



CRISPR and iPSCs: Recent Developments and Future Perspectives in Neurodegenerative Disease Modelling, Research, and Therapeutics

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Abstract

Neurodegenerative diseases are prominent causes of pain, suffering, and death worldwide. Traditional approaches modelling neurodegenerative diseases are deficient, and therefore, improved strategies that effectively recapitulate the pathophysiological conditions of neurodegenerative diseases are the need of the hour. The generation of human-induced pluripotent stem cells (iPSCs) has transformed our ability to model neurodegenerative diseases in vitro and provide an unlimited source of cells (including desired neuronal cell types) for cell replacement therapy. Recently, CRISPR/Cas9-based genome editing has also been gaining popularity because of the flexibility they provide to generate and ablate disease phenotypes. In addition, the recent advancements in CRISPR/Cas9 technology enables researchers to seamlessly target and introduce precise modifications in the genomic DNA of different human cell lines, including iPSCs. CRISPR-iPSC-based disease modelling, therefore, allows scientists to recapitulate the pathological aspects of most neurodegenerative processes and investigate the role of pathological gene variants in healthy non-patient cell lines. This review outlines how iPSCs, CRISPR/Cas9, and CRISPR-iPSC-based approaches accelerate research on neurodegenerative diseases and take us closer to a cure for neurodegenerative diseases such as Alzheimer's disease, Parkinson's disease, Huntington's disease, Amyotrophic Lateral Sclerosis, and so forth.

Keywords Neurodegenerative diseases · Induced pluripotent stem cells · Clustered regularly interspaced short palindromic repeats · Genome editing · Disease modelling

Introduction

Neurodegenerative diseases/disorders represent one of the major causes of death, disability, and increased social as well as economic burden on affected individuals and their families around the world. In the year 2016, neurological disorders/diseases were the leading cause of disability-adjusted life years and the second leading cause of death globally. Among these, Alzheimer's disease (AD) and other dementias ranked third for age-standardized disability-adjusted life years, whereas Parkinson's disease (PD) and motor neuron diseases (MND) ranked 11th and 15th, respectively (Feigin

et al. 2019). For decades, attempts to develop effective cures for various neurodegenerative diseases have been ongoing. However, no neurodegenerative disease is curable to date (Durães et al. 2018).

The first step to understand a complex disease process, as is the case with almost all neurodegenerative diseases, is to develop an effective disease model. A disease model is a system displaying the entire set or more frequently a definite subset of the pathological processes underlying an actual disease process. The contribution of animal models, and specifically mouse models, in understanding modern biology is undeniable and irrefutable. A wide variety of different transgenic mice models have been utilized in a diverse range of research applications over the decades. However, they have their drawbacks, especially in modelling neurodegenerative diseases. Mice differ significantly from humans in a variety of therapeutically relevant areas such as anatomical and physiological complexity, life expectancy, neurotransmitter function, anatomical complexity, metabolic rates, and even pharmacological response (Haston and Finkbeiner 2016). Therefore, many scientific investigators and pharmaceutical companies consider

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datasets obtained from established human cell lines to be more therapeutically translatable as compared to datasets derived from mouse models (Benam et al. 2015; Donowitz et al. 2020).

Traditionally, tissue samples had to be obtained from diseased subjects in order to set up primary cultures and consequently derive the cell line(s) harboring the disease-causing mutation(s) for research applications (Valadez-Barba et al. 2020). However, this approach has several drawbacks. The inherent risk of biopsies combined with the cumbersome and time-consuming procedures/protocols necessary to establish primary cell lines render patient tissue sample acquisition a tedious process (Verma et al. 2020). Post-mortem tissue samples are more likely to represent the terminal stages of a complex disorder/disease and, therefore, may obfuscate critical insights pertaining to early pathological markers, overall disease etiology, and pathophysiological hallmarks exclusive to specific disease stages (Siller et al. 2013). Furthermore, modelling complex polygenetic or multifactorial neurodegenerative disorders like AD, PD, or amyotrophic lateral sclerosis (ALS) requires multiple isogenic cell lines sourced from a diverse patient pool to obtain a comprehensive view of the summative and more importantly combinatorial effects of all the genetic aberrations responsible for the disease phenotype (Poewe et al. 2017; Penney et al. 2020; Trapecar et al. 2021). Finally, modelling a disease using traditional animal cell monocultures may yield inadequate results as the cell growth medium does not resemble the microenvironment of the tissue niche, nor does it account for the various interactions the cell type of interest has with other cellular and non-cellular entities (like the extracellular matrix) in its vicinity (Li et al. 2012; Bolognin et al. 2019; Lam et al. 2019). For example, non-neuronal tissues like astrocytes have been demonstrated to play vital roles in the pathogenesis of complex neurodegenerative processes like AD or PD (Lin et al. 2018; Penney et al. 2020). Therefore, studies aiming to study neurodegenerative disease processes in vitro using only neuronal cells may overlook vital clues pertaining to potential therapeutic targets arising due to the interaction between different cell types or their secreted factors in vivo (Bolognin et al. 2019). All these issues severely delimit attempts to model complex neurodegenerative disease processes. The combination of clustered regularly interspaced short palindromic repeats (CRISPR) and induced pluripotent stem cells (iPSCs) technology can represent a nexus of two promising technologies working in concert to ameliorate and possibly eliminate these challenges.

A Brief Summary of CRISPR and iPSCs

CRISPR

The term CRISPR coined by Jansen and colleagues provided a detailed description of the CRISPR loci derived from in

silico analysis of the genomic DNA of several bacterial and archaeal species (Jansen et al. 2002). Later, Barrangou and colleagues reported the first experimental evidence documenting the nucleic acid-mediated adaptive immunity conferred by the CRISPR machinery in a multitude of prokaryotic and archaeal systems (Barrangou et al. 2007). The authors demonstrated the acquisition of short stretches of phage DNA into the bacterial genome when exposed to a bacteriophage infection (Barrangou et al. 2007).

A CRISPR array comprises of short, direct repeats punctuated by stretches of variable DNA sequences, referred to as spacers. When bacteriophage or plasmid DNA is introduced into a bacterial system, they are processed by the Cas1 and Cas2 proteins and integrated as spacers into the CRISPR array. This step is referred to as protospacer acquisition and can be considered analogous to committing a new record in a database. Protospacer acquisition in CRISPR/Cas systems generally requires the presence of a short stretch of nucleotides in the foreign target nucleic acid, referred to as the protospacer adjacent motif (PAM). These spacers function as the basis of the memory required for a targeted host defense response to a subsequent infection. Following the protospacer acquisition, the CRISPR array is transcribed as a precursor transcript (pre-crRNA), which is either bound to a single, multidomain protein named Cas9 or to a multiunit effector complex, forming the crRNA–effector complex. The pre-crRNA is subsequently processed into smaller CRISPR RNAs (crRNAs). On re-exposure to foreign offending nucleic acids, these CRISPR RNAs recognize and guide either the Cas9 protein or the multiunit crRNA–effector complex to cleave and neutralize the infection (Makarova et al. 2015).

The native Cas9 system requires two separate short RNA strands, the mature crRNA and a transactivating crRNA (tracrRNA), to constitute the CRISPR/Cas9–RNA complex capable of generating DNA double-strand breaks (DSBs) at desired target sites. Successful attempts at fusing the tracrRNA and crRNA have enabled the generation of a single chimeric RNA strand called the single guide RNA (sgRNA), which can be effectively packed into any standard genetic payload delivery system. This breakthrough, alongside several others, provides researchers the capability to control the precise site-specific cleavage capabilities of the Cas9 protein and the flexibility conferred by specifically tailored sgRNAs to target nearly any locus in any target organism, including human cell lines and iPSCs. Although several different CRISPR/Cas systems have been repurposed for genome editing over the years, the most widely used CRISPR/Cas system is the *Streptococcus pyogenes*-derived type II CRISPR/Cas9 system. One of the prominent factors driving this popularity is the simple NGG PAM sequence requirement of the *S. pyogenes* II CRISPR/Cas9 system. However, this PAM sequence requirement is also one of the major limitations

of this system as the Cas9 protein screens and identifies the region immediately downstream of the sgRNA-target DNA duplex in search for an appropriate PAM sequence, failing which its DSB activity is aborted (Makarova et al. 2015; Adli 2018). Therefore, active research aiming to enrich our CRISPR/Cas toolbox is currently underway. Among these, notable mentions include the CRISPR from *Prevotella* and *Francisella* (Cpf1) endonuclease protein orthologs derived from *Acidaminococcus* sp. (AsCpf1) and *Lachnospiraceae bacterium* (LbCpf1), respectively (Bin Moon et al. 2018; Safari et al. 2019; Alok et al. 2020). Not only do Cpf1 protein naturally require only one sgRNA and recognize a broader range of PAM sequences as compared to the *S. pyogenes* derived Cas9, but they also generate 5' overhangs at the cut-site instead of blunt ends like CRISPR/Cas9 (Li et al. 2019).

CRISPR/Cas9 has two catalytic domains (HNH and RuvC), each of which mediates the cleavage of one strand. Together they are responsible for mediating DNA DSBs. A targeted point mutation in either one of the catalytic domains (D10A and H840A for SpCas9) results in the creation of a CRISPR/Cas9 nickase system capable of cleaving any one DNA strand in the target site. Point mutations in both the catalytic domains result in the complete ablation of DNA cleavage activity, resulting in creating a catalytically inactive or “dead” Cas9 (dCas9) complex. Therefore, a dCas9 complex possesses the target sequence specificity of the native CRISPR/Cas9 system but is devoid of the DSB capability. Researchers have exploited this strategy to modulate gene expression patterns in various systems by means of two approaches: CRISPR interference (CRISPRi) and CRISPR activation (CRISPRa). In the CRISPRi approach, scientists fuse the dCas9 protein to a potent repressor complex such as KRuppel-Associated Box (KRAB) and target it to a specific target site, resulting in strong gene repression activity. On the contrary, in the CRISPRa approach, the dCas9 protein is fused to a potent transcriptional activator such as VP64, which consequently upregulates gene expression. The VP64 transcriptional activator is composed of four tandem copies of the transactivation domain (VP16) of the Herpes simplex virus.

To summarize, the CRISPR platform has rapidly evolved with many advancements in the last few years for a wide range of applications, including neurodegenerative disease modelling as is discussed in the following sections.

iPSCs

Stem cells are a critical component of any complex living system which are capable of self-renewal and differentiation into several different terminal cell fates. They are classified as totipotent, pluripotent, multipotent, and unipotent based on the range of terminally differentiated cell lines they

can differentiate into. Totipotent cells can differentiate into any cell type affiliated to all three germ layers as well as extra-embryonic tissues like the placenta. Pluripotent cells can differentiate into cell lineages affiliated to all the three germ layers (ectoderm, mesoderm, and endoderm) but not the extra-embryonic tissues. However, both totipotent and pluripotent cells can only be found in the very early stages of embryonic development and hence cannot be utilized due to technical and ethical constraints (Mitalipov and Wolf 2009).

Cell differentiation was traditionally considered to be a unidirectional pathway that commits cells to a particular lineage with a specific set of functions and properties. However, in a culture dish, this idea was established to be incorrect when Shinya Yamanaka and his group were able to successfully reprogram somatic cells, a terminally differentiated cell line, into iPSCs in a culture dish (Takahashi and Yamanaka 2006). The authors utilized a cocktail of four reprogramming factors consisting of Octamer-binding transcription factor (Oct-4), Sex determining region Y-box 2 (Sox2), c-Myc, and Kruppel-like factor 4 (Klf4), which are now referred to as Yamanaka factors, to reprogram mouse and human fibroblasts (Takahashi and Yamanaka 2006; Takahashi et al. 2007). Another group led by James Thomson later generated stable iPSCs using a partially different reprogramming factor cocktail consisting of Oct4, Sox2, Nanog, and Lin28 to reprogram human fibroblasts (Yu et al. 2007). Since then, many genes (Dey et al. 2022) and cell types (Ray et al. 2021; Sundaravadivelu et al. 2021) have been identified that play a crucial role in generating iPSCs efficiently. Various technological advancements have been made with an aim to prospectively generate clinical-grade iPSCs for future biomedical applications (Dey et al. 2017, 2021; Saha et al. 2018a; Haridhasapavalan et al. 2019, 2020; Borgohain et al. 2019). Over the years since the discovery of iPSCs, the use of these cells in disease modelling applications has increased substantially (Young 2012; Singh et al. 2015; Tang et al. 2016; Saha et al. 2018b; Agrawal et al. 2021). Neurodegenerative disease modelling is no exception to this overarching trend, as is discussed in the following sections.

