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Organoid and Pluripotent Stem Cells in Parkinson's Disease Modeling: An Expert View on their Value to Drug Discovery

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Abstract

Introduction: Parkinson's disease is a devastating neurodegenerative disorder preferentially involving loss of dopaminergic neurons in the substantia nigra, leading to typical motor symptoms. While there are still no therapeutics to modify disease course, recent work using induced pluripotent stem cell (iPSC) and 3D brain organoid models have provided further insight into Parkinson's disease pathogenesis and potential therapeutic targets.

Areas covered: This review highlights the generation of iPSC neurons and neural organoids as models for studying Parkinson's disease. It further discusses the recent work using patient-derived neurons from both familial and sporadic forms of Parkinson's to study disease pathogenic phenotypes and pathways. It additionally provides an evaluation of iPSC neurons and organoid models for therapeutic development in Parkinson's.

Expert opinion: The use of Parkinson's disease patient-derived neurons and organoids provides us with the exciting opportunity to directly investigate pathogenic mechanisms and test drug compounds in human neurons. Future studies will involve generating more sophisticated models of brain organoids, studying neuronal pathways using larger patient cohorts, and routinely assessing therapeutics in these models.

Keywords

Induced pluripotent stem cells; Organoid; Parkinson's disease; Therapeutics; Dopaminergic neurons; Midbrain; Neurodegeneration; α-synuclein; GBA

1. Introduction

1.1. Induced Pluripotent Stem Cells

The discovery in 2006 of four transcription factors (Sox2, Oct3/4, c-myc, and Klf4) marked the development of pluripotent stem cells from mouse fibroblasts [1], and was later

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replicated in human somatic cells [2, 3]. Current research efforts have identified protocols for generating induced pluripotent stem cells (iPSCs) from dermal fibroblasts, hematopoietic stem cells, adipocytes, and peripheral blood mononuclear cells [4, 5, 6, 7]. Together, these findings have opened the field to new advances in patient-specific cell lines and circumvented the need for embryonic stem cells which require gene editing and are linked to ethical concerns [8, 9, 10]. Furthermore, pluripotency now allows researchers to selectively differentiate stem cells into any somatic cell type, resulting in the generation of disease relevant tissues for study. iPSCs additionally offer a strategy for disease modeling using patient-specific cell lines and disease-relevant genetic backgrounds, thus allowing for new opportunities in therapeutic development and drug screening applications (Figure 1).

1.2. IPSC-Derived Neurons

Due to the lack of access to human neuronal tissues [11] and the intrinsic differences in animals models from human pathologies [12, 13], iPSCs provide new methods for modeling disease pathology for multiple neurodegenerative diseases including Alzheimer's, Parkinson's, Amyotrophic Lateral Sclerosis (ALS), and Huntington's disease. Specifically, the identification of neural fate induction by TGFβ antagonists through dual SMAD inhibition [14] has led researchers to further develop protocols for differentiating iPSCs into multiple different neuronal subtypes (cortical, cholinergic, dopaminergic, GABAergic, hippocampal, hypothalamic, motor, serotonergic and Purkinje neurons) as well as glial cells (astrocytes and oligodendrocytes) [15, 16, 17, 18, 19, 20, 21, 22, 23, 24, 25, 26, 27, 28, 29, 30, 31]. Furthermore, iPSC differentiation protocols have also been optimized to produce mature electrophysiological neurons supporting basic synaptic functions [32, 33] and have also been transplanted into primates for potential therapeutic applications [34]. For Parkinson's disease (PD), the ability to generate patient-derived dopaminergic neurons has proved to be particularly insightful, with current differentiation protocols using dual-SMAD inhibition followed by Sonic Hedgehog and FGF8b signaling, and subsequent maintenance in BDNF, GDNF, ascorbic acid, and cAMP [35]. Importantly, multiple studies have been able to recapitulate key PD pathological features and shed light on new mechanistic pathways using patient-derived iPSC dopamine neurons, which will be discussed in further detail in this review.

1.3. IPSC-Derived Neural Organoids

Organoids are derived from stem cells in a 3-dimensional matrix such as Matrigel or animal-derived hydrogels, which allow for efficient cell growth and differentiation. The successful growth of organoids further relies on the innate ability of stem cells to self-organize and form ordered structures and cyto-architecture [36], as well as their cell-cell interactions, and their ability to differentiate into diverse cellular populations [37, 38, 39]. Organoids have been used to model systems ranging from kidneys, liver, intestine, optic cup, cerebral, and midbrain regions [40, 41, 42, 43, 44, 45], reflecting the pluripotent state of iPSCs. Furthermore, organoids have become a critical tool in disease modeling from early stages of development following endogenous temporal stages within cell populations [37, 43], providing readily available models that can replicate disease phenotypes. Indeed, cerebral organoids have been used to model microcephaly, while midbrain organoids have been used to model sporadic PD [43, 46]. Importantly, 3D brain organoids further provide the potential

to model complex circuity by generating assembloids (assemblies of different region-specific organoids) to better advance our understanding of the human brain. These include recent studies investigating cell migration *in vitro*, such as the migration of GABAergic neurons from the ventral forebrain to the dorsal forebrain [47] and neuronal circuity such as cortico-thalamic assembloids which demonstrate projections between deep layer cortical neurons and thalamic neurons [48, 49]. Of note, such organoids may be used to facilitate organ-on-a-chip technology to utilize patient-specific iPSC-derived neurons as an alternative to conventional preclinical models for drugs screening [50]. Thus, brain-region-specific organoids are a valuable asset for studying pathology of diseases during development, and further offer great potential for drug screening in tissue specific environments.

