derived from embryos and hiPSCs derived from somatic cells, have tremendous potential for human disease modeling and therapeutic development. Because hPSCs are pluripotent, meaning they have the ability to differentiate into any cell type in humans, they can be used to generate disease-relevant cell types, tissues, and organs to unveil cellular and molecular events underlying normal and abnormal neural development. In addition, because hPSCs are non-transformed human cells, they can be used to elucidate neurological disease pathogenesis in a human genetic background. Additionally, pure populations of specific neural cell types derived from hiPSCs can be used for the rapid screening of pharmaceutical compounds to accelerate drug discovery and advance treatments. The power of hPSCs to model human

brain development lies in the ability to generate specific neural cell types *in vitro* over a long period of time that corresponds to *in vivo* development and thus recapitulate many of the developmental steps. Methods of generating specific neuronal and glial subtypes from hPSCs that follow *in vivo* developmental principles and timing have been developed.<sup>29–31,36,40,292,294–302</sup>

A critical opportunity for hPSCs is their use in studying the developmental course of genetic disorders. hESCs can be derived from embryos that have been found to carry genetic mutations after pre-implantation genetic diagnosis (PGD).<sup>303</sup> By building upon studies of ESCs, the establishment of iPSC technology in humans<sup>289,291</sup> has resolved many difficult issues facing hESCs and revolutionized our ability to study human development and diseases. To date, hiPSCs can be derived from not only skin fibroblasts but also more accessible lymphocytes, hair follicle cells, and dental pulp cells, for example. Characteristics of a given genetic disorder can be defined at the molecular level with hPSCs that either carry the genetic defect (derived from affected individuals) or have been genetically engineered to carry the disease mutation. Unlike their mouse counterparts, hPSCs have been difficult to modify genetically. Gene editing of hPSCs is typically achieved by random transgene insertion due to inefficiency in homologous recombination and cellular cloning, and such transgenes are often downregulated upon stem cell differentiation, especially during differentiation to functional neurons. Technical difficulties in genetically modifying hPSCs have significantly impeded neuroscience research involving hPSCs. Recent technological advances enable more efficient genetic hPSC modifications, including homologous recombination based on zinc fingers, transcription-activator-like effector nucleases, and CRISPR-Cas systems.

#### **Human Stem Cells for Modeling Neurodevelopmental Disorders**

The unique ability of hPSCs to model human brain development has facilitated their use in defining human-specific aspects of cortical development and defining differences that occur in neurodevelopmental disorders. Human-derived hiPSCs have been generated for modeling both single-gene mutations and complex mutations that lead to intellectual disability in humans. PSC models of neurodevelopmental disorders caused by mutations in single genes have been established and include those for Rett syndrome (*MECP2* [MIM: 300005]),<sup>304–308</sup> tuberous sclerosis (*TSC1* [MIM: 605284]),<sup>309–311</sup> and FXS.<sup>312–316</sup> More complex genetic disorders have benefitted from the ability to retain the chromosomal defect in hiPSCs for disorders that result in deletions or duplications of chromosomal regions, such as 15q11.2 microdeletion syndrome (MIM: 615656),<sup>317</sup> Prader-Willi syndrome (MIM: 608636),<sup>318,319</sup> and DS.<sup>320–324</sup> Some phenotypes in neural cells derived from these cells link to disease symptomology, whereas other phenotypes do not, and there is variability in the phenotypes in different reports.

**Fragile X Syndrome.** Both hESC and iPSC models of FXS have been developed.<sup>312–316</sup> FXS hESCs are derived from embryos that are determined to have the full mutation by PGD.<sup>325,326</sup> Analyses of neurons derived from these cells confirm that the epigenetic mutation is preserved during reprogramming in the case of hiPSCs and during differentiation. The phenotypes of neural progenitor cells differentiated from these cells vary among reports; some have provided evidence that neurogenesis is aberrant,<sup>316</sup> whereas others have reported that neurogenesis is unaffected.<sup>312,327</sup> As discussed above, FXS-affected individuals have grossly normal brain formation, so neurogenesis does not appear to be affected. Neurons from FXS PSCs do have functional deficits,<sup>312,328,329</sup> but it is still unclear how these phenotypes link to disease characteristics. FXS-affected individuals are hypothesized to have synaptic and plasticity defects, as has been demonstrated in FXS mouse models, but assessment of synaptic function and plasticity in human FXS neurons has not been carried out primarily because of the limitations of the system, in which the human neurons in culture are immature. These discrepancies and limitations suggest that phenotypic modeling of FXS disease phenotypes with hiPSCs remains challenging. One way to overcome the immaturity of hPSC neurons in culture is to transplant FMRP-deficient human NPCs into immune-deficient mouse brains to study the development, maturation, and functions of human neurons without functional FMRP. As discussed below, these chimeras could enable the detection of synaptic differences in human FXS neurons.<sup>330</sup>