CRISPR and iPSCs in Disease Modelling

CRISPR-iPSC-based disease modelling eliminates the primary barrier surrounding the procurement of diseased tissue samples for traditional disease modelling applications: exposing patients to risky biopsies. This is significant as brain tissue is among the most inaccessible tissue systems in the body and brain biopsies are inherently risky. Cell lines derived from any easily accessible region of a patient's body can be converted into human iPSCs (Dey et al. 2017, 2021; Haridhasapavalan et al. 2019; Borgohain et al. 2019; Ray et al. 2021; Sundaravadivelu

et al. 2021). The iPSCs can subsequently be differentiated into the desired cell type by treating it with an optimized cocktail of growth factors and culture conditions (Young 2012; Hargus et al. 2014; Singh et al. 2015; Tang et al. 2016; Saha et al. 2018b; Agrawal et al. 2021). Using this approach, isogenic cell lines carrying one or multiple disease-relevant mutations can be derived from a single iPSC background, thus significantly eliminating genotypic variability (Beylina et al. 2021).

A pool of mutations has been implicated to underlie the pathology of most neurodegenerative disease phenotypes, with varying levels of penetrance. Moreover, multifactorial neurodegenerative diseases like AD, PD, or ALS are significantly modulated by a combination of both genetic and environmental factors (Barber 2012; Giri et al. 2016; Kim et al. 2020b; Tran et al. 2020; Vázquez-Vélez and Zoghbi 2021). Therefore, to model the entire gene pool of mutations responsible for a neurodegenerative disease process, it is necessary to procure cell/tissue samples and generate iPSCs from large cohorts of patients, demonstrating a significant degree of genetic heterogeneity with respect to the disease-causing mutations. Obligatory access to patient cell lines is a persistent limitation of iPSC-based disease models. This limitation is expunged by CRISPR-iPSC-based neurodegenerative disease models. CRISPR/Cas9 allows the seamless targeted editing of genomic DNA of any desired cell line, thus imparting the capability to introduce or correct mutations as desired. Non-patient-derived healthy iPSC lines, as well as patient-derived diseased iPSC lines harboring non-desired mutations, can also be utilized to generate the required isogenic disease models as mutations can easily be introduced or corrected by means of CRISPR/Cas9-mediated genome editing (Chen et al. 2020; Laverde-Paz et al. 2021; Hernández et al. 2021) (Fig. 1). Approaches like CRISPRa or CRISPRi can also be utilized to express or repress the production of specific gene products, respectively (Heman-Ackah et al. 2016; Tian et al. 2019; Inoue 2021). Thus, genome-edited iPSC-based disease models can be utilized to derive actionable insights regarding the mechanistic underpinnings of a disease process and its potential drug targets.

In this review, we will discuss some interesting examples of how CRISPR-iPSC-based disease modelling approaches are helping us generate scientific and clinical insights in a vast range of neurodegenerative diseases with unprecedented levels of flexibility, robustness, and reliability. This review also aims to present some of the emerging directions as well as perspectives of neurodegenerative disease research in a seamless narrative.

Alzheimer's Disease

Disease Pathology and Etiology

According to the National Institutes of Health, AD is the most prevalent neurodegenerative disorder inflicting a significant degree of medical, social, and financial burden on millions of individuals and their families globally (Selkoe et al. 1999). The entire pathological progression of AD can be segmented into three symptomatically characteristic epochs: preclinical AD, mild cognitive impairment due to AD, and dementia due to AD. Preclinical AD is characterized by measurable neurological changes such as abnormal amyloid β ($A\beta$) levels and decreased glucose metabolism, the early-stage pathological hallmarks of AD. As preclinical AD progresses to mild cognitive impairment due to AD, subtle cognitive and memory changes start to surface, which, although perceptible, does not interfere with an individual's ability to meet the cognitive rigors of everyday life. Finally, as the disease progresses further, the patient develops Alzheimer's dementia that is characterized by strong biomarker evidence as well as cognitive and memory decline, which noticeably impairs a person's ability to function normally in everyday life. AD is, therefore, a progressive condition, the symptomatic manifestations of which worsen with increasing age.

AD has two distinct forms: familial early-onset type AD (EOAD) and sporadic late-onset type AD (LOAD). EOAD is characterized by the onset of AD symptoms before the age of 65 years and is responsible for less than 5% of all reported AD cases (Wu et al. 2012). Over 270 highly penetrant mutations in the amyloid precursor protein (*APP*), presenilin 1 (*PSEN1*), and presenilin 2 (*PSEN2*) genes have been implicated in the pathogenesis of EOAD (Wu et al. 2012; Giri et al. 2016; Oksanen et al. 2017). The presenilin 1 (*PSEN1*) and presenilin 2 (*PSEN2*) genes encode components of gamma-secretase, which is the enzyme complex primarily responsible for sequentially cleaving the amyloid precursor protein (APP). Mutations in the *PSEN1* and *PSEN2* genes have been reported to alter gamma-secretase-mediated APP processivity. This alteration has been hypothesized to result in an increase in the intracellular concentration of the amyloidogenic (compound tending to produce amyloid deposits) $A\beta_{42}$ peptide, thus promoting EOAD pathogenesis (Carroll and Li 2016; Inoue et al. 2017).

Most individuals who develop AD are aged 65 or older and are affected by LOAD, which is primarily caused by the apolipoprotein E4 (*APOE4*) variant of the *APOE* family of fat-binding apolipoproteins (Liu et al. 2013;

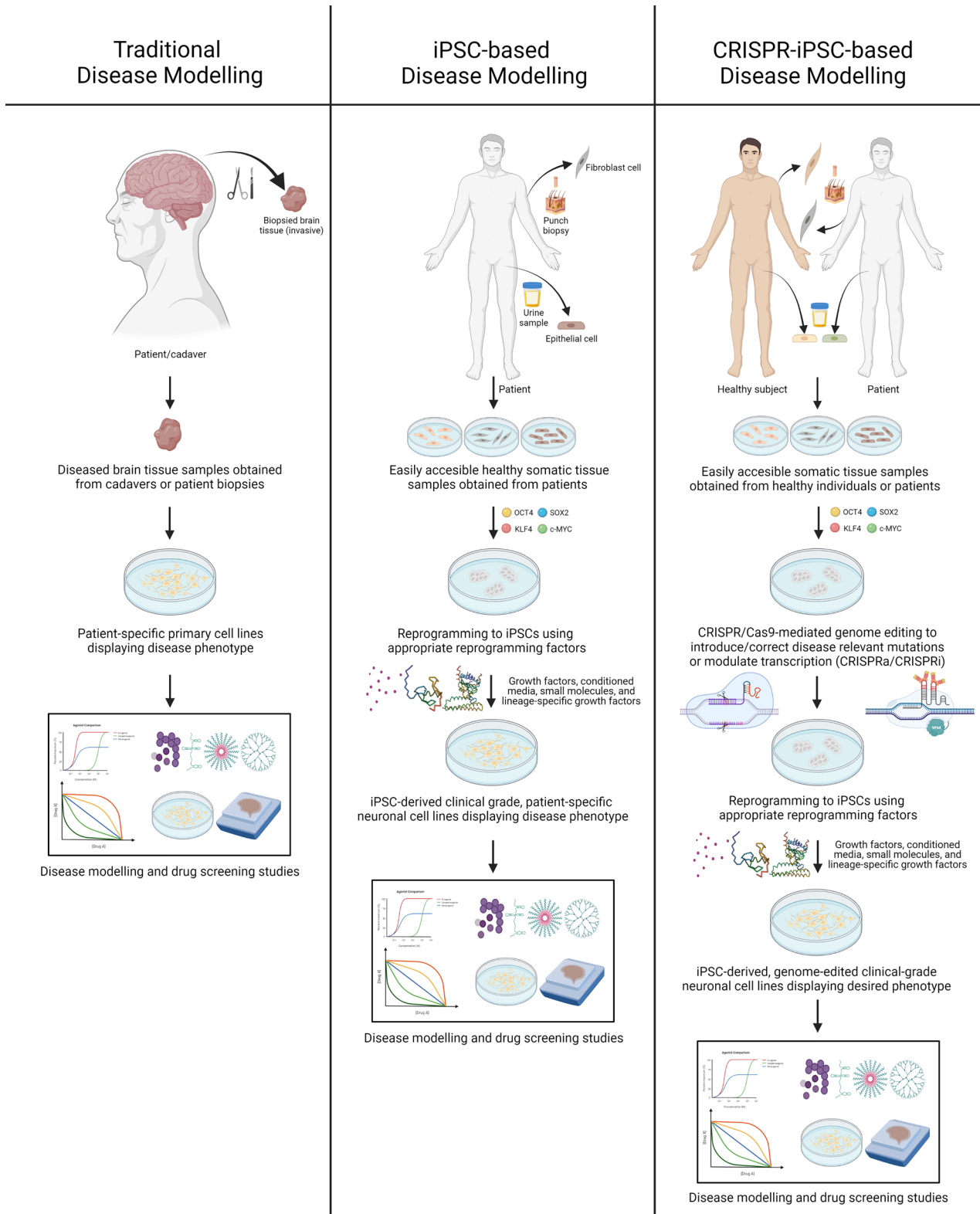


Fig. 1 A detailed comparison of traditional, iPSC-based, and CRISPR-iPSC-based disease modelling approaches

Oksanen et al. 2017; DeTure and Dickson 2019). *APOE2*, *APOE3*, and *APOE4* are the three polymorphic *APOE* alleles observed in humans among which *APOE3* has the highest frequency (77.9% of the general population), followed by *APOE4* (13.7% of the general population) and, finally, *APOE2* (8.4% of the general population) (Lee et al. 2020). Apolipoprotein E (*APOE*) is an important cholesterol carrier responsible for lipid transportation and injury repair in the brain (Liu et al. 2013). *APOE* polymorphic alleles are the main genetic factors that are indicative of LOAD risk. Individuals bearing the *APOE4* variant of the *APOE* allele are at an increased risk of developing AD than individuals with the more common *APOE3* variant (Liu et al. 2013). This is demonstrated by the fact that the occurrence of the pathological *APOE4* allele is significantly enriched in LOAD patients (Lee et al. 2020).

Both EOAD and LOAD pathology are characterized by the formation of intracellular neurofibrillary tangles (NFTs) and extracellular amyloid plaques (APs). NFTs constitute of hyperphosphorylated tau protein, whereas extracellular APs comprise of A β peptides. The extracellular A β plaques and neurofibrillary tangles tend to aggregate, propagating neuronal dysfunction and cell death, resulting in the pathological hallmarks of the condition (Israel et al. 2012; Wang et al. 2018; Penney et al. 2020; Valadez-Barba et al. 2020). Some of the other pathophysiological indicators of AD include inflammation, blood–brain barrier anomalies, gliosis, altered cellular and endocytic degradation pathways, and elevated DNA damage (Valadez-Barba et al. 2020).

