# Modeling neurodegenerative diseases and neurodevelopmental disorders with reprogrammed cells

**Summary:** Induced pluripotent stem cells and reprogrammed cells offer the opportunity to generate disease-relevant human cells from readily-available patient cells for studying disease and identifying therapeutics.

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The British statistician George E.P. Box reflected, “Essentially, all models are wrong, but some are useful.” Models represent a simplification of reality that enables analysis of complex systems. The underlying question remains, “What aspects of reality must be included to produce a useful model?”

Modeling of neurological disease has been impeded by the inaccessibility of disease-relevant human neural cells from patients. Even if accessible, the value of these primarily post-mitotic cells would be limited by the inability to expand the cells in culture. While post-mortem samples provide snapshots of the terminal points in the disease course, the ravages of degeneration often obscure molecular cause and effect. Further, post-mortem samples necessarily preclude experimentation by which to obtain mechanistic insight.

Genetically generated animal models of neurological disease have shed light on pathogenic mechanisms [1-4]. However, genetic and phenotypic differences between human diseases and rodent models persist. The tenuous correlation between human neuropathology and animal models of neurological disease may frustrate identification of the precise molecular and genetic mechanisms that render unique neural populations vulnerable to degeneration.

As an alternative to post-mortem samples and animal models, new technology in cellular reprogramming provides an exciting new avenue for investigating the mechanisms and causes of disease. Human pluripotent stem cells are characterized by the ability to self-renew and differentiate into an array of tissues, providing a readily-accessible source of rare, difficult-to-isolate stem cell types. As screening tools, cells differentiated from pluripotent precursors may serve to identify therapeutics that reduce the severity of neurological diseases.

In 2007, Yamanaka, Thompson, Daley, and colleagues demonstrated that the forced expression of four transcription factors expressed in embryonic stem cells (*KLF4*, *SOX2*, *OCT4*, and *C-MYC* or *LIN28*) can convert fibroblasts into human induced pluripotent stem cells (iPSCs). [5, 6]. iPSCs possess the two key functional properties of embryonic stem cells; the ability to self-renew and the potential to generate all of the cell types in the body [7, 8]. The gene expression and epigenetic profiles of iPSCs are highly similar to and in some cases, indistinguishable from embryonic stem cells, indicating that the conversion of somatic cells to the embryonic stem cell state by defined factors is a complete reprogramming process. Subsequently, iPSCs have been generated from a variety of adult cell sources, including blood cells [9], hair follicle keratinocytes [10], and neural stem cells [11] and by alternative, non-integrating methods such as transfection of episomal plasmids, mRNAs, and small molecules treatment [12-15]. Directed differentiation of iPSCs, in which externally applied morphogens mimic embryonic development and drive stem cells into specific somatic cell types, allows the generation of patient-specific cells of a variety of cell lineages [16].

Recent studies have shown that transcription factor-mediated reprogramming can be adjusted to convert skin fibroblasts directly into many somatic cell types including neurons, neural precursors, cardiomyocytes, and hematopoietic cells [17-20], skipping the generation of iPSCs. By expanding the range of genetic backgrounds to include any fibroblast donor, iPSCs and lineage reprogrammed cells fundamentally alter the possibilities for modeling neurological diseases at the cellular level.

For neurodegenerative disease and neurodevelopmental disorders, a constellation of factors (e.g. genetic background, environmental exposure, history of infection) influence the onset and course of neurological disease. The ability of patient-specific iPSC-derived and reprogrammed cells to exhibit the consequences of these factors is just beginning to be evaluated. Reassuringly, in proof-of-principle studies, iPSCs and reprogrammed cells recapitulate disease phenotypes for relevant patient-derived

cells. iPSC models of ALS, Huntington's, Parkinson's, and Alzheimer's diseases demonstrate changes in disease-related protein processing [21-30]. Neurons induced directly from the fibroblasts of patients with familial Alzheimer's disease also display altered processing of amyloid precursor protein [31]. RNA foci that are found in the brain tissue of ALS patients with a $G_4C_2$ repeat expansion in *C9ORF72* accumulate similarly in iPSC-derived neurons from these patients [32, 33]. *In vitro* experiments show that these foci possess cytotoxic activity that can be ameliorated by antisense knockdown [32, 33]. Down syndrome-derived cells display increased DYRK1A expression and reduced neuron formation, which is reversed upon silencing of the tertiary copy of chromosome 21 [34-36]. Thus, patient-derived iPSCs and reprogrammed cells are able to capture relevant disease phenotypes, suggesting the potential to use these cells as a tool to study neurological diseases. While these reports are promising, there remain many questions to answer regarding the construction of disease models.

In the following chapter we will discuss the methods for generating cells for particular neural subtypes for disease modeling as well as case studies from these models. We will conclude by presenting open questions and challenges for the field.

## Generating a model of neurological diseases with iPSCs and reprogrammed cells

Accurately modeling neurological disorders with iPSCs and reprogrammed cells relies on robust and reliable methods for efficiently differentiating the distinct neural subpopulations from stem or somatic cells. Studies of inductive pathways that initiate neurogenesis in developing chick and mouse embryos have guided protocols for defined factor differentiation of iPSCs [37]. Once iPSC cell lines are generated, extracellular cues direct cells toward various post-mitotic fates. Alternatively, direct conversion protocols introduce genetic factors intracellularly in order to directly convert somatic cells into neural lineages. Additionally, in three-dimensional culture, iPSCs are able to form cerebral, retinal, and inner ear organoids that possess many of the cell types and structural traits found in their natural organ counterparts [38-43].

### Description of processes for differentiation and lineage conversion into various neural types

*Directed differentiation of iPSCs*

Protocols to differentiate iPSCs into specific subpopulations of neural cells have been guided by studies of developmental morphogen patterning. In the developing embryo, neuralization is initiated by a combination of FGF signaling and BMP and Wnt inhibition in the neural tube to establish neural progenitors (Figure 1A). BMP is secreted from the dorsal ectoderm covering the spinal cord [44]. Rostral localization of BMP/Wnt inhibition induces forebrain development, while caudal localization of retinoic acid (RA) and Wnt initiates spinal cord formation. FGF8 intervenes along the rostral-caudal axis to establish the mid and hindbrain regions. Emanating from the notochord, a sonic hedgehog (Shh) gradient induces ventral fates including spinal motor neurons along the length of the spinal column (Figure 1B). During development, opposing RA and FGF gradients established along the spinal column interact with Hox genes to induce segmentation and confer anterior/posterior identities to spinal motor neurons.

Neuralization of iPSCs begins with the addition of small molecule inhibitors of TGF-β and BMP signaling [45]. TGF-β inhibition prevents the self-renewal of iPSCs and suppresses mesodermal differentiation while BMP inhibition prevents differentiation into trophoblast and endodermal lineages [45]. Following neuroectodermal induction, regional specific inductive signals are added to direct neural types specific to forebrain, mid/hindbrain, or spinal cord [46-48]. Alternatively, the addition of

FGF and EGF can expand and mature the neural progenitor cell population to allow differentiation into the glial lineage [49].

Forebrain development may be the default developmental trajectory. In the absence of inductive signals, pluripotent cells primarily generate forebrain progenitors and cortical pyramidal neurons [50]. Differentiation protocols utilize inhibition of BMP and Wnt to generate cell types in the forebrain including cortical neurons and basal forebrain cholinergic neurons which are affected in dementia and Alzheimer's disease, respectively.

In the midbrain, dopaminergic (DA) neurons such those affected in Parkinson's disease have been derived iPSCs treated with Shh and FGF8 following neuralization form dopamine-producing neurons [24, 51-54]. Timing of the addition of FGF8 may modulate rostral-caudal identity. While early treatment results in midbrain DA neurons, later treatment may direct cells toward forebrain DA neurons [53].

The first directed differentiation protocol was demonstrated for spinal motor neurons (MNs). Embryonic stem cells treated with RA and Shh yielded MNs with a unique pattern of expression including the presence of Hb9 a marker specific for spinal motor neuron identity [55]. RA caudalizes progenitor cells following neuralization, while Shh ventralizes cells, converting them to spinal motor neurons. Spinal motor neurons mature into one of three columnar fates: median (MMC), hypaxial, (HMC), or lateral (LMC) motor column. MMC neurons express Lhx3 and innervate axial muscles, LMC neurons that innervate the limb muscles, expressing FoxP1, and the HMC neurons innervate the hypaxial muscle and do not express either marker [56]. Treatment with RA generates MNs with cervical MMC identify. In spinal muscular atrophy (SMA), MMC as well as HMC MNs are primarily affected. Alternatively, RA-free differentiation generates LMC MNs, which are the first MNs affected in Amyotrophic lateral sclerosis (ALS). A small molecule protocol was recently demonstrated for differentiating LMC MNs from ESCs [48]. Finally, refinement of motor neuron identity to a more posterior type may be achieved by inhibition of Activin/Nodal signaling. Given the cell type specificity of neurodegenerative diseases, generating particular subpopulations of cells may be important for accurate disease-modeling and effective drug screening.