2. IPSC-Derived Neuronal and Organoid Modeling and Drug Discovery in Parkinson's Disease

2.1. Parkinson's Disease (PD)

Parkinson's disease is associated with the progressive loss of A9-dopaminergic neurons in the substantia nigra leading to a loss of dopamine and the dysregulation of fine motor control localized in the basal ganglia. Ultimately, the death of dopaminergic neurons clinically manifests in parkinsonian symptoms including bradykinesia, muscular rigidity, and resting tremors [51] and pathologically involves the presence of Lewy Body aggregates comprised of α-synuclein [52]. PD exists as a multifaceted disease that presents itself clinically with some degree of heterogeneity [53, 54]. Approximately 10% of PD cases are familial with a genetically inherited mutation, while the rest are idiopathic and have an unclear etiology. At the cellular level, PD has been linked to defects in multiple pathways including abnormalities in mitochondrial and lysosomal function, protein accumulation, synaptic and axonal dysfunction, ER stress, and increased oxidative stress [51, 55]. Genetically, multiple genes have been linked to either dominant or recessive familial forms of PD including SCNA, LRRK2, PINK1, PARK2 (parkin) and GBA1, as well as additional genes such as DJ-1, PARK9 (ATP13A2), SJ-1 and VPS35. Thus, iPSC-derived neurons and organoids obtained from PD patients harboring mutations in these genes, as well as from idiopathic PD patients have allowed for new models for understanding PD pathology (Table 1) and testing therapeutics.

2.2. IPSC-Derived Neuronal modeling in PD

2.2.1. α-Synuclein (SNCA) models—The SNCA gene encodes the 14kDa monomeric protein α-synuclein, which has been linked to multiple functions including lipid binding and regulation of synaptic vesicles, and is a major component of Lewy Body aggregates in PD patients [52, 56]. Additionally, α-synuclein is a flexible protein that takes on different confirmations dictated by cellular stress and ligand binding [57, 58]. Both N-terminal point mutations (A53E, A53T, A53V, A30P, E46K, H50Q, and G51D) [59] as well as genomic triplication of the SNCA locus [60] lead to autosomal dominant forms of familial PD [56].

iPSC-derived dopamine neurons (iPSC-DA neurons) generated from patients harboring mutant A53T α-synuclein or α-synuclein triplication have highlighted multiple pathways disrupted in patient neurons. These include increased nitrosative stress and mitochondrial

dysfunction [61], disrupted synaptic connectivity and transcriptional alterations in synaptic signaling genes [62] and reduced α-synuclein tetramer to monomer ratio [63] in mutant A53T α-synuclein neurons. In addition, iPSC-DA neurons carrying α-synuclein triplication which have elevated levels of α-synuclein [64] demonstrate increased oxidative stress markers [65], decreased lysosomal hydrolase trafficking and lysosomal GCase enzyme activity [66] and increased oxidized dopamine levels [12]. They have also been found to have increased mitochondrial permeability transition pore opening via α-synuclein aggregates that interact with the ATP synthase [67], increased unfolded protein response and ER stress [68], defective ER-mitochondria contacts via VAPB [69] and decreased neurite outgrowth and neuronal activity [70]. Moreover, both A53T and α-synuclein triplication neurons have shown defects in mitochondrial respiration, membrane potential, morphology and expression of genes linked to mitochondrial function [71], as well as abnormal accumulation of Miro on the outer mitochondrial membrane contributing to delayed mitophagy [72]. Additionally, α-synuclein duplication and oligomer forming mutants (E46K and E57K) lead to impaired axonal mitochondrial transport and synaptic degeneration [73]. Interestingly, iPSC-derived cortical neurons harboring A53T or α-synuclein triplication also result in nitrosative stress, accumulation of endoplasmic reticulum (ER)-associated degradation substrates, and ER stress [74], suggesting that certain pathogenic phenotypes may also be present in non-dopaminergic neurons.

2.2.2. LRRK2 models—Leucine-rich repeat Kinase 2 (LRRK2) is a 285 kDa multi-domain protein consisting of Ras-GTPase domain (Roc domain), kinase domain, and protein binding domains with roles in neurite development, phosphorylation of multiple proteins and endocytic sorting via interactions with Rab-GTPases [75, 76, 77, 78, 79, 80]. Mutations in LRRK2 have been identified in the kinase domain (G2019S and I2020T), GTPase domain (R1441G, R1441C, R1441H, and N1437H), and the Carboxy terminal of the Roc domain (Y1699C), leading to autosomal dominant forms of familial PD [81].

LRRK2 G2019S iPSC-DA neurons have been shown to have increased oxidized dopamine [12], increased levels of the lysosomal receptor for chaperone mediated autophagy [82], transcriptome profiles similar to that upon rotenone treatment [83], microRNA alterations [84] and global DNA hyper-methylation [85], and both LRRK2 G2019S and R1441G DA neurons also show impaired NF-κB signaling [86]. iPSC-DA neurons harboring the G2019S and I2020T mutations also show increased levels of apoptosis, reduced neurite outgrowth and length, a disruption of tau and tubulin phosphorylation, an increase in autophagosomes and autophagy genes, increased mitochondrial DNA damage, impaired Miro recruitment to the mitochondria and dysregulation of mitophagy, and upregulation of α-synuclein expression [77, 78, 87, 88, 89, 90, 91, 92, 93]. Interestingly, LRRK2 was also shown to regulate synaptic vesicle recycling, as it phosphorylates auxilin in its clathrin-binding domain at Ser627, leading to disrupted synaptic vesicle endocytosis and decreased synaptic vesicle density in LRRK2 R1441G/C mutant iPSC-DA neurons [94]. Thus, studies in iPSCs investigating LRRK2 function have helped to characterize its role in PD pathogenesis, and may identify additional targets for drug development in future studies.