Attempts to identify viable drug targets for AD have been ineffective thus far as no cure or therapeutic strategy for AD exists to date. The overwhelming reliance on mouse disease models to simulate the complex pathology of AD is considered to be one of the key factors behind this failure (Bolognin et al. 2019). This is due to several considerations such as the differences between mouse and human neurobiology, the significantly shorter lifespan of mice, and the significant amino acid composition differences of several protein orthologs implicated in neurodegenerative disease processes (Dawson et al. 2018; Ke et al. 2019; Penney et al. 2020). Furthermore, the lack of viable animal models capable of accurately recapitulating the pathological hallmarks of AD in humans has forced scientists to look for better alternatives such as iPSC-based human disease models (Vethe et al. 2017). To this end, several studies aiming to obtain a deeper understanding of the mechanistic underpinnings underlying AD pathology using the CRISPR-iPSC-based approaches have been reported, some of which are briefly discussed below.

CRISPR-iPSC in Alzheimer's Disease Modelling and Therapy

The first ever attempt to generate iPSC-based models of EOAD was reported in 2011 after a group successfully generated two EOAD patient fibroblast-derived iPSC lines containing either the *PSEN1* mutation or the *PSEN2* mutation (Yagi et al. 2011). Both the iPSC lines were subsequently differentiated into neuronal cells, which successfully recapitulated key aspects of EOAD pathology: increased A β peptide secretion and an elevated A β 42 concentration (Yagi et al. 2011). Subsequently, one group reported the generation of iPSC-derived neuronal disease models for LOAD as well as EOAD pathology (Israel et al. 2012). The authors reported several interesting insights pertaining to the complex nature of AD pathology, such as the magnitude of phenotypic variation that can exist between different iPSC-derived neuronal cell lines, thereby demonstrating the need for isogenic iPSC-based disease models (Israel et al. 2012).

In an attempt to better model the biochemical and pathophysiological implications of possessing the *APOE4* allele, one group generated iPSCs from patient skin fibroblasts and subsequently used them to produce *APOE4*-expressing neurons (Wang et al. 2018). The authors utilized a gene-editing approach to generate healthy *APOE3* isogenic controls from the diseased *APOE4* iPSC lines. Conversion of the disease-causing *APOE4* allele to the healthy *APOE3* allele eliminated the detrimental effects of *APOE4* and consequently rescued the cell from the disease phenotype, thereby implicating the role of the *APOE4* allele in the context of LOAD pathology. Furthermore, in this study, the authors also demonstrated the etiology of LOAD pathology, such as an increase in APOE fragmentation, tau phosphorylation, A β production, and GABAergic neuron degeneration. Another study utilized CRISPR/Cas9 to correct the *PSEN2* mutation in iPSC-derived basal forebrain cholinergic neurons (BFCNs) and reported the reversion of the diseased BFCN phenotype to the healthy BFCN phenotype (Ortiz-Virumbrales et al. 2017).

CRISPR-iPSC-based approaches provide interesting perspectives into how genetic mutations derange the healthy functioning of astrocytes. For example, in order to better understand how astrocytes with a deranged metabolism contribute to the AD pathology, one group generated isogenic diseased and control astrocyte lines from CRISPR/Cas9-edited iPSCs derived from EOAD patients having a mutant *PSEN1* gene (Oksanen et al. 2017). The diseased astrocytes demonstrated elevated A β production, modified cytokine release, Ca²⁺ homeostasis dysregulation, increased oxidative stress, depressed lactate secretion, and compromised neuronal function.

Dysregulated expression of a gene product due to copy number aberrations is known to be responsible for autosomal dominant disorders such as AD. A recent study aimed to generate monoallelic, biallelic, and triallelic knockouts of the *APP* gene in an AD patient-derived triallelic iPSC line, using a paired Cas9 nickase-based strategy (Ye et al. 2021). The genome-edited iPSCs were subsequently differentiated into cortical neurons, demonstrating APP dosage-dependent AD pathogenesis. The isogenic corrected lines (biallelic APP knockouts) also demonstrated a correction of the disease phenotype, thus demonstrating the research and therapeutic potential of gene dosage-manipulation of CRISPR-iPSC-based AD disease models (Ye et al. 2021).

Non-neuronal cells such as fibroblasts have been used to study several aspects of AD pathology over the past two decades (Govoni et al. 1996; Inoue et al. 2017; Iannuzzi et al. 2021; Inoue 2021). Although the premise might sound paradoxical at first glance, fibroblasts are an attractive non-neuronal proxy for modelling some biochemical aspects of AD pathology due to their availability, stability, and robustness. Furthermore, fibroblasts isolated from AD patients have been documented to demonstrate several alterations in their cellular physiology, such as altered ionic permeability, atypical signaling patterns, and increased production of the highly amyloidogenic A β 42 peptide (Inoue 2021). One group reported the use of a CRISPR/Cas9 synergistic activation mediator-based CRISPRa system to generate a fibroblast model capable of recapitulating aberrant gamma-secretase-mediated APP processivity, which is hypothesized to promote EOAD pathogenesis. In the study, the authors reported several interesting insights about intracellular APP processing and how CRISPRa based approaches can promote the utilization of non-neuronal AD models.

Recent studies have suggested that lipid droplets tend to accumulate in aged microglia, which in turn is associated with a dysfunctional proinflammatory phenotype (Marschallinger et al. 2020; Claes et al. 2021). The *TREM2* gene is deeply associated in the regulation of brain lipid biology and is known to encode a microglial lipid-sensor protein. Furthermore, the *TREM2* R47H mutation is associated with a significant elevation (up to threefold) in LOAD risk as it is one of the potent microglia-expressed risk factors (Claes et al. 2021). To improve our understanding of microglial lipid accumulation in AD pathogenesis, a group introduced a *TREM2* mutation (R47H) in a GFP-expressing iPSC line (AICS-0036 GFP line) using CRISPR/Cas9-based approach (Claes et al. 2021). The genome-edited, mutation-bearing iPSC line as well as its isogenic control was subsequently differentiated into hematopoietic progenitor cells and transplanted into an established chimeric AD mouse model. Interestingly, the iPSC-derived microglia which were xenografted in the chimeric AD mouse model demonstrated reduced in vivo lipid droplet accumulation as well as plaque

reactivity, CD9 expression, and plaque-associated APOE secretion (Claes et al. 2021).

Over the recent years, faults in the endolysosomal-autophagy network have been emerging as an important pathological process underlying AD pathology. Therefore, the role of aberrant endolysosomal function in AD development and pathology as well as its underlying genetics is currently an active area of investigation (Hung et al. 2021). The *BINI* (Bridging Integrator 1) gene is involved in the regulation of endosomal dynamics and has been strongly implicated in the development of AD pathology (Lambert et al. 2022). It is also known to demonstrate extensive differential splicing, giving rise to multiple splice variants having different tissue distributions. In order to better understand how different *BINI* isoforms contribute to AD pathogenesis, a group generated isogenic wild type as well as CRISPR/Cas9-mediated *BINI* knockout human iPSC lines, which were subsequently differentiated into neurons and astrocytes via an intermediate neural progenitor stage (Lambert et al. 2022). They reported that overexpression of *BINI* isoform 1 contributed to early-endosome size deregulation which is an early AD pathology marker, thus suggesting that tightly regulated *BINI* isoform 1 expression levels are required for proper neuronal endocytic trafficking (Lambert et al. 2022). The *SORL1* gene is responsible for encoding a sorting receptor protein involved in retromer-related endosomal trafficking, thereby regulating the anterograde as well as retrograde movement of APP between the early endosomes and trans-Golgi network (Yin et al. 2015; Knupp et al. 2020). Using a CRISPR-iPSC-based approach, a group generated *SORL1*-knockout human iPSC-derived neuronal and microglia-like cells to ascertain how *SORL1* ablation promotes endosome dysfunction and consequently AD pathogenesis. The authors reported that *SORL1* loss results in the induction of early-stage AD cytopathology (endosomal enlargement) in neurons but not the microglia-like cells, suggesting the presence of heterogeneous cell type-specific early AD pathology (Knupp et al. 2020). Using a comparable approach, another group reported the involvement of *PSEN1*, *APP*, and *SORL1* in a common pathway responsible for the regulation of the endolysosome system, which becomes dysfunctional in AD patients (Hung et al. 2021).

The *CLU* gene is yet another major genetic AD risk factor which, alongside *APOE*, regulates cholesterol transport and has a documented role in AD pathogenesis (Yu and Tan 2012; Robbins et al. 2018). Furthermore, A β treatment has been previously demonstrated to induce *CLU* expression in rodent neurons (Killick et al. 2014). To better understand the relationship between the *CLU* gene and AD, a group generated CRISPR/Cas9-edited, iPSC-derived *CLU* knockout cortical neurons as well as their isogenic controls and subjected both to A β treatment (Robbins et al. 2018). It was observed that the *CLU* knockout neurons were protected

from A β -induced phenotypes, while their counterparts underwent dose-dependent degeneration. The findings of the study indicated the role of the *CLU* gene product in A β peptide-mediated AD pathology and established it as a necessary effector of A β toxicity (Robbins et al. 2018).

Reports describing CRISPR-iPSC-based three-dimensional organoid culture-based AD disease models have also surfaced in recent years. In one such study, the authors utilized CRISPR/Cas9-mediated genome editing to introduce the faulty *APOE4* allele in iPSC-derived neurons, astrocytes, and microglia-like cells cultured in three-dimensional organoid systems (Lin et al. 2018). The results of the study suggested that *APOE4* impacts AD pathology through the impairment of astrocyte and microglia-mediated A β clearance (Lin et al. 2018).

The SARS-CoV-2 pandemic has promoted the emergence of interesting research perspectives exploring the intersection of virology and CRISPR-iPSC-based AD modelling. One such study tested the neurotropism of SARS-CoV-2 in iPSC models generated using the CRISPR/Cas9 nickase system and reported an increased rate of SARS-CoV-2 infection in neurons and astrocytes containing the *APOE4* allele. In the same report, the authors also demonstrated the remarkable effects of remdesivir in treating SARS-CoV-2 infection using CRISPR/Cas9-edited iPSC-derived brain cells, thus providing the medical fraternity with evidence in favor of treating neurological complications in COVID-19 patients using remdesivir (Wang et al. 2021).

Recent developments in iPSCs and CRISPR-iPSC-based disease models offer promising scientific insights in the search for a definitive cure for AD. How these scientific perspectives translate to an effective clinical intervention remains to be seen.

Parkinson's Disease

Disease Pathology and Etiology

Parkinson's disease (PD) is the second most prevalent neurodegenerative disease following AD. It is characterized by the progressive loss of dopaminergic neurons in a brain region known as the substantia nigra pars compacta due to the intraneuronal accumulation of misfolded α -synuclein aggregates (Vázquez-Vélez and Zoghbi 2021). The substantia nigra pars compacta is a midbrain structure that houses the dopamine secreting dopaminergic neurons. It plays a critical role in various cognitive activities such as motor behavior, motivation, reward, and working memory (Chinta and Andersen 2005). The death of the dopaminergic neurons due to α -synuclein aggregation disrupts the normal functioning of the basal ganglia, a brain structure responsible for orchestrating and executing movement (Shulman et al. 2011; Vázquez-Vélez and Zoghbi 2021). This ultimately results in

the clinical manifestation of PD, which is primarily defined by resting tremors, rigidity, bradykinesia, and postural instability. As the disease progresses, the pathology spreads beyond the confines of the basal ganglia, and more generalized nonmotor symptoms such as dyskinesias (involuntary and erratic body movements), distressing sensory experiences (pain), autonomic dysfunction (urinary incontinence, orthostatic intolerance), and neuropsychiatric manifestations (depression, hallucinations, and dementia) progressively emerge in the patient.

PD is categorized as familial or sporadic based on the family history of the affected individuals. Familial PD patients have a family history of PD symptoms and account for a small fraction (5–15%) of reported cases, whereas spontaneous PD patients develop the disease without any discernable hereditary predisposition (Klein and Westenberger 2012; Tran et al. 2020). The genetic architecture underlying PD pathology is complex and multifactorial. More than 90 variants of genes such as *SNCA* (Synuclein Alpha), *GBA* (Glucosylceramidase Beta), *LRRK2* (Leucine-Rich Repeat Kinase 2), and *MAPT* (Microtubule-Associated Protein Tau) have been implicated in PD pathology.