On the other hand, FXS hPSCs do provide a unique and much-needed model for studying *FMR1* inactivation and reactivation. Because the FMRP coding sequence in most FXS-affected individuals is intact, efforts have been made to reactivate the silenced *FMR1*.<sup>331</sup> Early attempts have involved FXS lymphocytes, which have limited expansion ability and non-neural lineage. With the development of gene editing, we created a FXS reporter line by inserting nanoluciferase into the 3' region of endogenous *FMR1* of FXS iPSCs.<sup>312,332</sup> The FXS reporter hPSC-derived neural cells are suitable for large-scale drug or genetic screening and will potentially lead to insight into the mechanisms of *FMR1* silencing and identify ways to reactivate the gene.

Furthermore, a number of ongoing projects in various laboratories are using hPSCs to study FMRP targets and validate molecular mechanisms of FMRP regulation of neuronal development. FMRP has been shown to target a large number of mRNAs in the mouse brain.<sup>333–335</sup> However, few of these targets have been validated in humans, and the identity of FMRP-bound mRNAs in humans remain unknown. FMRP targets most likely differ between humans and rodents, leading to different phenotypes when the protein expression is lost. As described above, an example is the dysregulation of *NOS1* in developing human FXS brains, but not in FMRP-deficient mice.<sup>95</sup>

Neuronal functions, specifically synaptic activities, have not been well studied because of the immaturity of hPSC-

derived neurons. Yet, analysis of this functional readout is critical for understanding underlying cortical dysfunction in FXS and other neurodevelopmental disorders. For example, about one-third of FXS-affected individuals exhibit cortical hyperexcitability.<sup>336</sup> If such hyperexcitability can be modeled in FXS iPSC-differentiated neurons, it will provide an excellent platform for understanding the molecular mechanism of the presence and absence of hyperexcitability and provide an opportunity to prescreen individuals for clinical trials. Therefore, hPSCs are essential for validating the molecular, cellular, and functional FXS deficits that we have known for years on the basis of animal studies. More importantly, hPSCs will help us to discover the hidden differences and molecular targets that we have failed to discover with animal models.

**Down Syndrome.** DS hESCs<sup>337–340</sup> and hiPSCs<sup>320–324</sup> have been established. The development of neurons has been primarily studied with iPSC-derived neurons, and these studies have revealed a common phenotype of oxidative stress and mitochondrial dysfunction that are consistent with many cell types from DS-affected individuals.<sup>320,323,324,341</sup> These results validate reports that neurons and progenitor cells derived from DS post-mortem fetal tissue have increased susceptibility to oxidative stress.<sup>286,342,343</sup> Recent bioinformatics analyses of human and mouse DS fetal-brain transcriptomes have indicated that specific common pathway abnormalities leading to oxidative stress, neuroinflammation, apoptosis, and cell-cycle delays are present in both as early as mid-gestation.<sup>344</sup>

In addition, studies using DS iPSCs have validated initial neurodevelopmental deficits, including those in neurogenesis and synaptogenesis, found in DS-affected individuals.<sup>323,341,345–347</sup> But, the inability thus far to analyze neuronal function and plasticity in human neurons with trisomy 21 has hindered the validation of hypotheses of cortical dysfunction described in mouse models. The use of DS hPSCs, especially from those DS-affected individuals with different genetic backgrounds and/or well-documented clinical history, will significantly help us to understand the genes that contribute to DS symptoms and identify targeted therapies for DS.

Altogether, the use of hPSCs is helping us to discover the hidden differences and human-specific molecular targets that we have failed to discover with animal models. Yet, there exist several challenges that currently constrain our ability to gain critical knowledge about foundational mechanisms of neurodevelopmental disorders.