2.2.3. PINK1 and Parkin models—PINK1 (phosphatase and tensin homolog (PTEN)-induced Putative Kinase1) is a mitochondrial Ser/Thr protein kinase, that is normally cleaved and released into the cytosol for degradation [95]. PINK1 localizes to the mitochondrial membrane upon its depolarization where it phosphorylates Parkin [96, 97, 98]. Parkin is encoded by the PARK2 gene and is an E3 ubiquitin protein ligase which translocates to the mitochondria to ubiquitinate mitochondrial substrates upon PINK1 activation. Together, PINK1 and Parkin regulate mitochondrial health and initiate mitophagy events, and mutations in either gene are associated with autosomal recessive and early onset forms of PD [97, 99, 100, 101].

iPSC-DA neurons from patients expressing PINK1 nonsense (Q456X) or missense (V170G) mutations show mitochondrial defects including impaired parkin recruitment to mitochondria, increased mitochondrial copy number, and upregulation of PGC-1α, a key regulator of mitochondrial biogenesis [102]. PINK1 G411S or Q456X mutant neurons also demonstrate reduced PINK1 kinase activity and mitochondria quality control [103]. In addition, S-nitrosylated PINK1 decreases Parkin translocation to mitochondrial membranes and disrupts mitophagy in iPSC-DA neurons [104]. Of note, partial genetic and pharmacological inhibition of fatty acid synthase was able to decrease toxicity in PINK1 mutant iPSC-DA neurons, potentially by increasing levels of the mitochondrial inner membrane-specific lipid cardiolipin [105].

Parkin mutant iPSC-DA neurons also show mitochondrial alterations [106] which can be rescued by induced expression of the parkin interactor mitochondrial Stomatin-like protein 2 (SLP-2) [107], as well as defective ER-mitochondria contacts and calcium transfer [108] and increased levels of soluble epoxide hydrolase which is involved in inflammation [109]. In addition, both Parkin A324 fsX110 and PINK1 Q456X mutant iPSC-DA neurons show increased α-synuclein accumulation [110] while iPSC-hypothalamic PINK1 and Parkin mutant neurons have excess ER-mitochondria contacts and abnormal lipid trafficking which may deplete phosphatidylserine from the ER to disrupt neuropeptide-containing vesicles production [111].

Interestingly, parkin V324A mutant iPSC-DA neurons also show increased levels of oxidized dopamine and decreased GCase activity [12]. Moreover, iPSC-DA neurons with knockout or mutant parkin have increased expression of monoamine oxidase (MAO) A and B and dysregulation of dopamine release and uptake via dopamine transporter (DAT) and aberrant dopaminergic regulation of presynaptic glutamatergic transmission [112, 113, 114]. Of note, mutant PINK1 Q456X neurons also show increased oxidized dopamine [12], suggesting a further role for both PINK1 and parkin in regulating dopamine homeostasis. Thus, PINK1 and Parkin contribute to a major component of mitochondrial homeostasis and dopamine metabolism, and their loss of function may help drive the loss of DA neurons in the substantial nigra.

2.2.4. GBA models—The GBA1 gene encodes a lysosomal enzyme called glucocerebrosidase (also known as GCase or β-glucosidase) that catalyzes the hydrolysis of glucosylceramide (GlcCer) to glucose and ceramide, as well as the hydrolysis of D-glucosyl-N-acylsphingosine to D-glucose and N-acylsphingosine. Homozygous or compound

heterozygote GBA mutations are known to cause Gaucher disease (GD), the most common lysosomal storage disorder. After the first report of GD patients with symptoms of Parkinsonism [115], other studies confirmed the link between PD and GBA mutation including insertion, deletion, frame shift and point mutations in GBA [116, 117, 118, 119]. Approximately 5–10% of PD patients carry GBA1 mutations [120] and the two most common mutations in GBA (N370S and L444P) account for ~3% of GBA-liked PD [115, 121]. Clinically, GBA mutation carriers tend to have an early onset [122, 123] and more cognitive symptoms in addition to severe motor symptoms [124, 125, 126].

Many studies have shown reduced protein levels and GCase activity in mutant GBA patient iPSC-derived neurons across multiple GBA mutations including N370S/N370S, N370S/c0.84dupG, N370S/WT, RecNcil/WT and L444P/WT [127, 128, 129]. In particular, abnormal GCase post-translation has also been observed in iPSC-derived neurons from N370S heterozygous patients [130]. Moreover, in addition to α-synuclein accumulation in GD-linked GBA iPSC-derived neurons [117], increased α-synuclein levels [127, 129, 130, 131] and its aggregation [128, 132] have also been reported in PD-linked GBA iPSC-derived neurons. Accumulation of lipids have also been identified as a major hallmark of mutant GBA iPSC-derived DA neurons including GCase substrates, glycolipids glucosylceramide (GlcCer) and glucosylsphingosine (GlcSph) which are increased in GBA mutant neurons [127, 128, 129, 130].

Additionally, defective function of cellular organelles including lysosomes, mitochondria and the ER have been demonstrated in mutant GBA iPSC-derived neurons. Increased size and number of lysosomes [129] which may result from the reported lysosomal degradation capacity impairment [130] have been clearly observed, along with evidence of autophagic defects [129, 130], potentially rendering neurons more vulnerable to apoptosis. Moreover, mitochondrial dysfunction including decreased oxygen consumption rate (OCR), reduced complex I activity and altered NAD+ metabolism together with altered mitochondrial morphology have also been reported in mutant GBA iPSC-DA neurons [133]. Furthermore, upregulation of ER stress was also observed in multiple studies, leading to defective downstream cellular mechanisms such as calcium homeostasis and the unfolded protein response (UPR) [129, 130, 133]. Lastly, the levels and uptake of dopamine were reduced in GBA N370S iPSC-DA neurons [127, 131], along with upregulated mRNA and protein levels of MAO-B [131], while 84GG/WT iPSC-DA neurons also showed elevated levels of oxidized dopamine [134]. Of note, single-cell transcriptomic analysis of GBA N370S iPSC-DA neurons have highlighted the transcriptional repressor histone deacetylase 4 (HDAC4) as a potential upstream regulator of ER stress and disease pathogenesis [135]. Overall, understanding the role of GBA mutations in PD pathogenesis may further provide possible therapeutic strategies relevant to other forms of PD as discussed below.

