● INVITED REVIEW

Using induced pluripotent stem cells derived neurons to model brain diseases

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Abstract

The ability to use induced pluripotent stem cells (iPSC) to model brain diseases is a powerful tool for unraveling mechanistic alterations in these disorders. Rodent models of brain diseases have spurred understanding of pathology but the concern arises that they may not recapitulate the full spectrum of neuron disruptions associated with human neuropathology. iPSC derived neurons, or other neural cell types, provide the ability to access pathology in cells derived directly from a patient's blood sample or skin biopsy where availability of brain tissue is limiting. Thus, utilization of iPSC to study brain diseases provides an unlimited resource for disease modelling but may also be used for drug screening for effective therapies and may potentially be used to regenerate aged or damaged cells in the future. Many brain diseases across the spectrum of neurodevelopment, neurodegenerative and neuropsychiatric are being approached by iPSC models. The goal of an iPSC based disease model is to identify a cellular phenotype that discriminates the disease-bearing cells from the control cells. In this mini-review, the importance of iPSC cell models validated for pluripotency, germline competency and function assessments is discussed. Selected examples for the variety of brain diseases that are being approached by iPSC technology to discover or establish the molecular basis of the neuropathology are discussed.

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Introduction

Yamanako (2008) showed that differentiated somatic cells could be reprogrammed to induced pluripotent stem cells (iPSC). Most commonly, fibroblasts or peripheral blood mononuclear cells (PBMC) are captured from patients with neurodegenerative diseases and used to make iPSC derived neural cells to study disease pathology. The reprogramming methodology requires four transcription factors (KLF4, c-MYC, OCT4 and SOX2) that reset the embryonic state (for details see Yamanaka, 2008). iPSC clones can then be transformed to selected differentiated cell types by adding the necessary growth/differentiation proteins and co-factors to the cell culture medium. iPSCs are particularly useful for studying human neurons and other brain cells that may only be available from autopsy material. Using patient derived iPSC allows the researcher to create defined neuron populations that can be scaled to meet research needs. Neurodegenerative and neurodevelopmental disorders are particularly approachable using iPSC technology as differentiated cell types provide the patient's genomic context to investigate the etiology of disease. iPSC derived cell models also bridge the gap when no appropriate animal model is available and they offer the capability to model neuron subtypes like glia and astrocytes. Consequently, cellular and molecular phenotypes driving neuropsychiatric, neurodevelopmental and neurodegenerative disorders may require a human neuronal cellular model that is able to recapitulate the genetic causes of the pathology and to produce the target cell type for study (Acab and Muotri, 2015).

In this short review, the approach and some criteria for obtaining fully characterized human iPSC and neurons to investigate pathology and molecular disruptions of function in neurodegenerative or neurodevelopmental diseases is outlined.

Characterizing Reprogrammed Human iPSC

Protocols that detail the steps necessary to obtain iPSC are available from many sources (Bohl et al., 2016). With rigorous culture practices, iPSCs are readily produced. The iPSC must be fully characterized to assure quality before transforming them to differentiated neurons or other brain cells. Characterization includes: immunocytochemistry for known pluripotency markers, confirmation that the iPSCs can differentiate to the three germ layers (endoderm, mesoderm and ectoderm), and gene expression profiles for known pluripotency markers. Common pluripotency markers are SOX2, OCT4, TRA1-60, NANOG and TRA1-81. Antibodies are readily available from several sources to conduct these assays. **Figure 1** shows a representative panel of pluripotency results from a Gaucher disease type 2 iPSC clone verification. High quality iPSC clones will report several pluripotency markers over the surface of the iPSC clone. The morphology of the iPSC clone should have clearly defined margins and no miscellaneous differentiated cells growing from the colony. Multiple, robust medium size colonies grow spread over the culture plate surface. The iPSC within the clone contain very little cytoplasm. A second round of verification includes

the determination of the iPSC clone's ability to differentiate into all three germ layers, endoderm, mesoderm and ectoderm. This can be done in two ways: 1) injecting iPSC cells into a mouse and waiting for a tumor (teratoma) to develop (Nelakanti et al., 2015) and then histologically analyzing the tissue types within the tumor or 2) differentiating the cells into the germ layer types using defined mediums containing the necessary differentiation factors. These bioassays confirm the pluripotency of the generated iPSC clones before using them to model disease.