From the glial lineage, oligodendrocytes and astrocytes have been differentiated from ESCs and iPSCs. Directed differentiation methods for astrocytes were undefined for years while protocols for other lineages were more rapidly developed. Initially, several weeks of neural culturing was required to obtain cells with detectable expression of the astrocyte markers GFAP and S100β [57]. More rapid and robust directed differentiation protocols have been recently demonstrated. Astrocytes have been obtained from PSCs cultured with astrocyte conditioned media [27] and RA/FGF8 or Shh followed by EGF and FGF2 [49]. Differentiated cells exhibit similar patterns of gene expression as primary astrocytes and are able to uptake glutamate, promote synaptogenesis, and form connections with blood vessels when transplanted into the mouse brain, demonstrating key astrocytic functions [49, 58]. Additionally, morphogens that provide dorsal-ventral and anterior-posterior specification to neurons (SHH, RA, FGF8, BMP, Wnts) were demonstrated to specify similar regional identities to astrocytes.

hESCs stimulated with a combination of glial restriction media (GRM), EGF, and FGF as well neurotrophic factors converted into oligodendrocyte-like cells capable of mylenating axons when transplanted into the *shiverer* mouse model of dysmyelination [57]. Oligodendrocytes are vulnerable cell type in multiple sclerosis which as yet does not have human cellular models from either iPSC or reprogrammed cells. Alternatively, oligodendrocytes may be used a cell-based therapy to repair spinal cord injury (SCI). Despite preclinical trials of treatment of oligodendrocytes precursor cells (OPCs)

showing improved outcomes in adult rat models of SCI [59], the first hES cell-based therapy for OPC administration was abandoned in phase I, showing little indication of potential efficacy in the safety study.

Direct lineage conversion provides a progenitor-free protocol for generating various neural types. Direct conversion relies on the overexpression of transcription factors to internally drive differentiation programs. Ostensibly, the forced expression of these factors replaces growth factors utilized in iPSC differentiation by directly activating downstream signaling pathways and genes. Additionally, either purified external cues or other cell types normally present *in vivo* are sometimes added to further guide the developmental trajectory and maturation of various cell types. Genetic neuralization, through introduction of *BRN2*, *ASCL1*, and *MYT1L* (BAM) to fibroblasts generates induced neurons (iN) [60]. The addition of *NEUROD1* further enhances this conversion [60]. From iNs, a secondary layer of transcription factors guide cells to particular neurons. Spinal motor neurons have been generated by adding *ISL1*, *LHX3*, *NGN2*, and *HB9* to the BAM factors [18]. Addition of *LMX1A* and *FOXA2* to the BAM cocktail results in dopaminergic neurons [61]. Oligodendrocyte precursor cells (OPCs) follow a separate glial lineage that is independent of BAM-mediated neuralization. Induced OPCs can be made by overexpressing either *SOX10*, *OLIG2* and *ZFP536* [62] or *OLIG1*, *OLIG2*, *NKX2.2*, *NKX6.2*, *SOX10*, *ST18*, *GM98*, and *MYT1* [63]. As in iPSC differentiation, inductive signals are added during direct conversion protocols to further guide cells to mature fates.

While these approaches offer unprecedented access to disease-affected human cell types, the lack of translation from some cellular disease models to clinical trials suggests that whole tissues may be needed to capture non-cell autonomous effects and accurately screen for therapies with high translational potential. Recent advances in heterogeneous cell culture and the generation of mini-organs may enhance the accuracy of modeling neurological disorders.

**Constructing co-cultures and organoid models**

To model non-cell autonomous effects and simulate whole tissues, recent studies have co-cultured multiple differentiated neural cell types or generated mini-organs called “organoids” by directed differentiation of pluripotent cells in three-dimensional matrices.

Co-cultures of mixed neural cell types, particularly spinal motor neurons and astrocytes, have highlighted unique mutation-specific cell autonomous and non-cell autonomous effects that may contribute to neurodegeneration in ALS. In ALS, mouse embryonic stem cell derived SOD1 mutant motor neurons showed reduced neuron death when cultured with wildtype versus familial or sporadic astrocytes, suggesting a role for non-cell autonomous effects in ALS neurodegeneration. Further, astrocytes derived from mESCs with an SOD1 mutation accelerate motor neuron degeneration *in vitro*, implicating glial toxicity in neurodegeneration [64]. In an interesting twist, astrocytes obtained from iPSCs derived from an ALS patient bearing a TDP-43 mutation did not reduce neuron survival when co-cultured. However, TDP-43 mutant astrocytes did express increased and mislocalized TDP-43 protein, resulting in reduced astrocyte survival. While the results from co-cultures of neurons with *SOD1*, *C9ORF72*, and *TDP-43* mutant astrocytes implicate a common theme of glial dysfunction, the distinct mutations and glial phenotypes argue for the potential for wide variance in the therapeutic response to drugs selected to distinct genetic forms of the disease.

Beyond two dimensional co-cultures, recent studies demonstrate that immersing differentiating pluripotent stem cell aggregates in a three-dimensional extracellular matrix extract induces the development and self-organization of organ-like structures *in vitro* [38-43]. Cerebral, retinal, and inner ear organoids have been generated, displaying a high level of inherent self-organization [38-43].

When mouse ESCs in serum-free culture of embryoid body-like aggregates (SFEBq) cultures were differentiated in Matrigel-supplemented media, retinal cells spontaneously emerged that organized into an invaginated structure resembling an optic cup [41, 42]. This same approach is effective at generating human optic cup organoids [42].

In the inner ear, mechanosensitive hair cells and sensory neurons translate movement, gravity and sound into neural signals which enable balance and hearing. In dish culture, attempts to generate inner ear cells from pluripotent cells resulted in inefficient or incomplete differentiation. Alternatively, culturing pluripotent cells in three dimensional matrices may more accurately recapitulate cellular interactions in the context of development. In SFEBq culture with matrigel, both mechanosensitive hair cells and sensory neurons formed [43]. Additionally, synapses were observed between the hair cells and sensory neurons which formed structures reminiscent of immature vestibular end organs.

Recently, several reports have shown how pluripotent cells can cultured as organoids to study human brain development [40]. Human iPSCs grown in suspension with rostral neuralizing factors generated organoids with a pattern of expression similar to that observed in the embryonic telecephalon. Additionally, cells mimicked *in vivo* development, organizing into layered structures containing a variety of layer-specific cortical neurons, polarized radial glia, and intermediate progenitors [40].

Culturing cerebral organoids in bioreactors can further their development to the point where they recapitulate aspects of cerebral cortical development that are specific to humans, including the formation of the inner fibre layer and outer subventricular zone [38]. Knoblich and colleagues recently demonstrated that human cerebral organoids that achieve this advanced developmental state can provide a window into differential developmental patterns between healthy and diseased states such as microcephaly [38].

Microcephaly is characterized by reduced brain size andresults from a variety of different mutations. Mouse models with similar mutations have failed recapitulate the significantly reduced brain size, perhaps because mice may lack the type of neural progenitor cells most affected by the causal mutations. Three dimensional culture from iPSCs with CDK5RAP2-dependent pathogenesis of microcephaly showed human-specific brain developmental pattern and morphology not observed in mouse cells [38]. Patient cells generated embryoid bodies of reduced size with more neurons and fewer neural progenitors indicating premature non-proliferative neuronal differentiation. Premature differentiation of progenitors may lead to microcephaly by depletion of progenitors, which could limit the total number of neurons produced over the course of development.

While organoids represent an exciting advance in studying development and disease, technical challenges limit the full potential of organoids for disease modeling and drug screening. Diffusion of oxygen limits the size of organoid cultures which lack necessary vasculature and blood supply to oxygenate and supply nutrients to the inner regions of tissues. Development of systems for delivery of oxygen and other nutrients will expand the potential to model diseases with organoids by extending their size, viability, and homogeneity.

**Pros and cons of iPSCs and reprogrammed cells**

Both iPSC and direct conversion protocols for generating specific neural lineages are powerful tools for disease modeling. Currently, published reports indicate that either approach can generate

specific somatic cell types that genuinely mimic their in vivo counterparts. Yet each comes with distinct advantages, considerations, and limitations.