2.2.5. Additional genetic PD models—Additional genes linked to familial PD such as DJ-1, PARK9 (ATP13A2), SJ-1 and VPS35 have also been used to model PD pathogenesis in patient-derived iPSC-DA neurons. DJ-1 is involved in regulating mitochondrial oxidant stress and its loss of function mutations result in autosomal recessive PD [136]. iPSC-DA neurons with mutant DJ-1 exhibit elevated mitochondrial oxidation, increased oxidized DA which is exacerbated over time, and decreased basal respiration, in

addition to decreased lysosomal proteolysis and GCase enzyme activity and α-synuclein accumulation [12].

PARK9 encodes a lysosomal type 5 P-type ATPase involved in cation homeostasis, ATP13A2. The loss of ATP13A2 function leads to lysosomal dysfunction and the accumulation of α-synuclein seen in Kufor-Rakeb syndrome (KRS), a rare form of juvenile-onset PD and familial PD [137, 138]. Recently, loss of function mutations of ATP13A2 in iPSC-DA neurons have exhibited α-synuclein accumulation as well as impaired lysosomal exocytosis due to the disruption of calcium homeostasis [139, 140]. Interestingly, ATP13A2 overexpression or activators of the lysosomal calcium channel TRPML1 (transient receptor potential mucolipin 1) were able to increase α-synuclein secretion and lysosomal exocytosis, and prevent neuronal toxicity [140]. ATP13A2 thus offers potential as a therapeutic target for PD-related synucleinopathies by increasing lysosomal exocytosis and neuronal secretion of intracellular α-synuclein.

In addition, the homozygous missense R258Q mutation in the Sac domain of Synaptojanin (SJ-1) results in autosomal recessive early onset PD [141, 142, 143]. iPSC neurons with the SJ-1 mutation exhibit accumulation of Atg18a on nascent synaptic autophagosomes, thus blocking autophagosome maturation and contributing to dopaminergic neuron loss [144], further suggesting a role for SJ-1 in synaptic macroautophagy. Finally, the D620N mutation in the retromer protein VPS35 leads to autosomal dominant PD [145, 146], and iPSC-DA patient neurons show defective synaptic transmission AMPA-type glutamate receptor (AMPAR) recycling [147], which may further contribute to dopaminergic neuron loss.

2.2.6. Idiopathic PD—The generation of iPSC-derived DA neurons from patients has for the first time, allowed for the possibility to model non-genetic forms of PD and provide important insights into sporadic etiology, which account for the majority of PD patients. Interestingly, idiopathic PD patient-derived DA neurons have been shown to have decreased basal mitochondrial respiration, increased oxidized dopamine levels and oxidized DJ-1, and decreased GCase enzyme activity [12] and maturation [132]. In addition, idiopathic DA neurons also demonstrate microRNA alterations [84], global DNA hyper-methylation [85], as well as impaired Miro degradation and mitochondrial motility [89], suggesting that pathways disrupted in genetic forms of PD may be similarly affected in idiopathic forms.

Of note, environmental factors have also been proposed to play a key role in driving PD pathogenesis [148, 149, 150, 151]. These include previous studies reporting the association between smoking and monoamine oxidase B (MAO-B) polymorphism [152], agricultural insecticides and polymorphisms in the Acetylcholinesterase/paraoxonase locus [153], pesticide exposure and the CYP2D6 polymorphism [154, 155], and mitochondrial toxin-induced defects in α-synuclein A53T iPSC-derived neurons [61]. Thus, although the role of gene-environmental interactions (GxEs) in PD are still not completely understood, the use of patient neurons with mutant genetic backgrounds together with environmental risk factors such as toxins may be useful in further testing the two-hit (or double hit) hypothesis in PD progression and further used for drug discovery models.

2.3. Neural Organoid Modeling in PD

Midbrain-like organoids were first characterized in 2016 and have been gaining interest with their potential to model PD [42]. In particular, organoids offer the advantage of providing a more appropriate 3D niche environment which can consist of multiple cell types, special organizational structure, and enhanced cellular maturity [156]. Further characterization of midbrain-like organoids have shown the presence of neuromelanin-like granules and the ability to form functional neural networks [42, 157]. Thus far, studies have found that midbrain-like organoids derived from LRRK2 G2019S iPSCs show upregulation of the thiol-oxidoreductase TXNIP [46] and an increase in the floor plate marker FOXA2 during organoid development [158]. Consequently, midbrain-like organoids need to be further investigated in studies of PD, as they have great potential for identifying neurodevelopmental and novel characteristics of PD that cannot be recapitulated in 2D neuronal systems.

2.4. PD Drug Discovery in iPSC Neurons

The overall drug discovery process using iPSCs has been reviewed recently [21] and our focus will be on discoveries made using iPSC models of PD (Table 2). Therapeutic strategies have currently shown potential in inhibiting overactive proteins, rescuing phenotypes with wildtype proteins, or using agonists to rescue the activity of associated proteins.

In particular, multiple studies have highlighted the potential use for targeting the GCase pathway in PD human iPSC-DA models. GBA chaperones NCGC758 and NCGC607 were found to restore GCase activity and reduce substrate accumulation in the lysosome in multiple PD models of iPSC-DA neurons [127, 132]. In addition, studies on GCase have identified quinazoline inhibitors that can be derived into activators that stabilize GCase activity within iPSC-DA and fibroblasts [159]. Moreover, inhibition of acid ceramidase using carmofur was shown to reduce α-synuclein accumulation in GBA-1 mutant iPSC-DA neurons [160], while reducing glycosphingolipids in GBA mutant (N370S/c.84dupG) or α-synuclein triplication neurons diminished pathology and restored physiological α-synuclein conformers that associated with synapses [161]. Finally, recent work has identified a novel chemical series of GCase activators, including a new small-molecule modulator (S-181) that increased wild-type GCase activity in iPSC-derived dopaminergic neurons from patients with 84GG-GBA1, as well as in LRRK2-, Parkin-, DJ-1-linked and sporadic PD [134]. Thus, GCase activity represents a major target for PD therapeutic treatment that is associated with multiple forms of PD, including both genetic and idiopathic cases.