Differentiating hiPSC to Neurons and Assessing Functionality

iPSC may be transduced to form most neural cell types (Begum et al., 2015), *e.g.*, cortical neurons, dopaminergic neurons, astrocytes or oligodendrocytes. This is done by supplying iPSC cultures with the appropriate differentiation and growth factors in culture medium and then maintenance growth in an appropriate defined neural maintenance medium, like Brain Phys (Bardy et al., 2015) while experimental evaluations are ongoing. Initial evaluations include analysis of biomarkers of neuronal differentiation (Nestin, NeuN, and others) by ICC or Western blot. Functional screening of neurons should include their ability to generate electrophysiological properties (see **Figure 2B** and **C**). All iPSC lines or iPSC derived neural lines should be authenticated to be free of human infectious agents and mycoplasma. They should also be karyotyped to show that no major chromosome rearrangements have occurred during the reprogramming or differentiation process. With these characterizations and authentication of the cell model accepted, it is appropriate to move to analyzing the specific disease pathology.

Studies Analyzing Neuropathology

The goal of an iPSC based disease model is to identify a phenotype that differentiates the diseased cells from the control cells. Patient derived or gene edited iPSC offer an unlimited source of patient cells for modeling specific neuronal diseases for assessment of cellular phenotype, for identification of molecular pathways implicated in disease or for assessment of cell therapeutics/drug efficacy. These applications offer a powerful and targeted approach for identifying new biological insights into pathology. While an in-depth discussion of the use of iPSC derived disease models to evaluate neuropathology is beyond the scope of this review, several examples demonstrate the utility and breadth of iPSC derived neuronal disease models and some of their significant findings.

iPSC and Inborn Errors of Neuron Metabolism

Lysosomal storage disorders

The lysosomal storage disorders represent a metabolic degradation pathway disrupted at many enzymatic junctions in the cell that can result in neuropathology. Neuron cell

models developed from patient samples point to the utility of evaluating human neuropathology by creating disease models employing iPSC derived neurons. They capture the patient's genomic background that may modulate the disease, provide an opportunity to study relevant pathological mechanisms in human cells and provide models to evaluate therapeutic approaches.

Neuronal ceroid lipofuscinoses (NCL)

NCL are inherited as recessive lysosomal storage diseases. An estimated 14 different genes (NCL1-NCL14) are involved where different mutations present in several forms of NCL in pediatric patients. A Finnish research team has focused on NCL5 (variant Jansky-Bielschowsky disease). The NCL5 protein is a lysosomal glycoprotein of, as yet, unknown function (Uusi-Rauva et al., 2017). It is suggested that NCL5 protein is involved in cholesterol and sphingolipid metabolism as well as endosomal sorting. A NCL5 mouse KO model is available (Kopra et al., 2004; Schmiedt et al., 2012) but it is uncertain if this model completely defines the human pathology since the mice do not replicate the characteristic seizures seen in NLC5 patients. To investigate the nature of the disease and to compare human neurons to animal model findings, iPSCs were produced from a NCL5 patient's fibroblasts with the Finnish variant mutation (c.1175_1176delAT, p.Y329X) and then differentiated to neurons for pathological evaluation. iPSC derived neurons contained an accumulation of autofluorescent material similar to findings in NCL5 patient fibroblasts. NCL5 iPSC derived neurons also sequestered the fluorescent synthetic glycosphingolipid, BODIPY-lactosylceramide, as previously shown in the animal model. This finding indicates a disruption in sphingolipid transport from the endo-lysosomes to the Golgi in these neurons (Uusi-Rauva et al., 2017). The NCL5 neurons also show ER stress, potentially due to retention of the mutant protein in the ER. However, the observed ER stress may be a result of a non-lysosomal function for the NCL5 protein. These studies of disease-relevant NCL5 neurons begin to unravel the role of NCL5 protein function and disease pathology in a previously unavailable human cell model.