As of now, there exists no pharmaceutical or surgical intervention capable of curing PD. The current lines of pharmaceutical (levodopa) or surgical (deep brain stimulation) interventions provided to PD patients attempt to improve his/her quality of life and decelerate the onset of motor deficits characteristic of the disease process (Kalia and Lang 2015; Poewe et al. 2017; Paff et al. 2020; Sonninen et al. 2020). Therefore, it is imperative that new lines of treatment be designed to bring us closer to a cure to PD.

CRISPR-iPSC in Parkinson's Disease Modelling and Therapy

One of the first attempts to generate iPSCs from sporadic PD patient-derived fibroblasts was reported in 2009 by Soldner and colleagues (Soldner et al. 2009). The authors of this study successfully differentiated the PD patient-derived iPSCs into dopaminergic neurons, thus validating the viability of iPSC-based approaches to generating PD models using patient-derived fibroblasts (Soldner et al. 2009).

α -synuclein dysfunction is a critical milestone of PD pathology and causes a penetrant, aggressive form of PD accompanied with dementia (Auluck et al. 2010; Stefanis 2012). α -synuclein, one of the three paralogs of the synuclein family, is a 140 amino acid long 14 kDa presynaptic nerve terminal protein that is encoded by the *SNCA* gene (Fares et al. 2021). It plays critical roles in several neurophysiological processes such as neurotransmitter biosynthesis and release, vesicular trafficking, calcium homeostasis, mitochondrial function, and gene regulation (Auluck et al. 2010; Perez 2020; Singh and Muqit 2020). There is strong

evidence suggesting that the aggregation of α -synuclein is promoted by the introduction of point mutations, increases in copy number, and regulatory changes of the *SNCA* gene at the promoter level (Vázquez-Vélez and Zoghbi 2021). These aggregates are observed in both cell bodies (Lewy bodies) and the processes (Lewy neurites) of neurons principally located in the substantia nigra pars compacta, a key regulatory center of the basal ganglia (Auluck et al. 2010). Therefore, investigations probing the mechanistic basis of neurodegeneration caused by the *SNCA* gene and its various pathological variants have been an active area of interest in the context of iPSC-based disease modelling (Ryan et al. 2013; Dettmer et al. 2015; Kouroupi et al. 2017; Ke et al. 2019). For example, one group reported a successful attempt at developing a PD model capable of recapitulating the pathological hallmarks of dysfunctional α -synuclein overexpression propagated neurodegeneration (Devine et al. 2011). The authors used midbrain dopaminergic neurons generated from iPSCs obtained from a spontaneous PD patient possessing three copies of the *SNCA* gene (Devine et al. 2011). Another study investigating the deleterious effects of *SNCA* triplications using iPSC-based disease models described how α -synuclein overexpression impairs the differentiation of neuronal progenitor cells from iPSCs (Oliveira et al. 2015). Similarly, iPSC-based PD models harboring other gene mutations responsible for promoting PD pathology such as *LRRK2*, *PINK1*, *PARK2*, *GBAI*, *GBA*, and parkin have also been developed and elaborately explored by several groups over the past decade (Nguyen et al. 2011; Jiang et al. 2012; Lin et al. 2016; Ke et al. 2019; Hu et al. 2020).

In 2016, using a CRISPR-iPSC-based indel approach, Soldner and colleagues deleted and replaced a region of the intron-4 enhancer element of the *SNCA* gene containing PD-associated single nucleotide polymorphisms (SNPs) with different allelic variants (Soldner et al. 2016). In doing so, they generated a collection of isogenic iPSC-derived neuronal cell lines representing an allelic series of the SNP of interest. On interrogating the entire set of allelic variants for PD pathology-specific markers, the authors identified a risk variant in a noncoding distal enhancer element of the *SNCA* gene responsible for upregulating α -synuclein expression and consequently promoting the pathological descent demonstrated by PD patients.

Besides the range of issues pertaining to heterogeneous genetic backgrounds, researchers working towards the generation of genome-edited iPSC-based disease models for polygenic diseases such as PD face another pertinent challenge. This challenge is the painstaking and time-consuming process of screening genome-edited cell lines to separate the desired clones containing the on-target gene knock-ins from the aberrant clones plagued by unwanted mutations such as random integrations, on-target indels, and second-allele indels. To streamline this challenging ordeal, an automated

high-throughput, fluorescent marker-based, genome-edited iPSC phenotype screening workflow was demonstrated by Arias-Fuenzalida and colleagues. The authors utilized donor vectors to deliver fluorescent protein tags alongside the desired *SNCA* mutation-bearing sequences inside iPSC-derived neuroepithelial stem cells, an early neurodevelopment disease model (Arias-Fuenzalida et al. 2017). Following CRISPR/Cas9-mediated integration at the appropriate locus, the desired clones could be seamlessly separated from the aberrant clones using fluorescence-activated cell sorting (FACS) (Arias-Fuenzalida et al. 2017). A recent study reported another innovative approach to FACS-based enrichment of dopaminergic neurons (Überbacher 2019). The group performed a study that generated novel CRISPR/Cas9-edited iPSC-derived dopaminergic neuronal lines endogenously expressing fluorescent tyrosine hydroxylase-enhanced green fluorescent protein reporter (Überbacher 2019). Tyrosine hydroxylase is the rate-limiting enzyme of the dopamine biosynthetic pathway and is commonly employed as a dopaminergic neuron-specific marker protein (Haavik and Toska 1998; White and Thomas 2012). Since a typical iPSC differentiation workflow yields a heterogeneous population of dopaminergic neurons contaminated with other unsuccessfully differentiated cell types, the reporter-based approach demonstrated significant improvements in the identification and sorting of dopaminergic neurons as compared to conventional manual approaches (Überbacher 2019).

Our understanding of the mechanistic underpinnings of α -synuclein aggregation, kinetics, as well as its associated pathology has improved considerably over the past few years due to the concerted efforts of many different groups employing a diverse range of chemical, biological, and biophysical approaches (Wördehoff et al. 2015; Fauerbach and Jovin 2018; de Oliveira and Silva 2019; Ruggeri et al. 2020; Srivastava et al. 2020). In one such approach, a group seeded exogenous fibrillar α -synuclein in neuroblastoma cells (SH-SY5Y) and iPSC-derived neurons, neither of which possessed PD-pathology promoting genetic mutations (Gao et al. 2019). Using CRISPR/Cas9, the neuroblastoma cells (SH-SY5Y) were genome-edited to obtain isogenic α -synuclein knockout and α -synuclein expressing control cell lines. The authors monitored inclusion body formation and investigated the temporal behavior of autophagy/lysosomal proteins in the exogenous α -synuclein fibril-treated neurons. They reported evidence suggesting a connection between the formation of cytoplasmic α -synuclein inclusion bodies and changes in the autophagy/lysosome markers. Furthermore, they also discussed the viability of autophagy-promoting interventions such as 5-AMP-activated protein kinase activation as potential therapeutic routes for managing PD (Gao et al. 2019).

LRRK2 gene mutations are responsible for causing both sporadic and familial PD (Sánchez-Danés et al. 2012; Qing et al. 2017). After the ground-breaking discovery of iPSCs

by Yamanaka group (Takahashi and Yamanaka 2006), several reports describing different aspects of PD pathology in *LRRK2* mutant iPSC-based disease models began to surface (Nguyen et al. 2011; Sánchez-Danés et al. 2012). However, as Qing and colleagues observe, many of the iPSC-based contemporary studies ignored the non-trivial differences between the genetic backgrounds of the patient and non-isogenic control cell lines (Qing et al. 2017). The authors articulated the possibility that these differences could potentially mask critical insights pertaining to PD pathogenesis and even obscure promising novel drug targets (Qing et al. 2017). The generated *LRRK2* mutation-bearing dopaminergic neurons demonstrated decreased neurite length (< 2000 μm) and branching. Furthermore, the neurons with longer neurite lengths (> 2000 μm) were positive for Serine-129 phosphorylated α -synuclein, thereby implying the involvement of this post translational modification in the formation and maintenance of long neurites (Qing et al. 2017).

Dopaminergic neurons are not the only CRISPR/Cas9-edited iPSC-derived products under active investigation due to their role in PD. Several genes with established causative roles in the pathogenesis of PD are also responsible for mediating critical processes in astrocytes (Sonninen et al. 2020). Therefore, several groups have explored the roles of non-neuronal cells such as astrocytes in PD pathology using CRISPR-iPSC-based astrocyte disease models (Booth et al. 2017; Santos et al. 2017; Ke et al. 2019). Using CRISPR/Cas9, one group FLAG-tagged the endogenous SNCA locus of PD-affected as well as healthy iPSC lines and subsequently differentiated them into astrocytes to monitor intracellular α -synuclein accumulation (di Domenico et al. 2019). They also used CRISPR/Cas9 to correct a particular pathological point mutation (G2019S) in the *LRRK2* gene of a mutant iPSC line to generate isogenic diseased as well as disease phenotype-rescued astrocytes. Using astrocyte-dopaminergic neuron co-culture models, the group demonstrated that the astrocytes in PD patients exhibit severely compromised lysosomal α -synuclein degradation, thus resulting in progressive neurodegeneration. They also discovered that diseased astrocytes accumulate and transfer α -synuclein to neighboring neurons, thus significantly contributing to the growing body of research exploring the role astrocytes play in PD pathology (di Domenico et al. 2019). Another group corroborated these findings using a similar approach involving isogenic iPSC-derived, CRISPR/Cas9-edited diseased and disease phenotype-rescued astrocytes (Sonninen et al. 2020). They reported that *LRRK2* (G2019S) and *GBA* (N370S) mutant astrocytes demonstrate upregulated α -synuclein expression, elevated astrocytic Ca^{2+} levels, increased inflammatory reactivity, compromised mitochondrial DNA maintenance, and metabolic homeostasis perturbation such as altered polyamine metabolism (Sonninen et al. 2020).

The *GBA* gene is responsible for encoding the lysosomal hydrolase glucocerebrosidase, an enzyme which hydrolyses the sphingolipid waste product glucosylceramide into ceramide and is a documented risk factor for PD (Sidransky and Lopez 2012; Mullin et al. 2019). The *GBA* gene is particularly interesting in the context of CRISPR/Cas9-based genome editing as it shares a 96% sequence homology with a pseudogene *GBAPI* (Hanss et al. 2020). The presence of pseudogenes having a high degree of sequence similarity with the target gene complicates genome editing attempts. This is due to the higher risk of off-target effects and accidental pseudogene modification, resulting in a potentially pathological imbalance in the gene/pseudogene transcript ratio. Hanss and colleagues described a detailed CRISPR-Cas9-based approach involving a screening strategy capable of gene vs. pseudogene discrimination, thereby enabling the insertion and correction of point mutations in the *GBA* gene without any concomitant alterations in *GBAPI* (Hanss et al. 2020).