**Advantages of iPSCs**

One advantage of iPSCs is that they can be derived from a variety of somatic tissues, including blood, fibroblasts and hair follicle keratinocytes [5, 9, 10]. This could be an important consideration given that blood is easy to obtain in a routine clinical setting.

The second major advantage of is that iPSCs are capable of limitless self-renewal and therefore provide a robustly expandable cell line for disease studies. This can be an important advantage for performing experiments that require large numbers of neural cells, such as genome-wide binding analyses and biochemical purification studies. This also allows the creation of create patient-specific isogenic control lines, which is important because every patient genome has hundreds of polymorphic loci that differ from the reference genomic sequence. Therefore, it is difficult to know if a phenotype observed in a patient culture is actually induced by the disease-causing mutation. Because iPSCs are capable of limitless self-renewal, one can use genome editing approaches such as CRISPR/Cas9 to specifically restore the disease-causing mutation to the non-pathogenic sequence and verify that this rescues disease phenotypes. This also allows the production of transgenic reporter lines which can be valuable tools for purifying iPSC-derived somatic cells. Several neurologic disorders such as autism spectrum disorders and schizophrenia are believed to be caused by developmental defects. When the precise stage of development in which the defects occur is unclear, it can be advantageous to use iPSCs to mimic full development in order to identify disease processes. In particular, phenotypes that affect brain size or architecture, such as microcephaly and agenesis of the corpus callosum, may only be adequately modeled in vitro using iPSC-derived 3D cultures.

**Disadvantages of iPSCs**

One disadvantage of iPSCs is that they are time-consuming, laborious, and expensive to generate. Establishing a typical iPSC line requires several months of daily manipulations. Therefore, it can be difficult to derive iPSC lines from more than 3-4 patients and controls at a time.

In addition, recent studies have shown that different iPSC lines from the same individual have different propensities for differentiation [8, 65] . Variability has also been observed in the somatic cells derived from different iPSC lines of the same patient [29, 30]. Although the advent of non-integrating approaches for producing iPSCs alleviates concerns over embedded transgenes inducing this variability, there may be unpredictable epigenetic heterogeneity amongst different iPSC clones that can propagate phenotypic variability.

A final concern regarding iPSCs is that long-term culture can induce mutations including chromosomal translocations. This could confound the interpretation of disease phenotypes or have negative implications for cell transplantation.

**Advantages of direct lineage conversion**

One major advantage of direct lineage conversion is that it does not require the generation of iPSCs. Because of this, it provides the fastest route to the generation of patient-specific somatic cells: for example, the process of conversion from fibroblast to motor neuron takes less than 30 days [18]. In addition to being faster, direct lineage conversion does not require laborious cell transfer steps.

Therefore, it is much easier to perform direct reprogramming for many patients in parallel, allowing rapid identification of patient cultures that exhibit disease phenotypes. Moreover, it allows for the comparison of many patient and control cultures for a given disease form, which minimizes the probability that an observed phenotype is due to genetic peculiarities of a small number of samples and not relevant to the disease. This could also enable the generation of patient-specific cultures from a diverse panel of individuals which could facilitate studies of diseases with complex etiologies including Alzheimer's disease, ALS and Parkinson's [66].

Another advantage is that it requires fewer cell divisions than derivation of patient-specific somatic cells through iPSCs. Fewer passages may help to preserve genomic integrity and may be especially important when studying repeat expansion mutations, such as those that cause Huntington's disease, ataxia, frontotemporal dementia and ALS, because they are prone to expansion and contraction.

The production of direct lineage-converted cells can be more reproducible than the directed differentiation of iPSCs because they typically do not rely on the step-wise integration of complex signaling cascades mediated by extrinsic factors (personal observation). Also, in contrast to iPSCs, direct lineage-converted cells are not clonal and are instead derived from a population of cells which eliminates concerns of clonal variation.

### Disadvantages of direct lineage conversion

A disadvantage of direct lineage conversion is that the inefficiency of conversion often yields insufficient numbers of cells for large-scale biochemical studies. However, direct conversion technologies are still in the early phases of development, and it is likely that chemical or environmental manipulation will allow more efficient reprogramming in the future.

Another disadvantage of this approach is that it has not been developed for starting cells other than fibroblasts except for one case in which hepatocytes were reprogrammed to neurons [67]. Furthermore, non-integrating approaches have not been demonstrated to be capable of replacing transgenic methods for inducing direct lineage conversion. However, it is likely that these problems will be overcome in the near future.

Our own data for in vitro-derived motor neurons indicate that, while both iPSC-derived and directly converted motor neurons are molecularly and functionally similar to primary motor neurons, each has their own unique characteristics that could influence their ability to model and treat disease. Therefore, a combination of both approaches would be optimal for translational studies.

## Case studies of disease modeling with hiPSCs and reprogrammed cells

### Neurodevelopmental disorders

| Disease | Cell | Method | Patient genotype | Phenotype | Notable | Reference |
|---|---|---|---|---|---|---|
| RTT | | | | | | |
| | Neuron | iPSC | Missense mutation to MECP2 | | | Hotta et al., 2009 |
| | | iPSC | 4 lines with distinct MECP2 mutations | Reduced VLGUT1 puncta | Reduced VLGUT1 puncta indicate MECP2 may regulate glutamatergic | Marchetto et al., 2010 |
| | | iPSC | | Cells with mutant allele showed smaller soma size | X-linked silencing of mutant allele allowed for isogenic comparison | Cheung et al., 2011 |
| FXS | | | | | | |
| | Neuron | iPSC | FMR1 CGG repeat expansion | FMR1 expression silenced | Differential FMR1 expression in ESCs and iPSCS | Urbach et al., 2010 |
| | | iPSC | FMR1 CGG repeat expansion | FMR1 expression silenced | Reversed epigenetic silencing | Bar-Nur et al., 2012 |
| | | iPSC | Various FMR1 CGG repeat expansions | Repeat length correlates with differential levels of epigenetic modification to FMR1 | FMR1 repeat length may change during reprogramming, vary by clone | Sheridan et al., 2011 |
| DS | | | | | | |
| | Neuron | iPSC | Trisomy 21 | | | Mou, et al. 2012 |
| | | | Trisomy 21 | DS-derived cells formed fewer NPCs and neurons. | | Lu, et al. 2013 |
| | | | Trisomy 21 | Fewer neurons, increased DYRK1A expression, reduced, increased apoptosis, increased astroglial and oligodendroglial lineages | Isogenic control, monozygotic twin with discordant trisomy 21 | Hibaoui, et al. 2013 |
| | | | Trisomy 21 | NPC proliferation defect | Isogenic line obtained by silencing of extra chromosome via Xist gene insertion | Jiang et al., 2013 |
| | | | Trisomy 21 | NPC proliferation defect | Isogenic control via drug selection for removal of extra chromosome | Li et al., 2012 |

**Table 1. iPSC and reprogrammed cell models of neurodevelopmental disorders**

## Rett syndrome

Rett syndrome, RTT, is a predominately female neurodevelopmental disorder. Preceded by normal development, RTT manifests in children around 18 months with symptoms including speech regression, ataxia, post natal microcephaly, hand dyspraxia, growth retardation, and autistic-like symptoms [68]. Genetically, RTT is characterized by mutations to MeCP2, methyl CpG binding protein 2, with over 100 different mutations in MeCP2 identified to cause RTT. The location and type of mutation dictates the impact on MeCP2 function and the severity of the disease. Given the spectrum of mutations and disease severity, therapeutics identified from screens may be specific to particular mutations.

With the advent of iPSC technologies, modeling of neurodevelopmental disorders such as RTT became possible with patient-specific cells [69, 70]. Embryoid bodies (EBs) were generated from patients with mutations in MECP2 using Noggin in the absence of FGF2. EBs were further differentiated to neurons by addition of RA. Additionally, neural progenitor cells (NPCs) were obtained from neural rosettes by FGF2 treatment. Patient-derived neurons showed reduced spine formation, fewer synapses, and electrophysiological defects compared to controls. NPCs showed normal proliferation rates suggesting abnormal progenitor proliferation may not contribute to the deceleration of brain growth that is observed in RTT patients.

Patient-derived neural cells provide a platform for discovering new candidate drugs or validating those identified in animal models before translation to clinical trials. Drug intervention with IGF1 administration was identified in a mouse model of RTT. Subsequently, IGF1 was shown to increase glutamatergic synapse formation from RTT patient-derived neurons, suggesting the possibility to reverse the RTT neuronal phenotype [71].