Batten disease

Batten disease, another neural ceroid lipofuscinosis (NCL3), results in premature death due to progressive motor and cognitive decline, retinal pigment degeneration and seizures (Lojewski et al., 2014). Abnormal pathology was replicated in multiple non-related CNL3 neural lines generated from patient iPSC. Functional deficits are observed for mitochondria, Golgi and the endoplasmic reticulum as well as lysosomal storage in the CNS. These critical defects were detected prior to the appearance of lysosomal storage, identifying a key cellular dysfunction (Lojewski et al., 2014). The authors point to these iPSC models as a way to screen novel drug therapies and they show proof-of-concept studies that evaluate the efficacy of
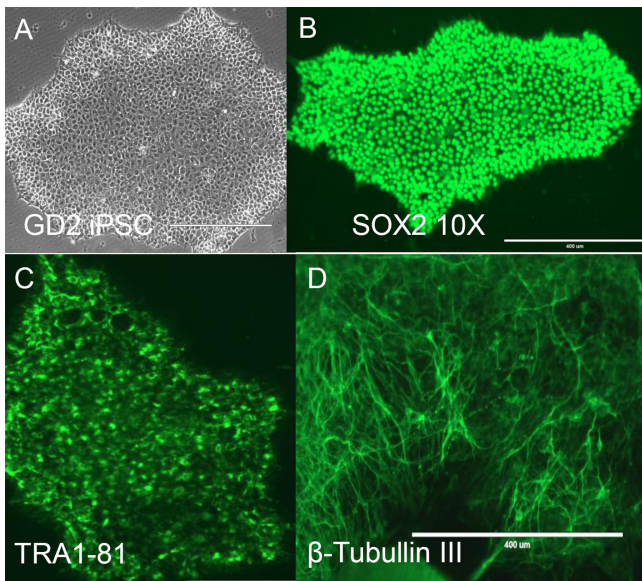
Figure 1 Characterizing induced pluripotent stem cells (iPSC) clones reprogrammed from patient fibroblasts.
(A) It is important to verify high quality iPSC clones by morphology (phase) and the expression of pluripotency biomarkers. (B) SOX2 is a nuclear transcription factor and (C) TRA1-81 is a cell surface marker. (D) Beta-tubulin III confirms the ability of the iPSC line to differentiate to neuroectoderm (mesoderm and endoderm markers not shown). All photos are 10× and sourced from the McKinney Lab. GD2: Gaucher type 2; SOX2: sex determining region Y-box 2; TRA1-81: tumor rejection antigen 1-81.

Figure 2 Characterization of Gaucher type 2 (GD2) neurons by biomarkers and functional analysis.
(A) Patient derived induced pluripotent stems (iPSC) differentiated to GD2 neurons using defined medium. (B) GD2 neurons express the reporter enhanced green fluorescent protein (EGFP) from a brain specific promoter (human synapsin) delivered by lentivirus. (C) GD2 neuron Patch-clamp analysis shows voltage sensitive Na^+ channels (arrow) in voltage clamp mode and (D) Multi-Electrode Array (MEA) analysis of GD2 neurons show a burst response at an electrode. Neurons are 32 days in culture after differentiation. These types of analysis confirm active neurons and the cells can be investigated for selected cellular functions.

the lipid lowering drugs, fenofibrate and gemfibrozil, on this NCL3 model.

