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Pluripotent Stem Cell Derived Neurons as In Vitro Models for Studying Autosomal Recessive Parkinson's Disease (ARPD): *PLA2G6* and Other Gene Loci

Renjitha Gopurappilly

Abstract

Parkinson's disease (PD) is a neurodegenerative motor disorder which is largely sporadic; however, some familial forms have been identified. Genetic PD can be inherited by autosomal, dominant or recessive mutations. While the dominant mutations mirror the prototype of PD with adult-onset and L-dopa-responsive cases, autosomal recessive PD (ARPD) exhibit atypical phenotypes with additional clinical manifestations. Young-onset PD is also very common with mutations in recessive gene loci. The main genes associated with ARPD are *Parkin*, *PINK1*, *DJ-1*, *ATP13A2*, *FBXO7* and *PLA2G6*. Calcium dyshomeostasis is a mainstay in all types of PD, be it genetic or sporadic. Intriguingly, calcium imbalances manifesting as altered Store-Operated Calcium Entry (SOCE) is suggested in *PLA2G6*-linked PARK 14 PD. The common pathways underlying ARPD pathology, including mitochondrial abnormalities and autophagic dysfunction, can be investigated ex vivo using induced pluripotent stem cell (iPSC) technology and are discussed here. PD pathophysiology is not faithfully replicated by animal models, and, therefore, nigral dopaminergic neurons generated from iPSC serve as improved human cellular models. With no cure to date and treatments aiming at symptomatic relief, these in vitro models derived through midbrain floor-plate induction provide a platform to understand the molecular and biochemical pathways underlying PD etiology in a patient-specific manner.

Keywords

Autophagic–lysosomal pathway · Calcium · Cellular reprogramming · Dopaminergic neurons · Lewy bodies · Mitophagy · PARK-14 · Phospholipase A2 · SOCE

R. Gopurappilly (✉)
National Centre for Biological Sciences, Tata Institute of Fundamental Research, Bengaluru, India
e-mail: renjithap@ncbs.res.in

Abbreviations

ARPD	autosomal recessive Parkinson's disease
DA	dopaminergic
ER	endoplasmic reticulum
ESC	embryonic stem cell
iPSC	induced pluripotent stem cell
LB	Lewy bodies
NSC	neural stem cell

PD	Parkinson's disease
PM	plasma membrane
ROS	reactive oxygen species
SNpc	substantia nigra pars compacta
SOCE	store-operated calcium entry
TH	tyrosine hydroxylase

1 Introduction

Parkinson's disease (PD) is the second most common neurodegenerative disorder characterized by motor symptoms such as resting tremor, bradykinesia, rigidity, postural instability, stooped posture and freezing, as well as non-motor symptoms including cognitive and behavioural symptoms, sleep disorders, autonomic dysfunction, sensory symptoms and fatigue (Tolosa et al. 2006; de Lau et al. 2006; Jankovic 2008; O'Sullivan et al. 2008; Kalinderi et al. 2016). The pathological hallmark of PD is the progressive loss of dopaminergic (DA) neurons in the substantia nigra pars compacta (SNpc) and the subsequent loss of dopamine inputs to forebrain striatal structures along with the appearance of protein inclusions called Lewy bodies (LB) composed of α-synuclein fibrils (de Lau et al. 2006; Jankovic 2008). PD is classified into two genetic subtypes including monogenic familial forms with Mendelian inheritance and sporadic forms with no underlying genetic factors (Karimi-Moghadam et al. 2018). The sporadic forms of PD are highly prevalent whereas familial PD accounts for only 5–10% of the reported cases. Monogenic familial forms of PD are rare, caused by highly penetrant disease-causing mutations. Parkinsonism caused by dominant mutations including *alpha-synuclein (SNCA)*, *leucine-rich repeat kinase 2 (LRRK2)*, *vacuolar protein sorting 35 (VPS35)* and the like are largely similar to the common, late-onset sporadic PD (Bonifati 2012, 2014). Autosomal recessive PD (ARPD) results from mutations in different loci which have clinical signs typical of PD or can exhibit a wide range of other complex symptoms. The common pathways underlying ARPD are mitochondrial quality control, protein degradation processes and oxidative stress responses, among others (Scott et al. 2017; van der Merwe et al. 2015).

The current knowledge of PD is mostly from postmortem studies and animal models. While the former represent only the end-stage of the disease, the latter fail to reflect human disease pathology due to interspecies differences. In this context, human pluripotent stem cells (both embryonic stem cells, ESC; and induced pluripotent stem cells, iPSC) are an excellent source of cells for differentiation to DA neurons in vitro. 'Disease modelling in a dish' by recapitulating the disease phenotypes in defined cell populations would make it possible to understand the cellular and molecular mechanisms of PD, along with providing a high-throughput drug screening platform (Marchetto et al. 2011; Badger et al. 2014; Martínez-Morales and Liste 2012).