Genes involved in the pathology of neurodegenerative diseases such as AD and ALS have been reported to be associated with PD-associated genes. For example, the *LRP10* (Lipoprotein Receptor-Related Protein 10) gene has been recently reported to interact with the AD susceptibility gene *SORL1* in a study involving both iPSC-derived cell lines (astrocytes and neurons) and CRISPR/Cas9-edited *LRP10* knockout cell lines (HEK293T and HuTu 80) but not a combination of the two (Grochowska et al. 2021). The study nevertheless provided interesting insights pertaining to the intertwined nature of the genetic architecture of different neuropathological conditions. In another interesting study, Harjuhaahto and colleagues demonstrated the viability of iPSC-derived motor neurons in which either the *CHCHD2* gene or *CHCHD10* gene had been knocked out using a CRISPR/Cas9-mediated genome editing approach (Harjuhaahto et al. 2020). This is an interesting finding because dominant *CHCHD10* mutations are known to promote a range of pathological motor neuron conditions, whereas dominant *CHCHD2* mutations are known to cause PD (Shi et al. 2016, p. 2; Harjuhaahto et al. 2020). The authors of the study noted that although both the *CHCHD2* and *CHCHD10* knockout iPSC lines were viable and pluripotent, compromised mitochondrial respiration (increased proton leakage and respiratory rate) was observed in both knockout iPSC and iPSC-derived neuronal cell lines (Harjuhaahto et al. 2020). Interestingly, reciprocal compensatory increases in *CHCHD2/CHCHD10* levels were observed in either of the knockout cell lines. Furthermore, both the knockout motor neuron cell lines demonstrated significantly overlapping transcriptome profiles when compared to isogenic controls. These findings are indicative of the fact that a *CHCHD2-CHCHD10* complex is required for efficient

mitochondrial respiration, a fact corroborated by other studies (Burststein et al. 2018; Harjuhaahto et al. 2020; Xiao et al. 2020). This study bears significance in both the context of PD and ALS, discussed later in the review.

Knockout studies conducted over the past few years have yielded a lot of information pertaining to the effects of several new genes implicated in PD pathology. In one such study, a group knocked out three different early-onset autosomal recessive PD-associated genes (*PRKN*, *DJ-1*, and *ATP13A2*) in three separate iPSC lines, all of which were subsequently differentiated into midbrain dopaminergic neurons (Ahfeldt et al. 2020). *DJ-1* is known to have various functions such as transcriptional, chaperone, protease, and mitochondrial regulation as well as antioxidative stress reaction (Ariga et al. 2013). *ATP13A2* is a lysosomal P5-type transport ATPase which is known to be involved in transmembrane cationic transport (Estrada-Cuzcano et al. 2017; Spataro et al. 2019). *PRKN* is a cytosolic ubiquitin E3 ligase which ubiquitinates a number of cytosolic and outer mitochondrial membrane protein targets (Seirafi et al. 2015). Using the knockout cell lines and their corresponding isogenic controls, proteomics as well as a transcriptomics datasets were generated by the authors for deeper evaluation of the molecular insights (Ahfeldt et al. 2020). Although all the three knockout cell lines experienced elevated oxidative stress levels as well as dysregulated lysosomal and mitochondrial function, only the *PRKN* knockout line demonstrated an increase in death rate. Furthermore, the authors uncovered evidence suggesting the presence of shared as well as distinct dysregulated oxidative stress and autophagy-lysosomal pathways among the three knockout cell lines. This study highlights the need for further studies studying individual as well as the combinatorial effects of different genes on PD pathology (Ahfeldt et al. 2020). In that regard, a very recent lab resource paper reported the generation of CRISPR/Cas9-edited homozygous *PRKN*, *PINK1* and double *PINK1/PRKN* knockout iPSC lines which can be differentiated into neuronal and glial cells for studying PD and related neuropathological conditions (Chen et al. 2022). The *PINK1* and *PRKN* genes encode proteins involved in mitophagy-mediated dysfunctional mitochondria degradation, and mutations in these genes are among the most common causes of early-onset familial PD (Bradshaw et al. 2021). *VPS13C* is another notable PD risk factor belonging to a gene family (the *VPS13* gene family) responsible for encoding lipid transfer proteins which localize in several distinct contact sites located in-between membranous proteins. Using a CRISPR-iPSC-based approach, a group generated *VPS13C* knockout iPSC-derived neuronal cell lines in which they studied knockout-mediated lysosomal function alteration. Combining these insights with that derived from *VPS13C* knockout HeLa cells and *VPS13C*^{-/-} mice,

the group demonstrated the perturbation of lysosomal lipid homeostasis in *VPS13C*-deficient cells, thereby suggesting the involvement of *VPS13C* in PD pathogenesis-relevant pathways (Hancock-Cerutti et al. 2022).

Simple two-dimensional cell culture-based disease models fail to recreate these complex physiological conditions, whereas three-dimensional cell culture-based disease models recreate in vivo conditions and consequently recapitulate disease processes much more effectively. To demonstrate this, Bolognin and colleagues developed a CRISPR-iPSC-based microfluidic PD disease model that explicitly illustrated the superiority of three-dimensional cell culture-based disease models over their traditional two-dimensional counterparts (Bolognin et al. 2019). The authors used both healthy and *LRRK2* (G2019S) mutation-bearing iPSC lines to generate diseased and disease-corrected isogenic pairs using a footprint-free piggyBac vector system-potentiated CRISPR/Cas9-based genome editing approach. The genome-edited iPSCs were subsequently differentiated into neuroepithelial stem cells, wherein the authors observed notable differences in the neuronal differentiation outcomes between the parallelly maintained two-dimensional and three-dimensional cultures. Exciting developments and advancements have been made in CRISPR-iPSC-based PD modelling. How these developments ultimately lead us to a cure for this complex yet debilitating malady remains to be seen.

Huntington's Disease

Disease Pathology and Etiology

Huntington's disease (HD) is an adult-onset, hereditary, monogenic, autosomal dominant, and fully penetrant neurodegenerative condition. It is characterized by protein misfolding and aggregation in the brain, resulting in progressive psychiatric disruption, cognitive decline, and loss of motor coordination (Schulte and Littleton 2011; Ross and Tabrizi 2011; McColgan and Tabrizi 2018). The first clinical manifestations of HD's motor, cognitive, and behavioral characteristics arise at a mean age of 35 (Roos 2010). The average life expectancy for HD is approximately two decades from the age of symptomatic onset (Roos 2010; Schulte and Littleton 2011).

HD is caused by a CAG triplet repeat expansion mutation in a gene located on the short arm of chromosome 4p16.3 called the Huntingtin (*HTT*) gene (Roos 2010; Ross and Tabrizi 2011). The *HTT* gene is an essential gene that is most strongly expressed in the brain, and surprisingly the testes, but is expressed at lower levels throughout the body. It is known to interact with over 200 different proteins and plays a vital role in critical cellular processes such as axonal trafficking, transcriptional control, and apoptotic regulation

(Schulte and Littleton 2011). The number of CAG repeats present in the mutant *HTT* gene of symptomatic HD patients is generally observed to lie between 36 and 100 and in some cases even over 100 (Langbehn et al. 2010; Roos 2010; Schulte and Littleton 2011). Healthy individuals have less than 27 CAG repeats, whereas reduced HD penetrance is observed in individuals having less than 40 repeats. However, full HD penetrance is observed for individuals having 40 or more CAG repeats (McColgan and Tabrizi 2018). The number of the CAG triplet repeats present in the mutant *HTT* gene is inversely correlated with the age of onset (Langbehn et al. 2010).

The expanded CAG repeat in the mutant *HTT* gene encodes an expanded polyglutamine stretch in the Huntingtin (HTT) protein (Ross and Tabrizi 2011). This polyglutamine expansion in the mutant HTT (mHTT) protein results in the presentation of a toxic gain-of-function phenotype. The polyglutamine stretch causes the mHTT protein to misfold, resulting in intracellular aggregates within the brain cells. The rate of protein aggregation is proportional to the magnitude of the polyglutamine expansion in the mHTT protein (Schulte and Littleton 2011). This is consistent with the inverse correlation between the age of symptomatic onset in HD patients and the number of CAG repeats in their respective mutant Huntingtin genes, as previously discussed. These protein aggregates tend to overload the cell's ubiquitin-proteasomal degradation system, thus resulting in a progressive deterioration of the affected cell's homeostatic integrity (Schulte and Littleton 2011). Currently, there are no effective lines of treatment that prevents the onset or progression of HD. The presently available therapeutic regimes can only provide symptomatic relief to HD patients.

CRISPR-iPSC in Huntington's Disease Modelling and Therapy

The limited clinical translatability of the insights derived from animal HD models is one of the notable reasons behind the failure of the research community to develop a cure. Moreover, most mouse HD models have a far higher number of CAG repeats in comparison to their adult HD patient counterparts, thus further complicating disease modelling efforts (Csobonyeiova et al. 2020). Therefore, it is not surprising that iPSC-based and CRISPR-iPSC-based HD models are experiencing substantial growth in research and clinical interest in recent years.

Some of the earliest reports of iPSC-based HD models go back as early as 2010 (Zhang et al. 2010). One such study aimed to better understand HD disease pathology by generating striatal neurons from an HD patient-derived iPSC line having 72 CAG repeats. This study is particularly interesting in the context of striatal neurons because the mHTT protein causes transcriptional and transport inhibition of

brain-derived neurotrophic factor, a factor critical for striatal neuron maintenance. The authors of the study reported several interesting findings, such as the stability of CAG stretches over multiple passages in HD patient-derived iPSCs, and growth factor-deprivation promoted elevation in caspase activity (Zhang et al. 2010). In another interesting study, a group packaged a healthy *HTT* gene flanked by homologous arms in a bacterial artificial chromosome and delivered it inside an HD patient fibroblast-derived iPSC line via nucleofection. The iPSC-derived neural stem cells were subsequently transplanted in the striatum of an HD mouse model's brain, which successfully underwent differentiation in vivo into neuronal and glial cells (An et al. 2012).

As the interest surrounding CRISPR, iPSCs, and their application in disease modelling applications developed, CRISPR-iPSC-based HD modelling approaches started gaining significant momentum. Very recently, a group recently reported a CAG repeat tract contraction strategy using an engineered Cas9 nuclease variant recognizing the NGN PAM sequence instead of the NGG PAM sequence (recognized by the wild-type variant of the Cas9 protein), thus enabling more precise targeting of the faulty *HTT* gene (Oura et al. 2021). In 2014, a group reported an attempt to generate isogenic iPSC lines containing 97 CAG repeats in the *HTT* gene using a CRISPR/Cas9-based knock-in strategy. They successfully demonstrated the viability of utilizing CRISPR-assisted homologous recombination in CRISPR-iPSC-based HD disease modelling applications (An et al. 2014). Another group reported a dual CRISPR/Cas9 guide RNA-based approach to selectively knock-out the mutant *HTT* gene in a fibroblast-derived iPSC line and an iPSC-derived neural progenitor cell line by exclusively targeting mutation-specific PAM sites (Shin et al. 2016). Since HD patients homozygous for the mutant allele are very rare and one functional copy of the *HTT* gene is sufficient to maintain cellular integrity, this proof-of-principle study demonstrated a potential CRISPR-iPSC-based route to model and eventually cure HD by completely ablating mHTT expression inside a cell (Shin et al. 2016). The authors also discussed the translatability of their approach to a wide variety of other devastating dominant gain-of-function disorders such as AD, PD, and ALS (Shin et al. 2016).

The existence of non-trivial genetic differences between different iPSC lines and their therapeutic and clinical implications is well reported and discussed in the literature (Kajiwarra et al. 2012; Wang et al. 2020). These differences can arise due to different factors ranging from genetic heterogeneities in the donor cell lines to qualitative and quantitative differences in the differentiation protocols utilized to derive different iPSCs (Kajiwarra et al. 2012). This problem is compounded for CRISPR-iPSC-based systems due to the additional involvement of the CRISPR/Cas9-specific molecular machinery such

as expression vector cassettes and transfection agents. These differences in genetic backgrounds among different iPSC lines are important considerations in disease modelling applications. Genome-wide expression analysis has revealed higher numbers of gene expression differences between iPSC-based HD disease models and their non-isogenic controls compared to isogenic controls, thus illustrating how heterogeneous genetic backgrounds can generate false insights about the disease transcriptome (Xu et al. 2017). To ameliorate this pertinent issue, fully removable selection cassettes such as the piggyBac transposon as well as selection cassette-free approaches to deliver the CRISPR/Cas9 payload into iPSCs have been explored by different groups (Yusa 2013; Xu et al. 2017; Malankhanova et al. 2020). Furthermore, isogenic HD disease models are being established by different groups using CRISPR-iPSC-based approaches, thus enriching the repertoire of tools currently available to effectively model HD. For example, a group established several homozygous HEK293T cell lines containing different numbers of CAG repeats generated using several different genome editing strategies involving both the wild-type CRISPR/Cas9 system and the CRISPR/Cas9 nickase system (Dabrowska et al. 2020). They also established a mutation corrected and *HTT* knock-out variant of an established HD patient-derived iPSC line (ND42222) to serve as effective isogenic controls in future proteome as well as transcriptome profiling studies carried using neuronal cell lines derived from the same iPSC line (Dabrowska et al. 2020).