Pompe disease
Pompe disease, another neural metabolic disease, is an acid alpha-glucosidase (GAA) deficiency. A neural model was generated from patient iPSC (Higuchi et al., 2014). GAA is the only enzyme that hydrolyzes glycogen to glucose in the acidic environment of the lysosome. Thus, GAA deficiency results in glycogen sequestering and subsequent enlargement of the lysosomes (Lim et al., 2015). Ultrastructure analysis of iPSC derived Pompe neurons recapitulated storage of glycogen granules in the cytoplasm. In a potential enzyme replacement approach, recombinant GAA treatment to correct iPSC derived Pompe neurons showed reduction of storagethat potentially opens a translational approach for therapy. Another lysosomal storage disease model reports iPSC derivation of Niemann-Pick type C1 neurons for study (Trilck et al., 2013).

iPSC and Neurodegenerative Disorders
Parkinson's disease
Parkinson's disease (PD) is a relatively common multifactorial neurodegenerative disease (Sanchez-Danes et al., 2012) characterized by deficits of motor skills. Movement loss is mapped to dysfunction of the dopaminergic neurons (DAn) in the substantia nigra. The loss of DAn is progressive and alpha-synuclein (SCNA) intra-neuronal inclusions, known
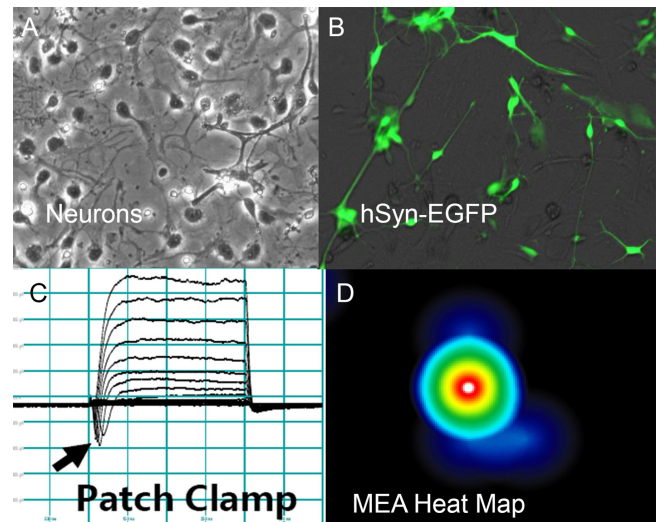
as Lewy bodies, are seen as the disease progresses. The majority of PD cases are sporadic and approximately 10% of cases are monogenic. The loci involved have documented mutations in the gene for Leucine Rich-Repeat Kinase 2 (*LRRK2*) and/or SCNA. Early onset PD is associated with mutations in the genes for Parkin, DJ-1, PINK1 and ATP13A2 (Lees et al., 2009). Many current animal models of PD do not demonstrate the key findings of the human disease; thus, PD remains poorly understood. The ability to reprogram patient samples (blood PBMCs or fibroblasts) to iPSC derived DAn provides a means for direct assessment of PD pathology. Several groups have now generated PD neurodegenerative disease models from iPSC that show pathology specific for the human disease (Sanchez-Danes et al., 2012; Fernandez-Santiago et al., 2015). Sanchez-Danes et al. (2012) report differences in neurite outgrowth between control and PD DAn, SCNA accumulation and alterations in autophagic flux (LC3-II assay). Fernandez et al. (2015) report that iPSC derived DAn from both sporadic and familial mutations in *LRRK2* demonstrate large DNA methylation changes in enhancers and transcriptome changes in a regulated gene network. Altered Ca^{2+} dynamics are also reported for PD neurons.