Additionally, other drugs have also been tested in iPSC-DA models which have been shown to help ameliorate several phenotypes. Mitochondrial antioxidants, mito-TEMPO and NAC, show reduction of oxidized dopamine leading to reduced insoluble α-synuclein levels and increased GCase activity in patient iPSC-DA neurons [12]. Activation of MEF2C using isoxasole was sufficient to rescue α-synuclein A53T iPSC-DA neurons from nitrosative stress via the MEF2C-PGC1α pathway by increasing mitochondria respiration and biogenesis [61]. In addition, LRRK2 G2019S iPSCs treated with the MEK inhibitor PD0325901 showed protection from oxidative stress [78], while knockdown of α-synuclein showed rescue of the phenotype and survival of DA neurons [92]. Interfering with α-

synuclein oligomerization in iPSC-DA neurons with NPT100–18A, NPT100–14A, and ELN48228 also rescued PD pathology [62]. Moreover, upregulation of Synoflavin or the ubiquitin ligase NEDD4 which it affects were shown to reverse accumulation of GCase and other glycoproteins in the ER in α-synuclein A53T and triplication iPSC-derived cortical neurons [74], while inhibition of stearoyl-CoA-desaturase was also shown to reduce α-synuclein inclusion formation caused by excess oleic acid in iPSC neurons [162]. Finally, treatment with coenzyme Q(10), rapamycin, or the LRRK2 kinase inhibitor GW5074 were previously shown to ameliorate mitochondrial dysfunction in iPSC neurons from multiple PD patients (LRRK2 R1441C; LRRK2 G2019S; PINK1 Q456X mutants) [163].

Of note, human iPSC-DA neurons have also been transplanted into *in vivo* models of PD to determine their ability to survive and function in future potential dopamine cell replacement therapy strategies for PD patients [164]. Initial studies found that human non-iPSC-derived DA neurons could efficiently engraft into PD rodent models [20, 165, 166], while later work showed that iPSC-derived DA neurons were also successful in integrating into PD rodent models [167, 168], including neurons derived from PD patients [169]. More recently, both human and non-human primate iPSC-derived DA neurons could function properly following transplantation back into PD non-human primate models [34, 170], highlighting the potential for success in future dopamine replacement studies based on iPSC-derived neurons.

3. Conclusion

iPSC-derived neurons and organoids generated from both familial and idiopathic patients have replicated key PD pathogenic phenotypes and are an important model for studying and identifying novel neuronal pathways involved in disease. While the majority of studies thus far have involved 2D neuronal cultures, the use of midbrain-like 3D organoids will be important for investigating pathologies and neuronal complexity that are not reflected in 2D models. Importantly, these technologies have added unique approaches to drug screens, and additionally provide new models to reassess current neuroprotective and neurotoxic compounds that are under consideration. Patient-derived cell cultures can thus play a key role in identifying disease mechanisms that can be therapeutic targets for multifaceted diseases such as PD. In addition, drug screens in iPSC-DA neurons thus far have been capable of identifying neuroprotective effects, and may additionally provide insight into the efficacy of compounds in human neurons. Finally, the use of organoids for drug discovery allow the possibility for studying how therapeutics modulate multiple cell types in a more physiological 3D model.

4. Expert Opinion

4.1. IPSC Derived Neurons for Parkinson’s Disease Modeling

Since 2006 and the discovery of the factors capable of rendering cells into a state of pluripotency, many advancements have been made in Parkinson’s disease research. One of the main benefits to using iPSCs to model disease is the genetic background of the patient cells, which allows for direct study of relevant disease mutations. Moreover, pluripotency allows for the generation of disease specific cell types, allowing for the study of disease mechanisms in human dopaminergic neurons. In addition, the ability to generate isogenic

controls from patient lines using gene corrective technologies has proven to be essential for identifying which phenotypes arise from specific disease mutations.

Importantly, human neurons have also provided a unique opportunity for screening and testing novel therapeutics that could not have been revealed by traditional cell culture or animal model experiments. Indeed, human PD iPSC-DA neurons in long-term culture show time-dependent onset of PD phenotypes such as early defects in lysosomal dysfunction followed by subsequent α-synuclein accumulation [140]. Patient-derived PD iPSC-DA neurons also exhibit multiple pathogenic phenotypes that are not observed in mouse models of PD, which display negligible levels of oxidized dopamine. In particular, human DJ-1 KO iPSC-derived neurons demonstrate decreased lysosomal GCase activity and reduced tyrosine hydroxylase (TH) in the SNc which are not observed in DJ-1 KO mice [12]. Thus, patient-derived iPSC-based PD modeling may allow for the study of distinct pathogenic phenotypes arising over time which were not previously found in animal or traditional cell models.

Of note, one important factor in studying neurodegenerative diseases is understanding the role of aging. Indeed, age represents the greatest risk factor for PD, with the incidence of PD at ~0.5 to 1 percent in people 65 to 69 years of age rising sharply to 1 to 3 percent in people over 80 years of age [171, 172]. Thus, studying aging in the context of iPSC-derived neuronal models has represented another key angle for PD research. Previous studies have used long term cultures [90, 173], artificial cellular aging induced by genotoxic stress [174] or expression of progerin [175] to induce age-related features of PD. Indeed, long-term cultures grown for hundreds of days have been used to study progressive PD-linked disease phenotypes in human iPSC-derived neurons [173]. Interestingly, age-related PD phenotype such as gradual loss of tyrosine hydroxylase (TH), mitochondrial dysfunction, dendrite degeneration, Lewy body formation, and accumulation of neuromelanin were accelerated in iPSC-derived dopaminergic neurons upon progerin-induced artificial aging [175].