A recent study aiming to develop and validate a model for the purpose of estimating the diagnosed prevalence of HD propounded that HD prevalence is increasing with time (Crowell et al. 2021). Therefore, it is essential that we develop better therapeutic approaches to manage and eventually cure HD. The CRISPR-iPSC-based disease models discussed provide some promising perspectives towards that common goal.

Amyotrophic Lateral Sclerosis

Disease Pathology and Etiology

ALS, also referred to as von Gehrig's disease, is a multisystem neurodegenerative disorder (Mejzini et al. 2019). ALS is primarily characterized by upper and lower motor neuron degeneration. The motor neuron degradation eventually leads to progressive muscular atrophy, weakness, and voluntary muscle paralysis resulting in the complete inability to initiate or control voluntary movements. Similar to AD, PD, and HD, no clinical intervention to effectively cure ALS is available to date (Mejzini et al. 2019). All lines of current ALS treatment are purely focused on delaying disease progression and providing symptomatic relief (van Es et al. 2017; Chen 2020).

ALS is a complex polygenetic multifactorial disorder with a combination of genetic, environmental, and age-related factors considered to be responsible for the symptomatic onset of the disease. ALS can be categorized as familial ALS or sporadic ALS, depending on whether the patient had any prior family history or not. Although over 90% of reported ALS cases are sporadic in nature, the remaining 10% reports of familial ALS almost always demonstrate an autosomal dominant pattern of inheritance. Many potentially causative or disease-modifying genes have been identified to potentiate the pathological hallmarks of ALS by genome-wide association studies, with varying levels of characterization and confidence (Mejzini et al. 2019). However, the identities of the most penetrant gene mutations such as the *SOD1* (superoxide dismutase 1), *FUS* (fused in sarcoma), *TARDBP* (TAR DNA-binding protein 43), *TBKI* (TANK-binding kinase 1), and *C9ORF72* (chromosome 9 open reading frame 72) genes are well characterized (Nguyen et al. 2018; Burk and Pasterkamp 2019; Mejzini et al. 2019; Masrori and Van Damme 2020; Amado and Davidson 2021).

CRISPR-iPSC in ALS Modelling and Therapy

Since the first *SOD1* mutant mouse disease models were developed in 1994, several animal models such as *Caenorhabditis elegans*, *Drosophila melanogaster*, *Danio rerio*, and even higher mammal models such as pigs and primates have been used by different research groups. However, clinical translatability of the animal model-derived insights to human trials has always remained dismal, as evidenced by the fact that no cure for ALS exists to date (Mejzini et al. 2019; Liguori et al. 2021; Braems et al. 2021). iPSC-based ALS disease models soon came into prominence given the advantages they presented over conventional animal-based disease models, as already discussed in the context of other neurodegenerative diseases.

Using iPSC-derived motor neuronal cell lines carrying mutations in the *SOD1*, *TDP-43*, or *C9ORF72* gene, one group demonstrated the influence of the cell's genetic background in determining the levels of intracellular protein aggregation (Seminary et al. 2018). They also established that protein aggregation itself is insufficient to trigger the cellular heat shock response (Seminary et al. 2018). Since then, a variety of genes with differing levels of penetrance are responsible for ALS. Similar attempts to recapitulate the pathological hallmarks of different mutations such as the *FUS* mutation or *C9ORF72* mutation using iPSC-derived motor neuron cell lines sourced from patients can be found in the literature (Bilican et al. 2012; Sareen et al. 2013; Japtok et al. 2015). The effects of ALS causing mutations on iPSC-derived non-neuronal cell lines such as astrocytes have also been reported. In one such report, the authors

demonstrated that *C9ORF72* mutation-bearing astrocytes secrete soluble neurotoxic factors, which aggravate oxidative stress in healthy motor neurons (Birger et al. 2019).

CRISPR-iPSC-based disease models and studies leveraging the combined potential of iPSCs and CRISPR/Cas9-based genome editing have been reported. One such CRISPR-iPSC-based study attempted to rescue iPSC-derived motor neuron cells from the deleterious effects of the *C9ORF72* mutation (Krishnan et al. 2020). The *C9ORF72* mutation occurs due to a hexanucleotide repeat expansion (GGGGCC) in the first intron of the *C9ORF72* gene, which is suggested to result in the formation of abnormal mRNA transcripts responsible for causing a range of downstream issues. Some of these issues include mRNA misprocessing and faulty transcription, protein sequestration and aggregation, and the translation of abnormal protein fragments that form neuronal inclusions (Mejzini et al. 2019). In the study, the authors carried out a CRISPR/Cas9-based minimal promoter knockout of the first exon of *C9ORF72* in an iPSC line containing around a thousand copies of the hexanucleotide repeat in the first intronic region located downstream of the first exon (Krishnan et al. 2020). The motor neuron cells generated thereafter from the edited iPSC lines demonstrated reduced neurodegenerative phenotypes, suggesting the application of CRISPR/Cas9-based promoter region targeting as a viable and potentially usable therapeutic approach to correct toxic gene product-triggered disease phenotypes (Krishnan et al. 2020).

Another CRISPR-iPSC-based study published used CRISPR/Cas9-corrected iPSC-derived motor neuron lines originally sourced from familial ALS patients positive for *SOD1* and *FUS* mutations (Wang et al. 2017). In this study, familial ALS patient-derived fibroblasts bearing a *SOD1* mutation (A272C) as well as a *FUS* mutation (G1566A) were each utilized to establish a single mutation-bearing iPSC line. Following this, mutation-corrected isogenic controls of the two mutant iPSC lines were generated using CRISPR/Cas9-based genome editing. Finally, the mutation-bearing iPSCs as well as their corresponding isogenic controls were differentiated into motor neurons. The authors further proceeded to perform genome-wide RNA-seq analysis of the iPSC-derived motor neuron cell line derived from the *SOD1* mutant and its corresponding isogenic control iPSC line (Wang et al. 2017). A total of 899 aberrant transcripts were uncovered including transcripts to genes involved in critical processes such as signal transduction, extracellular matrix organization, homeostatic equilibrium maintenance, and neurogenesis implicating that a few different pathways may be involved in *SOD1*-associated early ALS pathogenesis (Wang et al. 2017). Apart from identifying potential early pathological markers of motor neuron decay due to *SOD1* mutation, the authors also demonstrated the viability of using integration-free single-stranded oligodeoxynucleotide

templates instead of the more conventionally used integrating plasmid-based constructs to deliver the corrected template strand to the Cas9 protein in vivo (Wang et al. 2017).

The combination of whole-genome sequencing-based gene variant discovery and CRISPR-iPSC-based disease modelling techniques has accelerated the discovery of rare yet highly penetrant ALS-causing novel gene variants which only certain ethnic groups are predisposed to (Yun et al. 2020). A sizeable fraction of the published studies have reported the utilization of CRISPR/Cas9-corrected patient-derived iPSC lines to generate isogenic disease models. As previously discussed, the versatility of CRISPR-iPSC-based disease modelling applications renders access to patient-derived cell lines an optional requirement. One such attempt has been recently reported wherein a group generated CRISPR-iPSC-based isogenic ALS disease models harboring a *SOD1* missense mutation (G93A) by utilizing a CRISPR/Cas9-based targeted knock-in approach on healthy iPSC lines. The genome-edited iPSCs were subsequently differentiated into motor neurons, demonstrating the pathological signatures of intracellular misfolded *SOD1* aggregate accumulation, such as reduced axonal length and branching, presynaptic and postsynaptic abnormalities, and anomalous electrical activity (Kim et al. 2020a). In another study, a group generated CRISPR/Cas9-mediated *SQSTM1* knockout iPSC-derived cortical neurons and assessed its impact on neuronal physiology (Poon et al. 2021). *SQSTM1* is a multifunctional protein involved in a wide range of cellular processes such as oxidative stress response, metabolic reprogramming, and autophagy. The authors demonstrated that the *SQSTM1* knockout iPSC-derived cortical neurons demonstrated alterations in mitochondrial gene expression patterns and functionality, autophagy flux, and mitochondrial spare respiratory capacity. Based on their findings, the authors suggested the role of *SQSTM1* to be related to mitochondrial function (Poon et al. 2021).

The neuromuscular junction (NMJ) is a very specialized structure where a motor neuron nerve terminal forms a synaptic junction with a muscle fiber. It is responsible for converting neuronal electrical signals into muscular contraction-initiating electrical activity. The NMJ also happens to be one of the best investigated and disease-prone synaptic structures of the nervous system. In fact, NMJ dismantling has been observed in ALS patients (Rodríguez Cruz et al. 2020). There is a growing body of evidence that indicates the need to consider muscle fibers alongside motor neurons as well as their functional interface, i.e., the NMJ, as important players in ALS initiation, progression, and therapy (Cappello and Francolini 2017; Jongh et al. 2021). However, the particulars of NMJ degeneration in ALS, such as its temporal relationship with motor neuron death or the exact series of events culminating in the disassembly of the NMJ circuitry, are poorly understood. It is essential that human NMJ disease

models are utilized in functional and mechanistic studies as rodent NMJs are significantly different from human NMJs in terms of size, complexity, fragmentation, and temporal stability (Stoklund Dittlau et al. 2021). To that end, several iPSC-derived three-dimensional cell culture, organoids, and microfluidics-based ALS disease models aimed to model the NMJ have been reported (Cappello and Francolini 2017; Osaki et al. 2018; Faustino Martins et al. 2020). In one such study, a group utilized CRISPR/Cas9 to introduce point mutations in *SOD1*(G85R), *TARDBP* (G298S), and *PFNI* (G118V), thereby establishing three mutant iPSC lines (Pereira et al. 2021), which were subsequently used to derive organoid cultures. The authors observed some distinct disease phenotypes such as innervated NMJs in the *SOD1* and *PFNI* mutants and a decrease in innervated NMJ area in the *TARDBP* mutant, suggesting the need to identify the mechanistic links between specific genes, their mutations, and aberrant NMJ physiology in the context of ALS (Pereira et al. 2021). Another notable mention among such reports is a recent attempt by a group to co-culture isogenic diseased (*FUS* mutant) and CRISPR/Cas9-corrected iPSC-derived motor neurons and human primary mesoangioblast-derived myotubes in a compartmentalized microfluidic device (Stoklund Dittlau et al. 2021). Mesoangioblasts are vessel-associated mesenchymal stem cells that are isolated from adult skeletal muscle tissue (Yucel and Blau 2019). The authors reported several interesting insights pertaining to the impact of the *FUS* mutation on NMJ formation as well as potential therapeutic strategies to ameliorate the detrimental effects of the mutation. They also demonstrated the spontaneous formation of NMJs between the motor neurons and myotubes under the influence of the chemotactic and volumetric gradients created between the two compartments of the microfluidic device (Stoklund Dittlau et al. 2021).

Among the four neurodegenerative diseases discussed, ALS has the most rapid progression. Therefore, it is imperative that more efforts be concentrated to establish a cure for this debilitating disease. CRISPR-iPSC-based disease modelling approaches, as summarized above, are a positive stride in this front.

Neurodegenerative Diseases: The Big Picture

The deranged genetic and biochemical signatures underlying the pathological and clinical manifestations of a neurodegenerative disease process are highly complex and heterogeneous. However, the overarching pattern of neurodegenerative progression with respect to the pathological markers, molecular players, and disease markers bear interesting congruencies as well as overlaps across most neurodegenerative

diseases (Cornblath et al. 2020). For example, TREM2, a microglial receptor encoding gene responsible for stimulating phagocytosis and suppressing proinflammatory cytokine production, has been associated with AD, FTD, PD, and ALS (Lee et al. 2020).