Amyotropic lateral sclerosis (ALS) corrected motor neurons
ALS is a debilitating neurodegenerative adult onset disorder that results in a progressive loss of upper and lower motor neurons while cognitive function remains unchanged. No

cure or clinical treatment halts ALS progression. It is estimated that about 20% of ALS cases are familial and mutations are identified in more than 20 genes that operate in diverse cellular pathways (Andersen and Al-Chalabi, 2011; Sreedharan and Brown, 2013). Rodent models of ALS (Kobayakawa et al., 2015) are available (SOD1 mouse, G93A), have been studied extensively and have yielded insights into ALS disease pathophysiology. However, as is frequently the case, the relevance of the mouse SOD1 model to human ALS is unclear because of species-specific differences. For example, most rodent models overexpress the mutant ALS related proteins under evaluation at non-physiological levels (Calvo et al., 2012; Cozzolino et al., 2013). Using motor neurons derived from patient iPSC and an isogenic CRISPR corrected iPSC motor neuron line, Bhinge et al. (2017) addressed the question of why motor neurons are targeted in ALS and non-motor neurons are less affected. They report that JUN, a member of the AP1 complex, is expressed at much higher levels in motor neurons. This finding led the authors to suggest that JUN has a function in preserving motor neuron homeostasis. *In situ* hybridization data from human spinal cord tissue also shows JUN mRNA at high levels in motor neurons compared to other spinal cord cells and were even higher in spinal tissues from ALS patients (Virgo and de Belleroche, 1995). So, using gene corrected motor neurons derived from iPSC allowed a focused comparison of the diseased and disease corrected cell line to reveal elevated levels of a specific transcription factor (JUN) somehow related to the known SOD1 mutation.

iPSC and Neurodevelopmental Disorders
Autism spectrum disorders (ASD)
ASD are a polygenic set of neurodevelopmental behaviors that share some core symptomology (impairment of social interactions, repetitive behaviors and deficits of interpersonal communication) (Acab and Muotri, 2015). About 10–20% of ASD is classified by known genomic alterations, for example, Rhett Syndrome (Balachandar et al., 2016). Non-syndromic causes of ASD are characterized by *de novo* and other hereditary mutations. The X-chromosome contains many genes that appear to be involved with intellectual disability that are currently under investigation (Lubs et al., 2012). As with other neurological diseases, the absence of relevant disease models has hindered ASD investigation. Animal models are productive for the study of single gene defects but are currently unable to adequately address complex disease and many do not appropriately address the social and behavior disorders seen in autism. Many monogenic models of ASD are available but iPSC derived neural models from patient samples are especially valuable for non-syndromic ASD (idiopathic) precisely because known genetic mutations are yet to be defined. Griesi-Oliveira et al. (2015) report that iPSC neurons from a protein channel transient receptor potential canonical

6 (TRPC6) mutant contain morphologic and functional alterations when compared with control neurons. TRPC6 may also play an important role in many signaling pathways (*e.g.*, BDNF) and neuronal calcium/calmodulin dynamics (Griesi-Oliveira et al., 2015). Since ASD has a neurodevelopmental etiology, iPSCs can be used to study early human neurogenesis in culture. Investigators compared neurodevelopmental transcriptome profiles from RNA-Seq of iPSC derived neurons made from both fibroblasts and dental pulp (Chen et al., 2013). Several lncRNAs were seen to become down-regulated during the transition from iPSC to neural progenitor cells while coding genes were up-regulated including many of the HOX genes (DLX1 and POU3F3). These studies show another example of the utility of iPSC derived neurons to study neurologic disease progression using exomic sequencing to define molecular pathway analysis. These analyses potentially capture alternative splicing defects, up or down regulation of key transcription factors or relevant alterations in miRNA or lncRNA regulators.