Another angle used to study aging phenotypes has been the generation of induced neurons (iNs) which involve the direct conversion from somatic cells to functional neurons. Importantly, this strategy overcomes the problem of rejuvenation during iPSC reprogramming from somatic cells [176, 177, 178] which makes it hard for iPSCs to reflect a donor's age. Compared to iPSC-derived neurons, iNs maintain epigenetic features and aging phenotypes of donors which drive age-related pathologies in the disease of interest. Previous studies on PD iNs have shown that PINK1 Q456X iNs do not demonstrate pS65-ubiquitin accumulation upon mitochondrial damage [179] and that both p.G411S and p.Q456X PINK1 heterozygote iNs have reduced PINK1 kinase activity and pS65-ubiquitin level [103]. However, the use of iNs also has several limitations including the fact that while iPSCs are self-renewing, the somatic cells from which iNS are derived become senescent after multiple passages and are thus limited in their ability to be expanded over long periods [180]. Thus, taken together, multiple approaches may be required to enable better modeling of aging in PD drug discovery.

In addition, there are currently several key points that must be considered when using iPSC technology. First, there exists significant batch to batch variation across patient lines and even within identical clonal populations taken from the same patient, which must be taken

into consideration when deciphering and interpreting results from drug screens aimed at targeting pathogenic phenotypes in patient neurons. Multiple factors account for this variability, including health of the neuronal culture, as well as the formation of gradients within media which can lead to differences in 2D neuronal differentiations. Furthermore, comparative studies using patient-derived and healthy donor-derived iPSCs often do not distinguish between general individual variations caused by different genetic background [181]. To address this issue, multiple studies have now used genetic editing to create isogenic controls by specifically correcting the known PD-linked mutation in patient-derived iPSCs [61, 129, 134, 175]. Addtionally, to overcome batch by batch differences, more than 3 independent batches of differentiation are normally required for iPSC-based disease studies. Thus, given small sample sizes and high sample variability, there is a need to recruit multiple patient and healthy donors, and create extensive biobanks (ex. skin and/or blood samples) with multiple clonal lines with isogenic controls for each patient line that will aid in reducing the effect of patient to patient variability in drug screens and aid in the discovery of specific phenotypes.

Secondly, there is a need for standardizing differentiation protocols across researchers and strict guidelines for characterizing healthy and successfully differentiated neuronal cultures. One of the challenges of iPSC-based PD modeling is the efficient generation of high quality A9 DA neurons and their subsequent validation. Currently, common markers used for validation of iPSC-derived DA neurons are immunostaining or immunoblot levels of TH, FOXA2, LMX1 and NURR1 [20]. In addition, electrophysiological recordings have also been used to further support the functional specific characterization of DA neurons including oscillation of membrane potential (2~5Hz) [20] or voltage sag and short rebound delay upon injection of a hyperpolarizing current (−250 pA) [12]. Thus, standardizing the methods for validating the generation of DA neurons and further characterizing the genetic, functional and/or molecular similarities and differences between iPSC-derived DA neurons and actual DA neurons from human brains will be important for understanding both the effectiveness and limitations of this model in future PD drug discovery.

Thirdly, iPSC-derived neuronal cultures are extremely costly, due to the sheer cost of media, peptides, small molecules, supplements, and consumables that are required for iPSC maintenance and throughout the duration of neuronal differentiation. This is further exacerbated in studies on neurodegenerative and age-related phenotypes which may require even longer culture durations. Thus, identifying ways to decrease these costs will be important for moving forward. Practically, this may involve purchasing expensive media and growth factors in bulk at lower costs to be shared across multiple labs, or identifying companies which are able to generate these reagents at reduced prices. It may also be important to plan experiments in advance to ensure that all neurons from each differentiation are efficiently used to avoid wastage of cells, materials and/or incubator space. Alternatively, the generation of iNeurons via direct differentiation of cultured patient fibroblasts into neurons which bypasses the need to culture iPSCs may also help to reduce time and cell culture costs for patient-derived neurons [182, 183]. Of note, these difficulties further present a challenge for conducting high-throughput screens using iPSC-derived neurons, where there is a need to produce large-scale expansion of neuronal cells, which is further

limited by both time and cost, as well as incubator space for storing large batches of neurons needed for screening.

Thus, future studies and additional models may be required to further study the role of aging in patient neurons, how different time points in 2D cultures map to an aging patient cohort, and the role of epigenetic components in mediating PD onset and progression. Finally, further work using genome-scale networks in iPSC-derived neurons may help to further identify possible targets for therapies in PD [184].

4.2. Neural Organoids for Parkinson's Disease Modeling

The use of midbrain organoids thus far for studying PD pathology has been mainly restricted to only a few studies. However, organoids can provide key insights into developmental characteristics that might be implicated in disease progression. Current studies have shown that organoids cultured up to two years give rise to functionally mature neurons, have increased cell diversity, and demonstrate time points which parallel *in vivo* development at postnatal stages [185]. Moreover, organoids may be capable of representing key time points in development, as seen in cerebral organoids with the appearance of astroglial cells during differentiation [38]. Additionally, unlike mouse models, midbrain-like organoids are able to produce neuromelanin [12, 42], and thus allow for possible testing of therapeutic compounds on multiple cell types, including both those affected in PD and those that are spared. In addition, the use of CRISPR technology in organoids [186] is highly relevant for studying the role of PD familial genes and generating isogenic controls to which to compare pathogenic phenotypes, as well as for the application of organoids in personalized medicine.

Importantly, neuronal organoids have previously shown drug discovery potential in other diseases, such as the discovery of pharmaceutical modulators of the mutated L-type calcium channel associated with Timothy Syndrome which can restore the migration of cortical interneurons [47]. Furthermore, 3D brain organoids have demonstrated promise by recapitulating key β-amyloid and tau pathology phenotypes seen in Alzheimer's disease [187]. Finally, neuronal organoids have been able to recapitulate aspects of the blood brain barrier in co-cultures with endothelial cells and thus provide the potential for screening compounds for CNS drug targeting [188]. As assembloids offer an interesting tool for modeling the connections between different brain regions, this may be relevant for PD pathogenesis in elucidating how patient neurons might exhibit dysfunctional circuitry between midbrain dopaminergic and striatal neurons.