Given the constellation of factors that can influence the development of neurological disorders, isogenic controls are of significant importance to accurately extract information from models of neurological disease. X-linked disorders such as RTT may allow more rapid isolation of isogenic controls. During reprogramming, female human iPS cells maintain their pattern of X-chromosome inactivation in the original somatic cell. Since the starting patient cells are mosaic, iPS cells can inherit

inactivation of either the normal or mutant allele based on the pattern in the somatic cells from which they were derived, allowing a pair of isogenic normal and diseased lines to be generated. X-linked silencing was exploited to study RTT in an MECP2 mutant with exon 3 and 4 deletion. When differentiated to neurons, cells with mutant allele showed smaller soma size [72]. Further characterization of this line may reveal additional mutation-specific differences. It should be noted that erosion of X-inactivation can occur in later passages [73]. Nevertheless, these results highlight the potential to achieve isogenic controls during reprogramming in X-linked disorders.

**Down syndrome**

Down syndrome, a result of trisomy 21, is the most common genetic developmental disorder. Patients with Down syndrome experience reduced neurogenesis and synapse formation resulting in cognitive, learning, and memory deficits as well as early onset Alzheimer's disease [74]. Since mice do not carry chromosome 21, a precise genetic mouse model is not possible. However, mice generated with various aneuploidies recapitulate some of the anatomical, behavioral, and cellular phenotypes observed in Down syndrome patients [75]. Mice bearing a second copy of dual-specificity tyrosine-(Y)-phosphorylation regulated kinase 1A (DYRK1A), a neurodevelopmental gene with altered expression in Down syndrome, demonstrate neurodevelopmental delays and altered synapse formation [75].

A number of different models of Down syndrome have been generated using iPSCs [34-36, 76, 77]. iPSCs derived from amniotic fluid cells were differentiated into neural progenitor cells (NPCs) and neurons. Relative to the healthy control, Down syndrome patient cells formed fewer NPCs and neurons [77]. Similarly, iPS cells were generated from fetal fibroblasts obtained from monozygotic twins discordant for trisomy 21 [34]. Discrepancies of trisomy 21 in monozygotic twins is an extremely rare opportunity to study diseased cells with an isogenic control. Following neuralization with FGF, NPCs were subjected to neuronal differentiation in culture with B-27, BDNF, cAMP, and ascorbic acid. NPCs derived from the twin with Down syndrome (NPC-DS) showed reduced proliferation and increased apoptosis compared to the healthy control. Additionally DS-NPCs produced fewer Nestin-Sox2 double-positive cells following differentiation with an increase in the proportion of cells committed to the astroglial and oligodendroglial lineages. Further, Down syndrome patient cells displayed two-fold higher DYRK1A expression. To rescue the DS-NPC cells, DYRK1A was inhibited or knocked down with an shRNA to reduce its activity. Both inhibition and knockdown restored NPC proliferation, reduced apoptosis, and increased neuronal differentiation. These results highlight that some aspects of the trisomy 21 pathology can be reversed by silencing expression of a single gene.

Due to the pathological consequences of the tertiary copy of chromosome 21, silencing expression from this chromosome may represent an important therapeutic approach and provide isogenic controls for disease modeling. Removal of the extra chromosome is possible via introduction of a gene encoding thymidine kinase (TK) into the Amyloid Precursor Protein (APP) gene located on the long arm of chromosome 21. Cells were cultured in the presence of the pro-drug gancyclovir, which enables the selection of TK-negative cells. TK converts gancyclovir into a toxic product, killing TK-positive cells. Removal of the extra chromosome resulted in an increase cell proliferation compared to the isogenic control [35]. In a similar scheme the *XIST* gene was inserted into one copy of chromosome 21 in iPSCs derived from a Down syndrome patient [36]. *XIST* RNA silences the secondary copy of the X chromosome in females. The *XIST* gene was inserted into *DYRK1A* locus, silencing of expression from that chromosome. As expected, silencing of the extra copy of chromosome 21 reversed proliferation and differentiation defects observed in Down syndrome-derived cells. Silencing or removal of the secondary control provides an isogenic control for studies of the

molecular pathology of trisomy 21. While these approaches allow for the selection of cells *in vitro* that achieve normal levels of expression from chromosome 21, the difficulty of delivering genetic material to every cell *in vivo* would limit the therapeutic potential of these schemes. Alternatively, identification of drugs that silence DYRK1A expression may yield a readily translatable therapeutic for Down syndrome patients.

**Fragile X**

Fragile X is the most common inherited form of mental retardation. Fragile X patients exhibit cognitive deficits that often increase with age. Behavioral symptoms of Fragile X include attention deficit, stereotyped behavior, anxiety, social difficulties and other autistic-like behaviors. Fragile X is caused by a trinucleotide repeat expansion of more than 200 CGG repeats in the 5' UTR of the Fragile X Mental Retardation, *FMR1* gene. Repeat expansion induces silencing of FMR proteins (FMRP)expression in post-embryonic stages of development via hypermethylation of the promoter and CGG region. Neurons are characterized by immature dendritic spines in the cerebral cortex.

A study of Fragile X using iPSCs suggests that in rare cases, these cells may not fully recapitulate embryonic developmental processes due to incomplete epigenetic reprogramming at certain loci. iPSCs and ESCs obtained from a case of Fragile X showed differential expression of FMR1[78]. Unlike in ESCs, FMR1remained silenced in iPSCs with DNA methylation and histone modifications at the promoter region, indicating that FMR1 silencing did not reset during reprogramming. Therefore, because the iPSCs were derived from mature cells, they were not useful for studying the process of *FMR1* silencing *in vitro*. However, they did provide a platform for studying the silenced *FMR1* state. Therefore, comparison of iPSC and ESC models may highlight aspects that would be missed by either one alone.

Reversing FMR1 silencing represents one potential therapeutic target. To determine if FMR1 was reactivatable, 11 iPSC lines from three different patients were treated with 5-azacytidine (5-azaC), a demethylating agent [79]. Treatment with 5-azaC at a concentration approved for cancer treatment restored some FMR1 expression in all three lines tested in both iPSCs and cells differentiated to neurons. FMR1 reactivation persisted in cells following removal of 5-azaC from media. Clones that had undergone FMR1 reactivation showed longer, more complex neuronal processes relative to inactivated clones.

Capturing the genetic background and causative mutations that give rise to neurological disorders makes iPSCs and reprogrammed cells attractive lines for modeling mutation- and patient-specific pathology. However, accurate models rely on genetic stability which may not be guaranteed for repeat expansion disorders. Characterization of several iPSC lines from which two clones were isolated indicate that repeat length could vary among the clones [80]. Additionally, repeat length of *FMR1* in iPSCs varied from the fibroblasts from which they were derived and in several examples, decreasing in number. Further, these studies demonstrated that aberrant neuronal differentiation is directly correlated with epigenetic modification and FMRP expression. Given that methylation increases with increasing repeat length, stability and exact repeat number are important factors to control in modeling Fragile X. Extracting instructive conclusions from neurological models of Fragile X may require confirmation that clones isolated maintain repeat length. Alternatively, variation in repeat length among otherwise isogenic cells may be used to model varying levels of disease severity in a single isogenic model.