IPSC and Neuropsychiatric Diseases
Frontotemporal dementia (FTD)
FTD is a heterogeneous neurodegenerative disorder presenting with cognitive impairment that affects frontal and/or temporal lobes function of the brain associated with progressive brain atrophy (Rossor et al., 2010). FTD accounts for 6–8% of early onset (< 65 years old) dementia cases. A mutation in *CHMP2B* (charged microvesicular body protein 2B) found on human chromosome 3 is linked as a cause for frontotemporal dementia type 3 (FTD3) in some familial cases (Zhang et al., 2017). *CHMP2B* functions as a partner in the endosomal sorting complex required for transport (ESCRT) and when mutated it disrupts endosome-to-lysosome trafficking, and consequently, substrate degradation in the lysosome. Zhang et al. (2017) developed three FTD3 neuronal lines from reprogrammed patient iPSC and gene corrected the *CHMP2B* splice acceptor mutant (31449G > C) to create isogenic control lines. Independent (non-isogenic) controls were also included in the study. FTD3 and isogenic iPSC were differentiated to forebrain specific cortical neurons and further characterized. Electron microscopy showed enlarged endosomes in the FTD3 neurons as previously seen in patient cells. Mitochondria were examined and shown to be cristae-lessand to cluster in the perinuclear region of the neuron. CRISPR corrected FTD3 neurons and independent controls showed the normal distribution of mitochondria in the axons and dendrites as well as the perinuclear region. This finding correlated with FTD3 patient's increased axonal degeneration. Mitochondria in the FTD3 cells also exhibited oxidative stress. RNA-Seq analysis found that the FTD3 transcriptomes were clustered and showed gene expression differences in endosome genes and neurodegeneration genes, such as, *LRRK2*/Parkinson's disease and *MPO* and *APOD*/

Alzheimer's disease were down-regulated. In addition, genes involved in iron homeostasis (TRPC6, *HFE*) showed altered expression profiles. Thus, FTD3 cortical neurons in this study confirmed results from patient tissue including early endosome trafficking defects, mitochondrial stress and suggested that defects in iron homeostasis are part of the FTD3 spectrum.

In another FTD study (Almeida et al., 2013), skin biopsies from patients with the FTD linked repeat expansion GGGGCC in the noncoding region of *C9ORF72*, a gene of unknown function, were reprogrammed to iPSC and differentiated to post-mitotic neurons. These FTD neurons showed significantly elevated p62 levels and increased sensitivity to cellular stress induced by autophagy inhibitors. These findings showed that key neuropathological features of GGGGCC patients are recapitulated in iPSC-derived human neurons and suggested that compromised autophagy may be a common pathogenic mechanism in neurodegenerative disorders. Other psychiatric disorders that have been approached by iPSC analysis are reviewed in Paşca et al. (2014) where they address the use of iPSC derived neurons to attempt to uncover molecular phenotypes and effective drug treatments.

Conclusion

There is a need for human neuron cell models of disease because of potential species-specific differences with rodent KO and transgenic models. Also, the limited availability of viable neurons from patient brain tissue restricts investigation into the mechanisms of neural pathogenesis. Relevant human models for neurodegenerative, neurodevelopmental, neuropsychiatric disorders and others can be derived from patient somatic cells by reprogramming to iPSC and then deriving neurons. Neural cell models may be generated from the same patient by CRISPR correction of the mutation. Such genetically edited iPSCs are ideal isogenic controls for the patient-derived iPSCs neurons, probing the significance of the disease-causing mutation in the patient's own genetic context. iPSC neuron studies provide proof of concept models that allow experimental analysis of disease pathogensis linking them to molecular phenotypes. Human iPSC models provide invaluable accessibility for studying disease progression from early progenitor cells to aged or mature neurons.

Emerging findings in the iPSC derived brain cells suggest, that while mutations are diverse, there may be a shared cellular dysfunction in endolysosomal pathways. Finally, human iPSC derived neurons offer targeted development of drug screening platform(s) and, in the future, they may facilitate the development of new therapies (Griffin et al., 2015; Sugai et al., 2016) across a wide spectrum of neural disorders. These platforms and potential therapies might be able to treat neurologic disease before clinical symptoms appear and, with a prescreen in iPSC neurons, earlier success in clinical trials might be obtained.

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