## Challenges and Perspectives

### Clinical Variability

Despite their incredible utility, hPSC studies encounter challenges that significantly impede research progress in the field. Variability in disease characteristics can introduce variability in modeling of disease-related phenotypes in cells. Both FXS and DS have highly variable clinical presentations.<sup>132,158</sup> The considerable clinical variability could

complicate the investigation of underlying mechanisms that lead to intellectual disability, the identification of therapeutic targets, and the application of therapeutic strategies. For example, recent work has indicated that variability in behavior and cognition in FXS could be linked to variability in electrical activity in the brain of FXS-affected individuals, as measured by EEG.<sup>223,336</sup> One solution is the establishment of high-quality iPSCs from individuals with well-characterized and stratified clinical features. Stratification of affected individuals and cells could enable more effective linkage of disease characteristics with cellular phenotypes and better design of clinical trials.

### **Genomic Variability**

Unlike model organisms that tend to be inbred, genetic variation between hPSCs is a challenge that largely results from inherent genomic variability among human beings. Simply increasing the sample size does not readily compensate for this challenge because of the enormous time and cost associated with research involving hPSCs. To make disease modeling using hiPSCs even more difficult, cellular and molecular changes in human cells are often not as robust as those seen in transgenic animals, thus amplifying the effects of variability. Challenges in distinguishing biologically relevant changes leading to complex traits from those resulting from variability between hPSCs (as a result of differences in the contributing individuals' total genomic makeups) can be addressed with isogenic "control" cells. For monogenic traits, isogenic controls can now be generated through CRISPR-Cas9 gene-editing technology, which enables facile gene editing in hPSCs. Isogenic controls can be generated through correction of the gene mutation in disease cells or introduction of the gene mutation in control cells. Use of isogenic controls enables validation of disease-related phenotypes such as FXS.<sup>348</sup> Given that the phenotypic effects of some disorders, including trisomy 21, involve multiple genes, isogenic iPSCs cannot be developed for these conditions through current gene-editing technology. A strategy for silencing the extra chromosome has been developed but cannot yet be easily applied on a large scale.<sup>349</sup> An alternative method for teasing apart the biologically relevant changes attributable to a trisomic imbalance is to derive PSCs from people with mosaicism for trisomy 21 (mDs).<sup>324</sup>

### **Pure and Enriched Populations of Cells**

Pure populations of specific neural cell types can be derived from hiPSCs. These enriched, homogeneous cultures are particularly useful for the rapid screening of pharmaceutical compounds to accelerate drug discovery and advance treatments. They are also potentially useful for defining cell-intrinsic consequences of a given mutation. However, they do not recapitulate the makeup of the human brain and so might be less useful in mechanistic studies, especially those that rely on cell-cell interactions. For example, only about 20% of neurons in the cortex

are GABAergic and 80% are glutamatergic according to analysis of rodents. This proportion could be different in humans. Yet, many hPSC-derived cultures utilize pure cultures of each subtype and do not always include the critical presence of glia. Advancement in tissue engineering could allow for obtaining pure population of cells grown in the presence of tissue-like cues.

### **Neuronal Maturation**

A serious limitation of hPSC-derived neuron cultures for modeling both normal human brain development and function and maldevelopment and dysfunction is the fact that our current culture systems model immature, fetal neurons. Although these cells provide information about early developmental processes, they are less valuable for investigating neuronal function and plasticity, which is the basis of intellectual disability. This major limitation is being addressed in culture through the use of three-dimensional cultures, mixed neuron and astrocyte cultures, and the development of new media.<sup>350–352</sup> Chimeras, in which hPSC-derived neurons are implanted into the mouse central nervous system, address the challenge by providing an environment that stimulates the maturation of human neurons.

### **Self-Organization of Neural Tissue**

Some of the limitations of two-dimensional culture of hPSCs for modeling the development of organs, including the nervous system, have been addressed with self-organizing three-dimensional cultures, commonly termed organoids or spheroids. As was initially shown in the nervous system by Sasai's group, a simple cell-culture system can undergo self-organization to generate a complex structure without external cues.<sup>353</sup> Within the last 5 years, several groups have developed these methods to model the mixed cell types and increased complexity of the human cortex by beginning with the initial formation of dorsal forebrain from hPSCs.<sup>354–358</sup> Recent reviews, over 70 in the last 4 years, have praised the use of organoids for modeling some aspects of human brain development but have also elucidated considerable limitations of the technology.<sup>359–361</sup> Organoids do appear to mimic two critical aspects of human cortical development: timing and the presence of the oSVZ, which enables cortical expansion in primates.<sup>356,362,363</sup> Yet, the heterogeneity that is inherent in self-organization presents a considerable challenge for identifying significant, reproducible, and disease-relevant phenotypes in these systems. For example, the assembly of individual organoids under seemingly identical conditions results in variation between organoids.<sup>359,364</sup> Further, the maturation of neural cells within organoids is still limited because it is in two-dimensional culture, and neurons are similar to fetal rather than adult neurons, thus limiting the assessment of function. Finally, the diverse cell types that contribute to the structure and function of the brain, including astrocytes, vascular cells, and microglia, are only beginning to be