## Neurodegenerative disorders

| | Disease | Cell | Method | Patient genotype | Phenotype | Notable | Reference |
|---|---|---|---|---|---|---|---|
| Spinal cord | ALS | | | | | | |
| | | Upper spinal MNs | iPSC | SOD1 mutant | | First motor neurons (MNs) derived from patient fibroblasts with ALS c | Dimos, 2008 |
| | | | iPSC | SOD1 mutants | | Characterized expression and electophysiology of ALS-derived MNs | Boulting, 2011 |
| | | | iPSC | SOD1 mutant | | A small molecule screen in stem-cell-derived motor neurons identifies a kinase inhibitor, kenpaullone, as a candidate therapeutic for ALS | Yang, 2013 |
| | | | iPSC | TDP43 mutant | | Drug screening | Bilican 2012 |
| | | | iPSC | TDP43 mutant | | Drug screening | Egawa, 2012 |
| | | | iPSC | C9ORF72 CAG repeat expansion | C9ORF72 RNA foci formation | | Donnelly, 2013 |
| | | | iPSC | C9ORF72 CAG repeat expansion | C9ORF72 RNA foci formation | | Sareen, 2013 |
| | | | iPSC | TDP43 mutants | | Drug screening identified FDA approved Digoxin as potential threrapuetic | Burkhardt, 2012 |
| | | Astrocytes | iPSC | SOD1 mutant; C9ORF72 CAG repeat expansion | Normal motor neuron surival; mutant astrocytes induce cytotoxicity | | Meyer, 2013 |
| | SMA | | | | | | |
| | | Spinal motor neuron | iPSC | SMN1 deletion | Reduced soma size ; reduced number of motor neurons | Pharmacological rescue of SMN expression | Ebert, 2009 |
| | | | iPSC | SMN1 deletion | Reduced soma size ; reduced number of motor neurons | Apoptosis-mediated motor neuron death; Fas or caspase inhibition blocks cell death | Sareen, 2012 |
| | | | iPCS | SMN1 deletion | Reduced soma size ; reduced number of motor neurons | Non-viral conversion of SMN2 to SMN1-like increased SMN expression, increased surival in SMA mouse model | Corti, 2012 |
| Midbrain/ Hindbrain | PD | | | | | | |
| | | Dopaminergic neurons | iPSC | SNCA triplication | Increased alpha-synuclein | | Devine, 2011 |
| | | | iPSC | LRRK2 mutant | Increased alpha-synuclein, sensitivity to oxidative stress | | Nyugen, 2011 |
| | | | iPSC | SNCA triplication,LRRK2 mutation | Increased gene expression of stress responsive genes and α-synuclein, increased sensitivity to oxidative stress | | Byers, 2012 |
| | | | iPSC | Idiopathic | Increased alpha-synuclein, sensitivity to oxidative stress | Viral-free iPSCs | Soldner, 2009 |
| | | | iPSC | PINK1 mutant | Increased alpha-synuclein, sensitivity to oxidative stress | Mitochondrial Parkin recruitment is impaired | Seibler, 2011 |
| | | | iPSC | PARK mutant | Reduced DA uptake and spotaneous DA release | Parkin controls dopamine utilization in human midbrain dopaminergic neurons derived from induced pluripotent stem cells | H. Jiang, 2011 |
| | | | iPSC | PINK1 mutant; LRRK mutant | Mitochondrial defects | Pharmacological rescue of mitochondrial deficits | Cooper, 2012 |
| | | Cortical neurons | iPSC | A53T mutation in SNCA | Increased oxidative stress | DA neurons validate efficacy of NAB2, a drug that reduces α-synuclein toxicity | Chung, 2013 |
| Forebrain | HD | | | | | | |
| | | Striatal neurons | iPSC | HTT 72 CAG repeats | Increased caspase activity | No survival difference between HD and wt | Zhang, 2010 |
| | | Astrocytes | | HTT 50 and 109 CAG repeats | Astrocyte vaculolization | | Juopperi, 2012 |
| | FXN | | | | | | |
| | | Neuron | iPSC | FXN repeat expansion | Expansion of repeats during reprogramming | | Liu, 2011 |
| | | | iPSC | FXN repeat expansion | Expansion of repeats during reprogramming, culture | Repeat expansions mediated by a mismatch repair enzyme, MSH2 | Ku, 2010 |

**Table 2. iPSC and reprogrammed cell models of neurodegerative diseases.** ALS-Amyotrophic lateral sclerosis; SMA-Spinal muscular atrophy; PD-Parkinson's disease; HD-Huntington's disease; FXN-Friedrich's ataxia.

## Spinal muscular atrophy

SMA is devastating autosomal recessive disease that causes lower spinal motor neurons to degenerate in children, often resulting in death by age two. SMA is caused by a defect in the *Survival of Motor Neuron1* (*SMN1*) gene that reduces SMN expression. While *SMN2* also produces SMN, its splicing pattern yields less full-length protein [81]. SMN is expressed ubiquitously and facilitates splicing through its role in producing small ribonucleoproteins that mediate pre-splicing events. Given its ubiquitous role, it is unclear how low SMN expression selectively induces degeneration of lower spinal motor neurons.

Increasing SMN expression in the motor neurons of SMA patients represents a potential therapy. Fibroblasts obtained from a SMA patient and her mother were converted to iPSCs and subsequently differentiated to spinal motor neurons [82]. No difference was observed between the WT and SMA-derived cells in the number of cells stained with the neuronal marker, TUJ1. However, cells expressing both TUJ1 and the motor neuron marker CHOLINE ACETYLTRANSFERASE (CHAT) were reduced in the SMA-derived cells. Additionally, smaller soma were observed in the SMA neuronal cultures. To rescue the pathological defects from low SMN expression, Tobramycin and Valproic acid, drugs that increase expression of SMN2, were added to the cultured cells, leading to 2-3 fold increase in SMN2 levels. Going forward, it would be important to establish if increasing expression of SMN2 could rescue the morphological and differentiation defects observed in SMA-derived cells.

Neurodegeneration in SMA may be mediated by apopotosis, suggesting that inhibition of apoptotic pathways could limit motor neuron death. Several iPSC lines obtained from two patients with SMA were converted to CHAT-expressing spinal motor neurons and recapitulated the differentiation and morphological defects observed in other iPSC models of SMA (e.g. small soma, fewer motor neurons). Additionally, these cells exhibited Fas ligand-mediated apoptosis and an increase in caspase-3 activation. Inhibition of Fas through a blocking antibody or inhibition of caspase-3 blocked apoptosis [83]. Given the importance of apoptosis in tissue homeostasis and development, therapeutic inhibition of apoptosis to block SMA neurodegeneration would require therapeutic molecules that selectively target spinal motor neurons.

Gene correction of iPSCs provides a test bed for examining disease pathology with sufficiency of gene repair indicating a gene-specific mechanism and a potential route to therapy. Introduction of viral materials represents one challenge in translating such gene therapy into clinical application. A non-viral method to increase SMN expression utilized oligonucleotides to convert SMN2 into an SMN1-like gene by altering splicing [84]. Conversion of *SMN2* to an *SMN1*-like gene increased SMN expression. When cells were transplanted into a mouse model of SMA, cells engrafted and allowed for improved muscle connection, symptom reduction, and increased survival. Non-viral correction of iPSCs may represent one translatable therapeutic for SMN.

**Parkinson's disease**

Parkinson's is progressive neurodegenerative disorder characterized by shaking and an unsteady gate. Motor dysfunction results from a progressive loss of dopaminergic neurons in the substania nigra, a region of the midbrain. Current, therapy with dopamine agonists and levodopa improve early motor symptoms, but may result in severe dyskinesia, involuntary writhing movements. While a number of mutations have been identified (e.g. *LRRK2*, *PARK2*, *PARK7*, *PINK1*, *SNCA*, *SNCAIP*), most cases of Parkinson's are sporadic with unknown etiology. At the molecular level, Parkinson's is characterized by accumulation of inclusions called Lewy bodies, comprised largely of α-synuclein [85]. Multiple copies of the *SNCA* gene which encodes α-SYNUCLEIN, the primary protein component in Lewy bodies, has been identified as a cause of familial PD [86]. While these inclusions

are a hallmark of disease, it remains unclear if the inclusions are cytotoxic or if they represent a protective response to increased expression of α-SYNUCLEIN . Dopaminergic neurons derived from ES cells and obtained from a mouse model of PD indicate that these cells are more sensitive to oxidative stress than control cells [87].

By enabling the study of a range of mutations that contribute to the same disease, iPSCs may elucidate how divergent genes converge on a similar pathology, potentially accelerating the identification of a common mechanism. Neurons derived from a patient with a *SNCA* triplication showed double the expression of α-SYNUCLEIN compared to neurons derived from an unaffected relative [22, 26]. A similar results were shown for when iPSCs generated from a patient bearing a homozygous mutation in *LEUCINE-RICH REPEAT KINASE (LRRK2*), were generated and differentiated into dopaminergic neurons [22, 88]. Both *SNCA*-iPSCs and *LRKK2*-iPSCs recapitulated increased expression of oxidative stress genes and α-SYNUCLEIN, the pathological hallmarks of the disease. Additionally, both cell lines demonstrated increased sensitivity to oxidative stress induced by hydrogen peroxide.

Increased susceptibility to oxidative stress and aberrant α-SYNUCLEIN expression and accumulation are hallmarks of PD seen in several different mutations including the *SNCA* triplication, *LRKK2* mutants, *PARK2* mutants, and *PINK1* mutants. PARKIN is a component of E3 ligase which targets proteins for degradation through the ubiquitin-proteosome system. iPSCs derived from the mutant PARK2 gene which encodes PARKIN were differentiated into DA neurons. PARK2-DA neurons displayed mitochondrial dysfunction, oxidative stress, and α-SYNUCLEIN accumulation as observed in postmortem brain samples [89]. In mutant *PINK1* iPSC-derived neurons, mitochondrial PARKIN recruitment is impaired [90]. Overexpression of wildtype PINK1 restored PARKIN recruitment and normal levels of mitochondria. In another example, iPSCs derived from patients with PARKIN mutations showed aberrant utilization of dopamine (DA) [91]. PD-derived neurons spontaneously released DA while showing only limited DA uptake. These and other disease phenotypes were reversed upon lentiviral overexpression of wildtype PARKIN, suggesting PARKIN plays a critical role in dopamine regulations in DA neurons.