It is a well-established fact that pathological protein aggregation is among the primary drivers of any neurodegenerative disease process. There are, however, multiple concurrently acting mechanisms responsible for causing protein aggregation-mediated cellular dysfunction and degeneration (Davis et al. 2018; Cornblath et al. 2020). For example, the presence of A β , tau protein, α -synuclein, and TDP-43 protein aggregates have been reported in a variety of post-mortem brain tissue samples sourced from patients suffering from some form of neurodegeneration. This is a surprising finding as A β and tau protein aggregation is a hallmark of AD, α -synuclein is primarily implicated in PD, and TDP-43 mutations are best characterized in connection to ALS and frontotemporal dementia (FTD) (Strong 2012; Cornblath et al. 2020). Interestingly, the notable overlaps in the clinical, genetic, and pathological signatures of FTD and ALS have propagated the interpretation that these two diseases lie on a single spectrum (Ling et al. 2013; Ghosh and Lippa 2015). There is clinical evidence suggesting that a sparse subset of HD patients are susceptible to developing some clinical manifestations of ALS because mHTT may predispose motor neurons to TDP-43 associated pathology and promote the manifestation of some atypical pathological features (Tada et al. 2012). It has even been suggested that tau protein and α -synuclein together constitute a neurodegeneration-promoting deleterious positive feedback loop in which they promote the fibrillization and solubility of each other (Moussaud et al. 2014). Furthermore, in vitro and animal model-based studies suggest that these dysfunctional aggregate-forming proteins mutually interact among themselves, causing unique, concomitant patterns of neurological dysfunction and heterogeneous clinical manifestations of the disease markers (Cornblath et al. 2020). A thorough understanding of the underlying patterns and similarities between all the neurodegenerative disease phenotypes is therefore essential to design holistic, robust, and clinically translatable disease models leveraging the capabilities of the CRISPR/Cas9-mediated genome editing and iPSCs.

The four neurodegenerative diseases and disease modelling approaches covered in depth in the above sections are representative of the overarching trends, patterns, strategies, and emerging approaches in the domain of neurodegenerative disease modelling and research. In the following sections, four distinct groups of neurodegenerative diseases are discussed to further delineate the mechanistically as well as pathologically interrelated and interconnected nature of various neurodegenerative diseases.

Polyglutamine Disorders

HD belongs to a group of disorders known as polyglutamine disorders, all of which are characterized by an expansion of the glutamine-encoding CAG codon in different gene loci (Nóbrega and Pereira de Almeida 2018). In HD, the polyglutamine tract expansion occurs in the *HTT* gene, which eventually triggers the pathological hallmarks of the disease, as already discussed. Aside from HD, there are eight other polyglutamine disorders that are currently known to exist, which includes spinal and bulbar muscular atrophy (SBMA), dentatorubral-pallidoluysian atrophy, and six variants of spinocerebellar ataxia (SCA) (Banno et al. 2009). Since there are several fundamental similarities between these diseases, many of the disease modelling strategies and developments discussed for HD extend to other polyglutamine disorders as well.

Spinocerebellar ataxias (SCAs) are a collection of neurodegenerative diseases characterized by a progressive loss of balance, coordination, and speech slurring. Over 40 genetically distinct subtypes of SCA have been identified, which include both non-repeat mutations and repeat mutations (Klockgether et al. 2019). Therefore, only a subset of SCAs can be categorized as polyglutamine disorders. Several CRISPR-iPSC-based protocols for the generation of various SCA disease models such as SCA2 (CAG expansion) and SCA12 (CAG/CTG expansion) have surfaced over the years (Marthaler et al. 2016; Feng et al. 2021). However, to date, only one CRISPR-iPSC-based study involving polyglutamine SCAs has been reported for spinocerebellar ataxia type 3 (SCA3) (Ouyang et al. 2018). SCA3 is characterized by a polyglutamine tract expansion in the human *ATXN3* gene, which is an important component of the ubiquitin–proteasome protein degradation system (Zeng et al. 2018). In the study, SCA3 patient-derived iPSCs were subjected to a CRISPR/Cas9-mediated knockout of the expanded stretch of CAG repeats in exon 10 of the mutant *ATXN3* gene. The gene-corrected iPSCs retained pluripotent status and were subsequently differentiated into neural stem cells and, finally, neural cells. The gene-corrected *ATXN3* gene was observed to be able to express functional Ataxin-3 protein, capable of binding ubiquitin (Ouyang et al. 2018).

Spinal and bulbar muscular atrophy (SBMA), also referred to as Kennedy's disease, is a late-onset, X-linked, neuromuscular condition characterized by progressive lower motor neuron degeneration, primary myopathy, weakness, muscular atrophy, and complex multi-organ involvement resulting in non-neurological features like gynecomastia, testicular atrophy, reduced fertility, and erectile dysfunction (Grunseich et al. 2014; Querin et al. 2018). It is caused by an abnormal polyglutamine tract expansion in the *AR* (androgen receptor) gene located on the X chromosome. *AR* requires coregulatory proteins to affect a multitude of downstream

signaling pathways responsible for critical cellular functions like chromatin accessibility modulation, transcriptional machinery recruitment, and transactivation enhancement. However, mutant polyglutamine stretch-bearing *AR* receptors have been observed to cause transcriptional dysregulation resulting in the clinical hallmarks of SBMA. A group recently explored the possibility of using naturally occurring truncated *AR* isoforms lacking the expanded polyglutamine stretch as a genomic regulatory switch in androgen-responsive tissues (Lim et al. 2021). In the study, the authors identified the most abundantly expressed *AR* isoform from a pool of nine candidate *AR* isoforms using RNA-seq data obtained from dihydrotestosterone-treated iPSC-derived motor neurons as well as other cadaver-derived neuronal tissue samples. They also utilized CRISPR/Cas9-mediated genome editing to insert an in-frame FLAG tag 5' of the isoform encoding gene for immunofluorescence-based validation studies. This study represents a deviation from the general trend of CRISPR-iPSC-based neurodegenerative disease modelling studies, which typically involve studying and contrasting the characteristics of isogenic iPSC-derived, CRISPR/Cas9-edited diseased and disease corrected cell lines for a particular disease phenotype (Lim et al. 2021).

Motor Neuron Disease

Although the terms ALS and motor neuron disease (MND) are often used interchangeably, MND encompasses an entire family of clinical phenotypes characterized by progressive and selective motor neuron degeneration. Among MNDs, ALS is most prevalent in adults, whereas spinal muscular atrophy (SMA) is most prevalent in children (Strong 2012; Bowerman et al. 2018; Mejzini et al. 2019). Although there are some notable differences between the pathophysiological intricacies and progression patterns of ALS and SMA, animal model-based studies have suggested several overarching similarities such as selective motor neuron vulnerability, anomalous motor neuron excitability, and functional collapse of neuromuscular junctions due to motor neuron damage (Comley et al. 2016; Bowerman et al. 2018). Deeper molecular links between ALS and SMA involving the RNAP II/U1 snRNP machinery have also been reported (Chi et al. 2018). Furthermore, SMA is a monogenic disease, whereby aberrations or deletions of just one gene, the *SMN1* (survival motor neuron 1) gene, are responsible for over 98% of reported cases (Li et al. 2020). Therefore, SMA is much simpler to model compared to ALS, which is known to be precipitated by a plethora of genetic and environmental factors. Interestingly the *SMN1* gene has a paralog, *SMN2* (survival motor neuron 2), which is present in all SMA patients. However, the *SMN2* gene is unable to compensate for the aberrant *SMN1* because it possesses a translationally silent C-to-T transition in its seventh exon, which flips an exonic

splicing enhancer into an exonic splicing silencer. Thus, the ensuing alternative splicing causes over 90% of the translated protein to be truncated and hence non-functional (Li et al. 2020). One group reported a CRISPR/Cpf1-based genome editing strategy to convert the offending T in the seventh exon back to a C, thus making the edited *SMN2* gene transcriptionally equivalent to the *SMN1* gene. The gene correction was carried out in a patient-derived iPSC cell line, which was subsequently differentiated into motor neurons demonstrating significantly rescued SMN protein expression. This was also the first report wherein a group demonstrated efficient CRISPR/Cpf1 homology-directed repair-mediated genome editing in human cells (Zhou et al. 2018). A study published later reported the proof-of-principle of a novel therapeutic strategy to correct SMA involving the utilization of CRISPR/Cas9-mediated splicing-regulatory element disruption of SMA patient-derived iPSCs (Li et al. 2020). The splicing-corrected iPSCs were differentiated into spinal motor neurons, demonstrating appreciable disease phenotype decreases. This was shown by restored SMN protein expression and decreased apoptosis as compared to the unedited iPSC-derived motor neurons functioning as the negative controls (Li et al. 2020).

Neuronal Ceroid Lipofuscinoses

Neuronal ceroid lipofuscinoses (NCL), also referred to as Batten disease, are a collection of neurodegenerative lysosomal storage disorders characterized by an overaccumulation of lipofuscin, a fluorescent mixture of partially digested proteins and lipids, that progressively builds up with age in the lysosomes of various cell types, including neurons (Adler et al. 2015; Tang et al. 2021). Most neurodegenerative diseases tend to be present in adults and develop with advancing senility. However, an exception to this trend is juvenile neuronal ceroid lipofuscinosis (JNCL), the most common form of NCL. Batten disease typically presents between 4 and 7 years of age and is characterized by blindness followed by severe CNS deterioration resulting in epilepsy, motor deficits, cognitive decline, and ultimately death, all within the third decade of life (Gomez-Giro et al. 2019). The vision loss characteristic of JNCL is due to the degeneration of the light-sensing photoreceptor cells present in the outer neural retina, the exact mechanism of which is not well characterized (Gomez-Giro et al. 2019; Tang et al. 2021).

JNCL is caused by mutations in the *CLN3* gene. Although the exact function of the *CLN3* gene is still not fully understood, it has been observed to be active in the lysosomes and endosomes of a cell, suggesting that it plays a crucial role in interconnecting cytoskeletal dynamics to endocytic membrane trafficking (Mirza et al. 2019). A total of 78 different *CLN3* mutations have been reported as per the NCL Mutation and Patient Database (<https://www.ucl.ac.uk/ncl/>

[CLN3mutationtable.htm](#)) (Gomez-Giro et al. 2019). Since JNCL is a pediatric neurodegenerative disease, patient tissue samples are particularly difficult to source. Therefore, the requirement for standardized non-patient JNCL disease models is particularly high. A very recent iPSC-based study using patient iPSC-derived retinal pigment epithelium (RPE) disease models has reported the importance of *CNL3* gene in photoreceptor cell integrity via photoreceptor outer segment phagocytosis (Tang et al. 2021). A group reported the generation of a JNCL disease model harboring one such pathologic variant (*CLN3*Q352X) from healthy iPSC lines using CRISPR/Cas9-mediated genome editing (Gomez-Giro et al. 2019). Using the genome-edited iPSC disease models, the authors were able to recapitulate JNCL hallmarks in iPSC-derived endothelial cells. Half of the cerebral organoids generated from the genome-edited iPSCs aborted growth and development before reaching the differentiation endpoint. The organoids which managed to reach the differentiation endpoint demonstrated several pathological markers relevant to the early stages of the disease process. The authors also documented the manifestation of early pathological markers associated with synaptic formation and neurotransmission (Gomez-Giro et al. 2019). Another group reported a successful attempt at correcting a very common 1.02 kb deletion spanning exons 7 and 8 of the *CLN3* gene, using a CRISPR/Cas9-based HDR approach in iPSCs derived from homozygous as well as compound heterozygous patients. The attempt was successful, as exemplified by the presence of mutation corrected *CLN3* and wildtype *CLN3* transcript expression in both the genome-edited iPSC lines (Burnight et al. 2018).