However, there still exists several limitations to the use of neuronal organoids as a model for drug discovery. Similar to 2D cultures, 3D neuronal organoids are also highly variable even if generated from the same clonal cell line. Thus, recent studies have sought to reduce the variability between organoids [37, 189], in addition to controlling the nutrient flow and cell gradient, and further reducing the spatial and temporal variability of the cell culture environment, as seen in organ-on-a-chip technology. Moreover, organoids present additional costs compared to 2D cultures, as they often require spinning bioreactors and may require even longer differentiation times to develop age-associated phenotypes. Additionally, as much of the current progress in organoid technology has been developed using cortical and

forebrain organoids, further work needs to be conducted characterizing and standardizing the differentiation of midbrain organoids which will be relevant for PD research.

Moving forward, it will thus be critical to further develop brain organoid models by improving vascularization to avoid neuronal death on the inside of the organoid, and to test the ability of compounds to cross the blood brain barrier. As organoids often represent multi-cell type cultures, it will also be important to adapt assays from 2D cultures which are used to screen singular cell types to multi-cell type cultures, such as the use of fluorescence-activated cell sorting to examine phenotypes in individual cell types after organoid dissociation. In addition, efforts to further optimize organoid differentiation protocols from patient cells may provide new insights into PD pathology.

4.3. Future of Stem Cell modeling in Parkinson's Disease

Future work using iPSC-DA neuron and organoid modeling in PD will be highly valuable for elucidating neuronal pathways and identifying relevant therapeutic targets, as well as providing important models for testing future therapeutics. Moving forward, it will be imperative to develop biobanks that host iPSCs from specific patient lines and disease linked mutations, as well as to establish standardized differentiation protocols and markers for midbrain DA neuron generation. More sophisticated brain modeling including the incorporation of different neuronal subtypes, as well as glial cells into organoid systems will be important. Moreover, studies involving multiple clones and patient lines, possibly comparing different PD genes and mutations, will help advance our understanding of PD pathogenesis. Finally, further studies involving idiopathic PD patient-derived iPSC neurons will be crucial for shedding light on the pathways contributing to PD onset in sporadic patients which represent the vast majority of PD cases and aid in the development of future therapeutics.

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Article Highlights box

- iPSC-derived dopamine neurons represent an important model for studying familial and idiopathic Parkinson's disease mechanisms.
- Parkinson's patient-derived dopamine neurons recapitulate key pathogenic phenotypes including α-synuclein accumulation, decreased GCase activity and organelle dysfunction.
- iPSC-derived midbrain-like organoids may offer more sophisticated and physiologically relevant 3D models for studying Parkinson's.
- iPSC-derived neurons and organoids are useful models for drug discovery, identifying therapeutic targets, and compound screening and may also be relevant for future dopamine replacement studies.
- Several studies have used iPSC-derived neurons as models for assessing the efficacy of Parkinson's disease therapeutics.

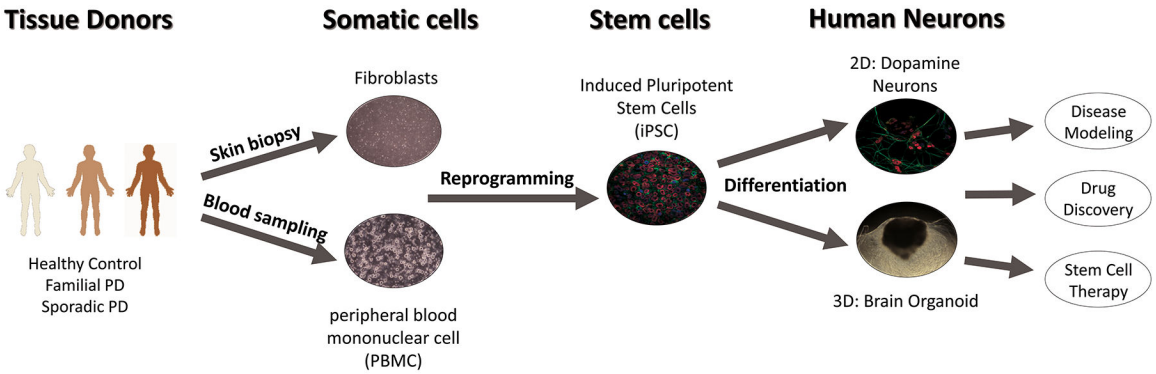

Figure 1. Application of human induced pluripotent stem cell-derived neurons for disease modeling and drug discovery in Parkinson's disease.

Human somatic cells such as fibroblasts or peripheral blood mononuclear cell (PBMC) from healthy control, familial and sporadic PD patients are reprogrammed into human iPSCs. Human iPSCs are further differentiated into dopamine neurons or 3D brain organoid depending on the purpose. Differentiated tissues enable replication of Parkinson's disease *in vitro* and can be further used for disease modeling, drug discovery and dopamine replacement stem cell therapy.