In addition to understanding the mechanisms of PD pathology, PD-iPSCs have recently been utilized to validate a candidate drug identified in yeast screens. In yeast, α-SYNUCLEIN toxicity induces nitrosative stress which amplifies the toxic effect [23]. The small molecule NAB2 was identified to reduce nitrosative stress in yeast and shown to rescue α-SYNUCLEIN toxicity in PD-derived cortical neurons. Interestingly, in yeast, NAB2 was shown to modulate a network of ubiquitin genes. NAB2 and α-SYNUCLEIN appear to inversely affect the ubiquitin network. These results combined with previous models suggest that the spectrum of PD-associate mutations may converge on the ubiquitin pathways responsible for protein degradation. PARKIN's role in protein degradation and dopamine utilization may suggest a mechanism for the selective degradation of DA neurons in PD. Future studies will determine if dopamine neurons from patients with idiopathic PD also exhibit prominent disease phenotypes and if they are affected by the same pathogenic mechanisms.

**Huntington's disease**

Huntington's disease (HD) is an autosomal dominant neurodegenerative genetic disorder that affects muscle coordination and causes cognitive and psychiatry problems [92]. HD is caused by a repeat expansion of CAG in exon 1 of *HUNTINGTIN* (HTT). Individuals with repeats numbers above 35 are at increased risk for the disease. At above 40 repeats, disease onset is certain. Increased repeat number also intensifies disease severity and decreases the age of onset. Despite being ubiquitously expressed, mutant HTT aggregates in medium spiny neurons located in the striatum and leads to death

of these neurons in early disease stages. Over time, the disease induces changes in other regions of the brain.

iPSCs were derived from a patient with 72 CAG repeats in HTT [52]. Cells were differentiated into neural stem cells via embryoid bodies and the addition of combinations of morphogens and nuerotrophic factors in two stages [28]. Derived cells expressed markers indicative of mature striatal neurons including β-III tubulin, calbindin, GABA, and DARPP-32. Notably, despite increasing caspase activity during neutrophic deprivation, HD-derived striatal neurons showed similar survival compared to the control.

In many diseases including HD non-cell autonomous effects linked to glial cells are implicated in mediating selective neuronal death. Fibroblasts obtained from a juvenile HD patient bearing 109 repeats, her father with adult HD and with 50 repeats, and a non-related control were converted to iPSCs and subsequently differentiated to neurons and astrocytes [27]. Neural progenitors from HD-iPSCs engraft when transplanted and yield neurons. Additionally, TUJ1-positive neurons derived from HD-iPSCs showed normal electrophysiology and did not display a HTT phenotype. Astrocytes were generated from iPSC neurospheres that were passaged in astrocyte conditioned media until GFAP and S100β expression was detected. Unlike the neurons, astrocytes displayed a unique cellular phenotype that correlated with HTT repeat number. With increasing repeat number, the number of cytoplasmic vacuoles and the number of cells with vacuoles increased. As a next step, the mechanism of how vacuolization of astrocytes could mediate neurodegeration should be explored.

**Friedrich's ataxia**

Friedrich's ataxia (FRDA) is the most common ataxic disorder. FRDA is characterized by a progressive muscle weakness, leg spasticity, scoliosis, and lack of voluntary motor coordination in the limbs. FRDA is caused by a GAA repeat expansion in intron 1 in the *FRATAXIN* (*FXN*) gene. Normally, cells contain 6-34 GAA repeats. Increasing repeat number above 66 leads to silencing of *FXN*. Additionally, *FXN* silencing and disease severity correlate with increasing repeat number. FXN is critical in synthesis of iron-sulfur clusters and thus respiratory chain complex. Silencing of *FXN* results in mitochondrial dysfunction and iron accumulation in neurons and cardiomyocytes is observed.

iPSCs represents a potential therapeutic test bed for evaluating molecular and genetic interventions to alter disease phenotypes. However, in diseases with repeat expansions, reprogramming has been shown to alter repeat length, both increasing and decreasing repeat number [80, 93, 94]. Thus, it is important to verify repeat number in reprogrammed cells and determine if the relevant phenotype can be captured in these cells. iPSCs from two FRDA patients were generated that maintained low FXN expression despite reprogramming inducing variations in repeat length [93]. Similarly, iPSCs derived from a different set of FRDA patients show repeat instability [94]. Repeat expansion showed instability in culture, changing with passages. Global gene analysis indicates that FRDA-iPSCs cluster with cells from healthy controls. However, a significant difference was seen in the expression of genes known to be important for mitochondrial function, DNA repair, and damage.

Mismatch repair enzymes (MMR) such as MSH2 have been previously implicated in repeat instability in HD. In FRDA-iPSCs, MSH2 expression is elevated and MSH2 occupancy upstream of *FXN* repeat expansion was increased relative to the controls [94]. Addition of MSH2 targeting shRNAs reduced repeat expansion relative to the non-targeting shRNA control, suggesting that limiting *MSH2* expression may be a potential target for inhibiting the primary molecular mechanism in FRDA.

## ALS

Amyotrophic lateral sclerosis (ALS; or Lou Gehrig's disease) is a neurodegenerative disease characterized by progressive loss of muscle control and motor neuron death. Average age of onset for ALS is 50 with most patients surviving fewer than five years following diagnosis. Several mutations have been identified in familial and sporadic cases including mutations in *SOD1*, *TARDP43*, and *FUS/TLS*, and a repeat expansion in *C9ORF72*. While animal models of ALS have helped to shed light on the development of the disease, they are limited primarily to the *SOD1* form, and therapeutics identified in mouse models fail to significantly improve patient outcomes.

### TDP-43 ALS

Abnormal protein accumulation is characteristic feature of neurodegenerative diseases. In ALS, several species of proteins (e.g. TDP-43, C9ORF72, PURα) have been shown to accumulate in patient cells. iPSC-derived MNs from patients with TDP-43 ALS contain cytosolic TDP-43-containing aggregates similar to those seen in post-mortem tissue. They also possess shorter neurites, a phenotype that has also been observed in mutant TDP-43 MNs in zebrafish [29].

TDP-43 binds nuclear DNA and RNA, mediating RNA processing. Studies from patient genotypes of ALS reveal that TDP-43 mutations that lead to longer protein half-lives correlate with earlier disease onset [95]. Curiously, TDP-43 is known to self-regulate stability, suggesting mutations may inhibit proper regulation of TDP-43 feedback loop. Stabilization of TDP-43 by addition of DD, a destabilizing tag that stabilizes proteins in response to a small molecule, induces cytotoxicity through abnormal proteostasis and RNA dysregulation. Recently several studies have the investigated the role of TDP-43 in mediating neurotoxicity using ALS iPSC-derived MNs [29, 96, 97]. Detergent-soluble and -insoluble aggregates accumulate in TDP-43 MNs despite similar mRNA levels between controls and TDP-43 mutant, suggesting post-transcriptional events affect the processing of TDP-43 protein. Notably, TDP-43 MNs do not show decreased neuron survival *in vitro*. However, introduction of cellular stress through PIK3 inhibition induces cytotoxicity unlike ER stress or MAPK inhibition [96]. The systemic effects of TDP-43 mutations may impact particular pathways, allowing normal stressors to induce TDP-43 cytotoxicity.

Pharmacological intervention with agents that reduce TDP-43 aggregation may reduce cytotoxicity. Neurons derived from TDP-43 mutant patient lines displayed increased TDP-43 expression, TDP-43 accumulation, abnormal RNA metabolism, and increased sensitivity to cell stressors [29]. Of four drugs tested that had been previously shown to modulate gene expression in stress-induced MN cytotoxicity, anacardic acid significantly reduced TDP-43 mRNA expression. Additionally, anacardic acid treatment reduced the amount of TDP-43 in the insoluble fraction, but not the soluble fraction, and increased neurite length.

In a similar approach to drug screening, iPSCs were generated from a patient with sporadic ALS characterized by TDP-43 aggregates in MNs. Small molecules with the ability to act as TDP-43 aggregate inhibitors were screened in TDP-43 ALS MNs. Digoxin, an FDA-approved cardiac glycoside reduced TDP-43 aggregation. [97] Determining if neuron survival can be rescued represents an in important next step in validating Digoxin as a potential therapeutic for ALS treatment.

### C9ORF72 ALS

Two papers in 2011 determined that the expansion of a GGGGCC hexanucleotide repeat sequence in an intron of *C9ORF72* causes frontotemporal dementia (FTD) and ALS [98, 99].