Frontotemporal Dementia

Frontotemporal dementia (FTD) is another example of a group of heterogeneous disorders typically characterized by cognitive impairment impacting the frontal and temporal lobes of the brain, resulting in progressive brain atrophy. Other motor disorders such as supranuclear palsy, parkinsonian disorders, corticobasal syndrome, and MNDs share a number of clinically overlapping features with FTD (Lines et al. 2020). The gene mutations underlying FTD have significant overlaps with the mutations responsible for causing other neurodegenerative conditions as well (Abramzon et al. 2020). For example, over 40 mutations in the *MAPT* gene, one of the genes implicated in PD pathology, have been linked to FTD (Espay and Litvan 2011; Lines et al. 2020). FTD shares conspicuous similarities with other prominent neurodegenerative conditions as well, making it an interesting yet challenging disorder to treat. To that end, various iPSC and CRISPR-iPSC-based disease models have been developed by different groups over the years.

ALS and FTD are considered two ends of a single spectrum disorder where pure FTD and pure ALS represent

two distinct polarities of an otherwise indiscrete continuum (Ling et al. 2013; Ugboode and West 2021). Therefore, CRISPR-iPSC-based disease models aiming to model the deleterious effects of the *C9ORF72* mutation in the context of ALS also bear relevance for FTD. A hexanucleotide (GGGGCC) repeat expansion in the first intron of the *C9ORF72* gene is a well-established cause for ALS (Braems et al. 2020). The mutation results in the expression of glycine-arginine and proline-arginine dipeptide repeat proteins, which tend to bind to a large number of intracellular proteins, thereby promoting aggregate formation and consequently DNA damage (Babu et al. 2021). A study aiming to better understand the mechanism of dipeptide repeat protein-mediated DNA damage using a CRISPR/iPSC-based *C9ORF72* disease model revealed that dipeptide repeat proteins such as proline-arginine repeat proteins compromise the proper functioning DNA double-strand repair pathways by inhibiting the action of key proteins such as nucleophosmin (Andrade et al. 2020).

Mutations in the *CHMP2B* gene, responsible for encoding the charged multivesicular body protein 2B, have been identified across the entire FTD-ALS spectrum and are responsible for causing FTD-3 (FTD linked to chromosome 3) (Zhang et al. 2017; Ugboode and West 2021). Mutations in the *CHMP2B* gene, responsible for encoding the charged multivesicular body protein 2B, have been identified across the entire FTD-ALS spectrum. This protein is a core component of the ESCRT (endosomal sorting complexes required for transport) machinery and therefore plays a critical role in orchestrating a cell's topologically unique membrane budding and scission events (Schmidt and Teis 2012; Ugboode and West 2021). It is in fact a component of the ESCRT-III complex and is therefore directly involved in cell surface receptor maintenance (Zhang et al. 2017). Since mutations of the *CHMP2B* gene are rare, a deep understanding of the cellular and molecular events underlying FTD-3 is non-existent (Zhang et al. 2017; Ugboode and West 2021). To better understand the same, a group used a CRISPR-iPSC-based approach in which the global gene expression profile of iPSC-derived FTD-3 neurons was compared to their CRISPR/Cas9-edited disease phenotype rescued isogenic controls (Zhang et al. 2017). The diseased neurons exhibited atypical endosomes as well as several mitochondrial defects such as faulty cristae formation, mitochondrial respiration deficiencies, and elevated reactive oxygen species concentration. Furthermore, they observed evidence indicative of perturbed iron homeostasis in the diseased neurons. The authors were able to successfully recapitulate and ablate the disease phenotype-specific histopathological markers in the diseased and disease-rescued cell lines, respectively,

thus establishing the validity of the disease model. In this study, not only did the authors develop a CRISPR-iPSC-based disease model capable of recapitulating the disease phenotype of this rare disorder, but they also established a new tool to model intracellular iron accumulation in the context of neurodegenerative diseases (Zhang et al. 2017).

Mutations in the *PGRN* gene results in the haploinsufficiency of progranulin which accounts for up to 10% of all documented FTD cases (Lines et al. 2020). In fact, the disease phenotype for *PGRN* mutations manifests in a dose-dependent manner. Patients heterozygous for *PGRN* mutations experience decreased progranulin expression and are consequently affected by familial FTD. However, patients homozygous for the mutant *PGRN* gene experience a complete cessation of progranulin expression and consequently experience symptoms corresponding to that of the lysosomal storage disorder, NCL (Smith et al. 2012). One group developed patient-sourced *PGRN* mutation-bearing iPSC-derived neuronal models to study the effects of progranulin haploinsufficiency in FTD patients (Almeida et al. 2012). Using the model, the authors recapitulated several key markers indicative of the disease phenotype, such as the elevation of cytoplasmic TDP-43 levels, a finding in agreement with past reports (Lines et al. 2020). To better understand how the dose-dependent nature of *PGRN* mutations contribute to this duality of disease outcome, another group generated FTD patient-sourced *PGRN* mutation-bearing iPSC-derived human cortical neurons. They also generated a mutation-corrected isogenic control line using CRISPR/Cas9-based genome editing. The authors observed that the *PGRN* mutant neurons exhibited histopathological changes common to both FTD and NCL pathology such as lipofuscin accumulation, enlarged electron-dense vesicles, and granular osmiophilic deposits. Furthermore, they uncovered evidence suggesting that progranulin and its cleaved product, granulin E, are responsible for the activation of cathepsin D. Cathepsin D is a lysosomal protease capable of degrading a wide range of long-lived substrates, including disease-associated proteins such as α -synuclein, APP, and tau protein. Some variants of the cathepsin D-encoding gene, *CTSD*, have been linked to increased risk of AD, PD, and NCL (Valdez et al. 2017; Bunk et al. 2021). Therefore, the authors hypothesized that the dose-dependent, *PGRN* loss-propagated decline in cathepsin D activity compromises normal lysosomal function, a key factor underlying both FTD and NCL pathology (Valdez et al. 2017).

CRISPR-iPSC-based disease modelling approaches are also deepening our understanding of the mechanistic underpinnings of FTD and propelling us closer to a viable medical intervention for this yet incurable disease (Zhang et al. 2017; Jiang et al. 2018; Hofmann et al. 2019; Lines et al. 2020).

Conclusions and Future Perspectives

The immense potential of CRISPR/Cas9-mediated genome editing, iPSCs, and the combination of the two in the context of neurodegenerative disease modelling and therapeutics is undisputed. iPSC-based neurodegenerative disease models have experienced a significant increase in adoption over the past half-decade due to the advantages and convenience it offers over other more traditional disease modelling approaches, as already discussed. With the accumulation of research and the consequent maturation of this domain, the quality of disease models generated by the scientific community at large is improving impressively. For example, when iPSC-based disease modelling was at its nascency, insights derived from diseased iPSCs were often compared to non-isogenic control iPSCs derived from healthy family and/or gender-matched control subjects (Doss and Sachinidis 2019). This soon raised concerns regarding genetic background heterogeneity and its resulting confounding effects on the obtained datasets, prompting a methodological improvement in study design. As a result, most recent studies report the use of isogenic controls in the generated datasets and reported insights.

There are still several challenges in the road ahead. Prolonged *in vitro* culture can result in iPSCs developing passage-induced mutations by accumulating genetic aberrations such as chromosomal anomalies, genetic instability, copy number variations, and heterozygosity loss. Moreover, pre-existing random mutations extant in a small fraction of the parental somatic cells can get expanded because of the clonal expansion step present in any iPSC generation workflow. There have also been reports of genetic mutations and genomic instability arising in iPSCs as an unintended consequence of the reprogramming process, which raises concerns regarding their tumorigenicity (Yoshihara et al. 2017; Doss and Sachinidis 2019). The immense heterogeneity across different iPSC lines as well as their derivatives in terms of parameters such as differentiation potential, residual epigenetic memory, immunogenicity, tumorigenicity, parental cell line health, lineage commitment preference, batch to batch experimental variability, reprogramming protocol used, differentiation efficiency, and co-occurring contaminating cell populations bring into question valid safety concerns regarding their therapeutic application in a clinical setting (Yoshihara et al. 2017; Paik et al. 2018; Attwood and Edel 2019; Doss and Sachinidis 2019; Huang et al. 2019). Also, the technical and practical limitations in current high-throughput sequencing methodologies may obfuscate oncogenic aberrations in generated iPSC lines (Attwood and Edel 2019).

Like the current distributed consensus with respect to the therapeutic viability of iPSCs, concerns surrounding the safety and efficacy of the CRISPR/Cas9 genome

editing platform have also surfaced over the past several years. Concerns regarding potential off-target edits caused by the CRISPR/Cas9 system have been voiced by multiple groups (Zhang et al. 2015; Modell et al. 2022). Other concerns such as *in vivo* payload delivery, immunogenicity, tissue system targeting, and unwanted genetic mosaicism (due to concurrently occurring cell division and genome editing) also remain prominent matters of contention in the scientific community (Mehta and Merkel 2020; Nishizono et al. 2020; Gohil et al. 2021; Modell et al. 2022).

Attempts to resolve these pertinent issues surrounding both iPSCs and CRISPR/Cas9 technology are currently under active exploration. For example, highly precise surface-marker and fluorescence-based cell sorting approaches to yield pure populations of iPSCs and iPSC-derivatives have been reported by several groups (Lehnen et al. 2017; Arias-Fuenzalida et al. 2017; Schmitt et al. 2017; Hoshina et al. 2018; Patterson et al. 2020; Sriram et al. 2021). Several recent reports have also described promising approaches to improving the efficiency and reliability of the CRISPR/Cas9 system while minimizing the occurrences of unwanted off-target edits. For example, very recently, a group developed a synthetic light-sensitive sgRNA system that fragments on light irradiation, thereby offering scientists more granular spatio-temporal control of the entire genome editing process (Carlson-Stevermer et al. 2020). Another group recently reported an innovative competitive inhibition-based method of shielding off-target sites from the Cas9 endonuclease by co-delivering off-target site-directed short guide RNAs alongside the on-target guide RNA (Coelho et al. 2020). Highly precise single base editor CRISPR/Cas9 systems, an area of active research, now enable the seamless execution of targeted nucleotide edits without the need to introduce double-stranded DNA breaks at the target site. This development considerably improves the efficiency and simplicity of introducing as well as correcting SNPs for disease modelling and therapeutic applications (Cheng et al. 2019; Chen et al. 2021; Li et al. 2021).

Over the past few years, iPSCs have slowly started entering the realm of clinical application. In August 2018, the Kyoto University Hospital conducted the world's first clinical trial to treat Parkinson's disease patients using human iPSC-derived dopaminergic progenitor cells. Dopaminergic progenitor cells were derived from clinical-grade iPSC cell lines, subjected to a battery of quality checks and ultimately transplanted into the bilateral putamen of PD patients (Takahashi 2020). On January 14, 2022, the Keio University announced the successful transplantation of human iPSC-derived neural progenitor cells into the participant of a clinical study aiming to treat subacute spinal cord injury (<https://www.keio.ac.jp/en/press-releases/2022/Jan/14/49-92092/>) (Nagoshi et al. 2020; Sugai et al. 2021). In 2020, a study published a systematic multi-database analysis

of iPSC-based clinical trials featuring a total of 131 studies being conducted (Deinsberger et al. 2020). More are expected to start in the coming years. However, clinical trials featuring genome-edited iPSCs are yet to see the light of day. As the protocols for the generation of CRISPR/Cas 9-edited iPSCs are standardized and the current technical issues are resolved, it is reasonable to assume that with time, CRISPR-iPSC-based therapeutic protocols will enter mainstream clinical application.

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Author Contribution TS and RPT were responsible for conception and design, collection and/or assembly of data, data analysis and interpretation, manuscript writing, and final approval of the manuscript. All the authors gave consent for publication.

Declarations

Ethical Approval This article does not contain any studies with human participants or animals performed by any of the authors.

Conflict of Interest The authors declare no competing interests.

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