included in organoid models.<sup>330,365–368</sup> Despite these limitations, the ability to model human-specific early development advocates for the use of organoid modeling in neurodevelopmental disorders where there is an expectation that early events in cortical development are affected, such as DS. It is likely that as organoid technology matures, so will the ability to use these cultures to model aspects of human cortical development beyond early developmental processes.

### Chimeras

Implantation of PSC-derived neural progenitors into the developing or adult mouse brain enables the analysis of human cells in an *in vivo* setting, where complex external cues are present.<sup>369–372</sup> This strategy of “humanized rodents” shows that human neurons can influence and be influenced by the mouse neural tissue. Recently, Mansour et al. demonstrated that hPSC-differentiated forebrain organoids can survive in rodent brains and form connections with rodent neurons.<sup>330</sup> In this paradigm, human cells can mature and form connections in a manner that is not observed in culture. Combining transplantation with methods such as optogenetics (which uses light-sensitive channels and pumps) or chemogenetics (which uses chemically engineered receptors and exogenous molecules specific to those receptors) to manipulate the activity of neurons enables control over the transplanted human neurons.<sup>373–377</sup> These technical developments could lead to the ability to manipulate and analyze complex functions of human neurons, such as plasticity, in an *in vivo* setting and could provide better understanding of cognitive function and intellectual disability. Further, in an effort to move potential therapeutic strategies that target human-specific aspects of cells from *in vitro* proof of principle to *in vivo* testing, human cells can be transplanted into the mouse brain and mice can be treated with potential therapeutics. The current focus on reactivation of *FMR1*, for example, cannot be carried out in mouse knockout models and necessitates chimeras for testing reactivation strategies *in vivo*. It is therefore likely that chimeras will better inform human clinical trials.

### Looking Forward

Fundamental differences in the development and function of the neocortex between humans and the animal models outlined above include expansion of the neocortex, neuronal subtype complexity, and human-specific aspects of gene expression and regulation. These critical differences limit the ability of animal models to recapitulate human brain development and, more importantly, to identify underlying cellular and molecular mechanisms of disorders that lead to intellectual disability. The failure of animal-study-based clinical trials for intellectual disorders further underscores the limitations of animal-model-focused human disease research. Human stem cell models will better inform clinical trials in neurological disorders generally by identifying disease-associated human neural

cell phenotypes that have not been identified in mouse models. For example, hPSC models of amyotrophic lateral sclerosis exhibit phenotypes that are more consistent with affected individuals than several mouse models.<sup>378</sup>

Human-stem-cell-based models conceptually provide a valid alternative to rodent models of human brain development. It is important to remember that the use of human stem cells to model neurodevelopmental disorders must be achieved through their ability to recapitulate phenotypes that are found in affected individuals, including molecular, morphological, and genetic deficits. The phenotypes that are currently modeled in human-stem-cell-derived neurons are limited to those that are developmentally early, such as neural progenitor proliferation and differentiation. We anticipate that continual development of biotechnology and innovative bioengineering methods will allow for long-term and developmentally appropriate differentiation of neurons and other brain cell types to mimic neural circuit formation and human brain development in three dimensions. In addition, emerging imaging technology (e.g., fluorescent, optical, and voltage imaging) with high time and space resolution, as well as live imaging, will be applied to *in vitro* hPSC-derived human models. Furthermore, high-sensitivity and single-cell genetics, as well as sophisticated bioinformatic network analysis and computation models, will allow us to understand the dynamic changes in gene expression in different cell types during human neuronal development. These exciting new methods will allow us to analyze the phenotypes that manifest in human developmental disorders related to neuron maturation, functional integration, and plasticity. Realizing the need for human models is the first step toward this future.

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