Subsequent studies verified that this is now the most common known cause of ALS [100]. At least three mechanisms may explain the motor neuron degeneration in these patients; a loss-of-function of C9ORF72 protein, cytotoxicity caused by RNA foci induced by the repeat expansion [99], or toxicity of dipeptide repeat proteins generated by repeat associated non-ATG (RAN) translation of the GGGGCC expansion [101].

Control iPSC MNs did not degenerate or display disease phenotypes when C9ORF72 levels were suppressed by shRNA [32], suggesting that a loss of C9ORF72 function is not toxic to MNs. Conversely, C9ORF72 ALS MNs displayed prominent RNA foci and degenerated more rapidly than controls when challenged with elevated levels of glutamate, which is thought to contribute to ALS pathogenesis [32]. The addition of antisense oligonucleotides complementary to *C9ORF72* RNA eliminated the RNA foci and glutamate-induced excitotoxicity, indicating that the foci may be toxic to MNs. RNA bearing the $G_4C_2$ repeat expansion was shown to bind ADARB2, PURα, and hnRNPA1, potentially titrating and sequestering these components from their native functions in RNA metabolism. In addition to the nuclear RNA foci, C9ORF72 RAN translation proteins aggregated in the cytoplasm of patient iPSC MNs, suggesting it will be possible to measure the toxicity of these species in MNs [32].

### SOD1 ALS

Although studies analyzing cell-autonomous effects of *SOD1* mutations in patient-specific MNs have not been completed, studies using primary mouse glia [102] or reprogrammed human astrocytes [103] have demonstrated that *SOD1* ALS glia exhibit toxicity towards control human MNs. Rubin and colleagues used mouse ESC-derived motor neurons to identify small molecules that promote motor neuron survival [104]. To screen for small molecules that could rescue survival, cells were tracked following withdrawal of neurotrophic factors. Of the 5000 drugs screened, kenpaullone increased survival 2.5 fold and decreased mutant SOD1 protein levels. They found that kenpaullone can not only protect mESC MNs against cell death, but also supports the survival of normal human and *SOD1* ALS MNs [104].

## Frontotemporal dementia

Frontotemporal dementia (FTD) is the second most common dementia in patients under 65 years old, and is characterized by the degeneration of neurons in the frontal and temporal lobes, which leads to profound changes in behavior, speech, and/or cognition [105]. The variability of degenerative patterns and phenotypic changes observed in patients – some do not develop language problems while others become completely unable to speak - suggest that FTD may comprise several different pathogenic mechanisms. However, little is known about why cortical neurons degenerate in these patients.

Loss-of-function mutations in the *GRN* gene cause FTD. iPSC neurons derived from patients with an S116X nonsense mutation in *GRN* displayed cytoplasmic TDP-43 aggregates and an increased sensitivity to small molecule-mediated PI3K/Akt and MEK inhibition, suggesting that PRGN deficiency may perturb or necessitate these pathways in neurons [106]. In addition, both *GRN* and sporadic FTD neurons displayed increased sensitivity to inducers of ER stress and proteasome inhibitors while retaining normal rates of apoptosis in the presence of mitochondrial or oxidative stress, which are commonly implicated in neurodegenerative diseases. Importantly, the forced expression of PRGN rescued the disease phenotypes in *GRN* FTD neurons, validating that the observed differences were due to the *GRN* mutation. These results suggest that prosurvival signaling

pathways are weakened in *GRN* FTD neurons and that therapeutic avenues may include the delivery of neurotrophic factors in addition to the restoration of normal PRGN levels.

### Alzheimer's disease

Alzheimer's disease (AD) is a common neurodegenerative disorder that is characterized by neurofibrillary tangles and increased amyloid plaques in the brain [107].The disease consists of rare, familial, early-onset forms and more common sporadic late-onset cases, implicating a myriad of different genes as causal factors [108].

Abeliovich and colleagues used direct conversion to generate neurons from fibroblasts of patients with familial AD or unaffected controls [31].Induced neurons with PRESENILIN 1 or 2 mutations displayed altered localization of the Amyloid-β precursor protein (APP) and an increased $A\beta_{42/}$ $A\beta_{40}$ ratio, both reflective of phenotypes previously linked to AD in patients [31]. These results indicate that neurons generated by direct conversion can recapitulate disease processes.

iPSC neurons derived from two familial AD patients carrying a duplication of APP possessed higher levels of $A\beta_{40}$, phosphorylated tau, and active GSK-3β than controls, indicating that AD processes were occurring [109]. Interestingly, neurons from one of two sporadic AD patients also displayed these phenotypes, suggesting that this approach could be used to study sporadic and late onset forms of AD. In an unexpected twist, treatment with β-secretase inhibitors, but not γ-secretase inhibitors, caused significant reductions in phosphorylated tau and active GSK-3β. These results suggest a direct relationship between APP proteolytic processing in GSK-3β activation and tau phosphorylation in human neurons [109]. Together, these studies indicate the reprogrammed neurons can recapitulate processes associated with late onset diseases *in vitro*.

## Open questions and challenges in the field

### Relevance of models to disease

The CNS comprises many distinct neuronal subtypes, each possessing unique qualities that contribute to human thought, sensation, or action. However, these properties also leave these cells differentially sensitive to disease stimuli, and it is this selective vulnerability that results in the stereotyped manifestations of CNS disorders. Therefore, if we are to understand the mechanisms of these diseases, then we must study them in the cellular subtypes that they affect *in vivo*.

Cellular reprogramming allows the investigation of CNS diseases using relevant human neural cells. Reprogrammed cells are an improvement over transformed human cell lines because they have the same cellular identity as those targeted by the disease. The advantages of this approach over animal models are that it uses human cells that possess the same genome as the patient, not an approximation engineered using transgenic approaches.

The ability to generate *bona fide* disease-relevant neural cell types using lineage reprogramming or directed differentiation is critical to the success of this approach. To date, a handful of the neuronal subtypes affected by CNS diseases are accessible through stem cells or reprogramming [16], but the developmental or transcriptional logic remains unclear for many others. In addition, the accurate interpretation of disease studies will require better characterization of the human neural cell types that can be produced *in vitro*. Thus far, most have not been validated by rigorous comparisons of genome-wide transcriptional and epigenetic profiles to primary cells, and therefore it is unclear how similar *in vitro*-derived cells are to their natural counterparts. One concern is that the *in vitro*-derived

cells are slow to reach maturity, which is presumably important for recapitulating adult-onset diseases. Moreover, the cellular heterogeneity amongst stem cell-derived or reprogrammed somatic cells is unknown. A key focal point for the field will be to expand the number of somatic cells that can be produced *in vitro* and to define the similarities and differences between *in vitro*- and *in vivo*-derived cells.

How closely do patient-specific disease models recapitulate human disease? This is still a very open question in the field that may be difficult to answer until *in vivo* studies emerge validating *in vitro* findings. However, there are several aspects of patient-specific disease models that should be emphasized in the future in order to strengthen the approach.

For every patient-specific disease model, there is a chance that an observed phenotype is actually caused by genetic variants that are outside of the locus being studied, and therefore may have little to nothing to do with the real disease process. One way to eliminate this concern is to use isogenic control iPSC lines that only differ by the causal variant in order to definitively show that it induces the observed phenotypes. TALEN and CRISPR/Cas9 technology have made iPSC genome editing inexpensive, rapid, and inexpensive. Although one concern is that the additional passaging and transgenic genome editing tools alter the genome outside of the locus of interest, validating phenotypes using 2-3 isogenic lines that are generated using different TALENs or CRISPRs minimizes this risk. When investigating a disease with an unknown genetic cause, increasing the number of patients and controls being compared can ensure statistical confidence that an observed phenotype is disease-relevant. The number of patients required can be predicted by using a power analysis, and the high patient volume approach may be better suited for direct lineage conversion rather than the iPSC method.

For many CNS diseases, only certain cell types are perturbed or lost. Therefore, demonstrating that disease phenotypes are restricted to the cell types that are selectively vulnerable in patients would increase one's confidence that the disease model is relevant. To date, almost no patient-specific disease modeling studies have investigated this and in fact, several have used unspecialized neurons to recapitulate diseases that specifically affect only certain cells [30-32]. To be fair, one caveat of this assumption is that many cell types could be affected by the disease-causing variants, but most are rescued by compensating mechanisms that are present *in vivo* but absent *in vitro*. Alternatively, many cell types could be affected at a low level *in vivo* but some are severely affected. If either of these are the case, then we would expect to see relevant disease processes even in neural subtypes that are not overtly affected in patients. Therefore, it is somewhat unclear exactly how relevant the findings of these studies are to their respective diseases. It is likely that some aspects of CNS diseases can be studied even in neurons that are not strongly affected *in vivo*, but some critical mechanisms may only be apparent in the right cell types. It is likely that some aspects of CNS diseases can be studied even in neurons that are not strongly affected *in vivo*, but many critical mechanisms may only be apparent in the right cell types. Regardless, these initial patient-specific disease studies are leading the way in showing that *in vitro*-derived patient cells can recapitulate hallmark disease phenotypes.