Table 1:

Key Pathogenic Phenotypes associated with Parkinson's Disease Mutant iPSC Neuron and Midbrain-like Organoid Models

Protein/Gene	Mutation	Phenotype	Reference
α-synuclein / SNCA	SNCA Triplication	Increased oxidized dopamine	Burbulla et al 2017 [12]
	SNCA Triplication	Decreased lysosomal hydrolase trafficking Decreased lysosomal GCase enzyme activity	Mazzulli et al 2016 [66]
	A53T, E46K	Reduced tetramer to monomer ratio of α-synuclein Decreased α-synuclein solubility Increased neurotoxicity	Dettmer et al 2015 [63]
	A53T	Inhibition of MEF2C-PGC1α leading to mitochondrial dysfunction. Increased nitrosative and oxidative stress Increased vulnerability to mitochondrial toxins Aggregation of α-synuclein	Ryan, SD et al 2013 [61]
	A53T, SNCA Triplication	Increased nitrosative stress, ER stress, and UPR GCase accumulation in the ER	Chung et al 2013 [74]
	SNCA Triplication	Aggregation of α-synuclein Overexpression of oxidative stress markers Increased sensitivity to peroxide induced oxidative stress	Byers et al 2011 [65]
LRRK2/ LRRK2	R1441C/G	Decreased activity dependent synaptic vesicle endocytosis Decreased GDP/GTP cycling Increased auxillin phosphorylation	Nguyen et al 2018 [94]
	G2019S	Increased oxidized dopamine	Burbulla et al 2017 [12]
	G2019S	Decreased neurite length Upregulation of several autophagic markers	Borgs et al 2016 [88]
	Q456X G2019S/R1441C	Mitochondrial dysfunction and increase of mitochondrial reactive oxygen species Increased sensitivity to Valinomycin	Cooper et al 2012 [190]
	G2019S	Accumulation of α-synuclein Upregulation of oxidative stress response genes Increased vulnerability to neurotoxins Elevated kinase activity	Nguyen et al 2011 [93]
	Midbrain Organoid: G2019S	Upregulation of TXNIP genes and a-syn accumulation	Kim, H et al 2019 [46]
	Midbrain Organoid: G2019S	Increased FOXA2 expression	Smits et al 2019 [158]
Pink1/PINK1	Exon 3,5 Deletion Exon 3 Deletion	Increased spontaneous Dopamine release Decreased DA uptake and DAT-binding Increased MAO transcripts and Oxidative stress	Jiang et al 2012 [112]
	Q456X; V170G	Impaired parkin recruitment to mitochondria Increased mitochondrial copy number Upregulation of PGC-1α	Seibler et al 2011 [102]
Parkin/PARK2	KO	Increased sensitivity to Oxidative stress Deficient glycolysis and lactate metabolism Mitochondrial elongation and enlargement Decreased Neuron Survival	Bogetofte et al 2019 [191]
	V324A	Increased oxidized dopamine Decreased GCase activity	Burbulla et al 2017 [12]
GCase/GBA1	84GG/WT	Increased oxidized DA	Burbulla et al 2019 [134]
	N370S/WT	Reduced protein level and activity of GCase Accumulation of GSL and α-synuclein	Kim et al 2018 [128]

Protein/Gene	Mutation	Phenotype	Reference
	RecNcil/WT, L444P/WT, N370S/WT	Defective mitochondrial function: Altered cristae morphology Increased mitochondrial diameter Reduced oxygen consumption rate(OCR) Reduced complex I activity Altered NAD+ metabolism Increased ER stress and UPR	Schondorf et al 2018 [133]
	N370S/c0.84dupG	α-synuclein accumulation in cell body and neurites	Mazzuli et al 2016 [132]
	N370S/WT	Abnormal GCase post-translation Lipid profile change Upregulation of ER stress Autophagic disturbance Impaired lysosomal degradation capacity Enlargement of lysosomes Increased extracellular α-synuclein	Fernandes et al 2016 [130]
	N370S/N370S N370S/c0.84dupG	Reduced protein level of GCase Reduced DA uptake Elevations in GlcSph, GlcCer Increase α-synuclein protein level but not mRNA level	Aflaki et al 2016 [127]
	PD: RecNcil/WT, L444P/WT, N370S/WT GD: Type 1: N370S/ N370S Type 3: L444P/ L444P	Reduction in GCase level and activity Increase in GluCer and a-syn Defected Calcium homeostasis and increased vulnerability to stress response Alteration in the autophagy Increased size and the number of late endosome/lysosome (Lamp1 +)	Schondorf et al 2014 [129]
	N370S/WT	Increased α-synuclein level Elevated mRNA and protein levels of Monoamine oxidase B (MAO-B) and lower DA level	Woodard et al 2014 [131]
DJ-1/PARK7	KO	Elevated mitochondrial oxidation Increased oxidized DA Decreased basal respiration Decreased lysosomal proteolysis and GCase enzyme activity α-synuclein accumulation	Burbulla et al 2017 [12]
ATP13A2/ PARK9	1550 C>T 3176 T>G and 3253 del C	Disruption of lysosomal Ca^{2+} homeostasis Reduced lysosomal Ca^{2+} Storage Increase in cytosolic Ca^{2+} levels Overall impaired lysosomal exocytosis	Tsunemi et al 2019 [140]
Synaptojanin/S J1	R258Q	Accumulation of Atg18a on nascent synaptic autophagosomes Decreased autophagosome maturation Dopaminergic neuron loss	Vanhauwaert et al 2017 [144]
VPS35/VPS35	D620N	Defective synaptic transmission AMPA-type glutamate receptor (AMPAR) recycling	Munsie et al 2015 [147]

Table 2:

Drug Discovery in Parkinson's Disease iPSC DA Neurons

Tested drug	Target	Mode of action	Reference
NCGC758 NCGC607	β-Glucocerebrosidase (GCase)	Small-molecule chaperone	Aflaki et al 2016; Mazzulli et al 2016) [127, 132]
Quinazoline inhibitors	β-Glucocerebrosidase (GCase)	Selective stabilization of GCase	Zheng et al 2019 [159]
Carmofur	Acid ceramidase	Inhibitor	Kim et al 2018 [160]
S-181	β-Glucocerebrosidase (GCase)	Increased wild-type GCase activity	Burbulla et al 2019 [134]
mito-TEMPO NAC	Mitochondria	Antioxidants	Burbulla et al. 2017 [12]
Isoxasole	MEF2C	Activator	Ryan et al. 2013 [61]
PD0325901	MEK	Inhibitor	Reinhardt et al 2013 [78]
NPT100–18A NPT100–14A ELN48228	α-synuclein	Interfering oligomerization	Kouroupi et al 2017 [62]