Compelling evidence suggests that non-cell autonomous effects contribute to several neurodegenerative diseases including ALS [64] , AD [102, 110], Parkinson's [111], and Huntington's disease [112, 113]. Therefore, neurodegenerative disease mechanisms include changes that are both intrinsic and extrinsic to neurons. Neutralization of both types of disease stimuli is likely to lead to the most effective treatments. Thus, co-culture experiments involving patient-specific versions of multiple relevant cell types will be required to gain a complete understanding of disease mechanisms. This could be done either in monolayer culture or in organoid form [38]. Three dimensional organoid cultures also seems to foster the spontaneous differentiation of many different cell types within the

target tissue, which could greatly improve the relevance of disease models [38, 41-43]. Organoids also provide the ability to assay for phenotypes that are impossible to evaluate in monolayer cultures such as microcephaly [38]. Other than neural cells, the immune system may be involved in many CNS diseases, although it is unclear if this can be effectively modeled *in vitro.* It may be possible to incorporate this aspect if efficient protocols for generating or harvesting patient-specific immune cells are developed.

If *in vitro* models accurately recapitulate disease mechanisms, then they should exhibit the same spectrum of disease severity observed *in vivo* with different causal variants. In other words, variants that cause more severe forms of disease should manifest more aggressive phenotypes *in vitro*. Although this requires the collection of a more comprehensive patient cohort, this would be powerful if it could be demonstrated. Not only would the system enable the testing of hypotheses of why certain variants are more deleterious than others, but it could also provide a measurement of how much change in phenotypic severity *in vitro* is required to produce a given difference *in vivo*. This ability would allow more accurate predictions of how efficacious a drug might be in patients depending on the magnitude of their effects *in vitro*.

Aside from these aspects of disease modeling that the field is striving to improve on, a concern that often arises is that it may be difficult to model late-onset diseases *in vitro*. However, promising studies on diseases such as Alzheimer's, Parkinson's disease, and ALS in which convincing phenotypes were identified have begun to mitigate this concern [29-31, 89]. One possible reason why it is possible to recapitulate late onset disease processes in short cell culture experiments is that the causal variants actually affect their neural cell targets throughout the life of the patient and compensatory mechanisms such as regeneration or synaptic plasticity delay disease presentation. Alternatively, the disease processes could be present early on but are increased substantially in later life by environmental stimuli. Thirdly, *in vitro* conditions are far less supportive than the *in vivo* niche, and this serves to amplify disease phenotypes in cell culture. This hypothesis is consistent with the fact that even neurons derived from healthy individuals degenerate within 1-2 months in culture, whereas they survive for decades *in vivo*. Still, it is likely that we are still failing to recapitulate certain disease processes that develop slowly over time. In these cases, perturbing specific cellular processes with small molecule stressors [29, 106, 114] may sensitize disease models.

**Drug Screening**

Lead compounds validated in animal models of CNS diseases have generally failed in clinical trials, and the hope is that patient-specific cell culture models of disease will provide more promising drug targets. Until the first success story emerges, it is still too early to tell if this will be the case. However, it would be worth addressing the following questions now: 1) If molecules that went through clinical trials are tested in patient-specific *in vitro* models, do they accurately predict efficacy? 2) Does screening drugs in *in vitro*-derived hepatocytes or cardiomyocytes accurately identify those that either passed or failed toxicology testing *in vivo*?

Developing robust, efficient protocols for generating large pools of patient-derived somatic cells is one current challenge for the field, but this is rapidly being worked out [104, 115]. A possible work-around could be to perform a high-throughput screen in a more amenable setting, such as yeast, and then validate hits in a patient-specific model (24).

A future goal for drug development will be to use patient-specific models as an *in vitro* clinical trial platform that predicts the efficacy and toxicity of drugs in a personalized manner. Moreover, chemical screens in combination with patient-specific models may be capable of identifying genetic-environmental interactions that combine to induce disease.

## Conclusions

Since the discovery of iPSCs in 2006 [5], cellular reprogramming and disease modeling technology have evolved at a ferocious pace. Recently, it was discovered that a 30 min acid treatment can reprogram somatic cells to a totipotent state, which is likely to propel the use of patient-specific cells forward at an even faster rate [116, 117]. In the last two years, the use of patient-derived tissue for CNS investigations has transitioned from a drizzle to a downpour as more and more laboratories adopt reprogramming and stem cell technology. A critical factor in this progression is indeed that reprogramming is robust and technically simple. However, the promise that this approach holds is perhaps the most important driving force**.**

## Figure

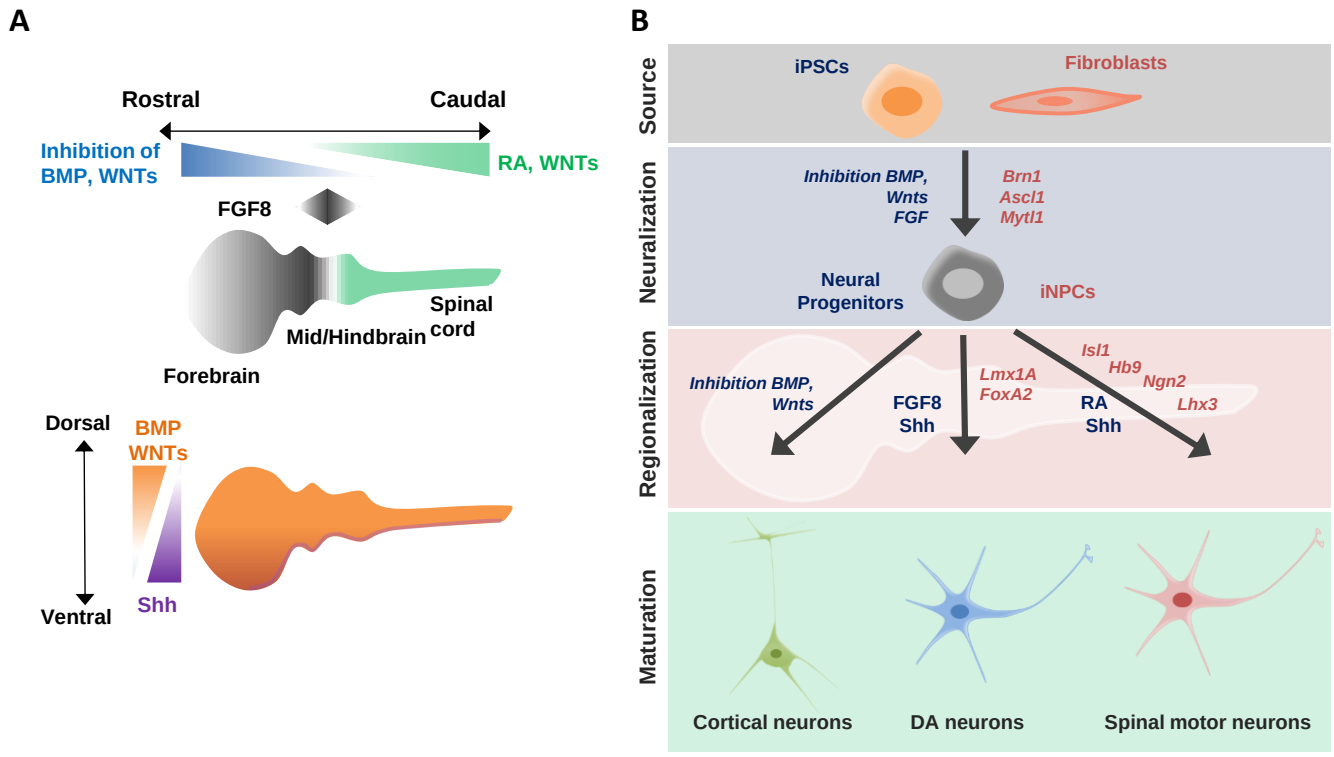


**Figure 1. Differentiation protocols guided by developmentally-relevant morphogens.** A. Developmental patterning of morphogens to establish regions of the brain and spinal cord as well as

dorsal-ventral axis. Adapted from Petros et al. 2011 [37]. **B.** Differentiation from iPSCs with inductive factors.

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