2 Autosomal Recessive Parkinsonism

The hereditary forms of parkinsonism which are transmitted in an autosomal recessive fashion are given in Table 1. Mutations have been identified most commonly in three genes in several ethnic groups spanning different geographical locations: *parkin* (*PRKN*, PARK2), *PTEN- induced putative kinase 1* (*PINK1*, PARK6), and *Parkinson protein 7* (*DJ-1*, PARK7). Point mutations, large genomic rearrangements, leading to deletions or multiplications presenting as homozygous or compound heterozygous variations are reported, particularly for *parkin* (Lucking et al. 2000). Recessive mutations in several genes, including *ATPase type 13A2* (*ATP13A2*, PARK9), *phospholipase A2, group VI* (*PLA2G6*, PARK14), *F-box only protein 7* (*FBXO7*, PARK15), *spatacsin* (*SPG11*), and *DNA polymerase gamma* (*POLG*), cause young- or juvenile-onset PD. These present with other clinical manifestations like dystonia, dementia and other disturbances (Bonifati 2012). *DNAJ subfamily C member 6* (*DNAJC6*, PARK19), *synaptojanin-1* (*SYNJ1*, PARK 20) and *vacuolar protein sorting 13C* (*VPS13C*, PARK23) are also reported to

Table 1 Genes associated with autosomal recessive Parkinson's disease (ARPD)

Locus	Gene	Protein
PARK2	*Parkin*	E3 ubiquitin-ligase
PARK6	*Pink1*	Phosphatase and tensin homolog-induced putative kinase1
PARK7	*DJ-1*	Parkinson protein 7, oncogene DJ-1
PARK9	*ATP13A2*	Lysosomal P5-type ATPase
PARK14	*PLA2G6*	Phospholipase A2, group VI
PARK15	*FBXO7*	F-box only protein 7
PARK19	*DNAJC6*	Putative tyrosine-protein phosphatase auxilin
PARK20	*SYNJ1*	Synaptojanin-1
PARK23	*VPS13C*	Vacuolar protein sorting 13C
	SPG11	Spatacsin
	POLG	DNA polymerase gamma

Genes mapped to different PARK loci and associated with ARPD are listed together with the involved protein. Rarely, mutations in *spatacsin (SPG11)* and *DNA polymerase gamma (POLG)*, cause autosomal recessive parkinsonism with juvenile onset, mostly with atypical features

have mutations causing autosomal recessive PD (Karimi-Moghadam et al. 2018).

2.1 *PLA2G6* (PARK14)

Phospholipase A2 group 6 (*PLA2G6*, *iPLA2β*) gene encodes a calcium-independent group 6 phospholipase A2 enzyme, which hydrolyzes the sn-2 ester bond of the membrane glycerophospholipids to produce free fatty acids and lysophospholipids (Pérez et al. 2004). Various mutations in this gene have been discovered in patients with neurodegenerative disorders such as infantile neuroaxonal dystrophy (INAD), atypical neuroaxonal dystrophy (ANAD), adult-onset dystonia-parkinsonism (DP) and autosomal recessive early-onset parkinsonism (AREP), together known as *PLA2G6*-asscociated neurodegeneration, PLAN (Gregory et al. 1993). PLAN can be classified as neurodegeneration with brain iron accumulation II (NBIA II); however, a wide range of clinical variability is exhibited in these phenotypes with most PD cases devoid of brain iron deposition or cortical atrophy (Guo et al. 2018). iPLA2β protein contains an N-terminal domain, Ankyrin repeats and catalytic domains (Fig. 1). iPLA2β is predominantly localized in the cytosol but can translocate to the Golgi, ER, mitochondria and nucleus under stimulation (Turk and Ramanadham 2004; Kinghorn et al. 2015; Balsinde and Balboa 2005; Ramanadham et al. 2015). Two distinct 85 kDa (VIA-1) and 88 kDa (VIA-2) human iPLA2β isoforms have been discovered along with many N-terminal truncated forms due to proteolytic cleavage and alternate splicing (Ramanadham et al. 2015). It is highly expressed in the human brain including SNpc (http://www.proteinatlas.org). The *PLA2G6* gene mutations was associated with parkinsonism almost a decade ago, with R741Q and R747W being the first to be reported in adult-onset levodopa-responsive dystonia-parkinsonism (Paisan-Ruiz et al. 2009, 2010; Sina et al. 2009). p.R741Q has also been indicated in early-onset PD without dystonia (Bohlega et al. 2016). Though PD-associated mutations in this gene are mostly homozygous, some of them are rare and specific to geographic areas (Gui et al. 2013; Shen et al. 2018; Kapoor et al. 2016; Lu et al. 2012) while others are compound heterozygous (Shen et al. 2019; Chu et al. 2020; Ferese et al. 2018). The mutations that are pathogenic and causal for PD in *PLA2G6* are detailed in Table 2.

Widespread LB pathology is seen with *PLA2G6*-linked PD (Paisán-Ruiz et al. 2012; Miki et al. 2017). The loss of PLA2G6 in *Drosophila* results in impaired retromer function, ceramide accumulation and lysosomal dysfunction, leading to age-dependent loss of neuronal activity (Lin et al. 2018). Dysfunction of mitochondria and increased lipid peroxidation have also been reported in *PLA2G6*-deficient *Drosophila*

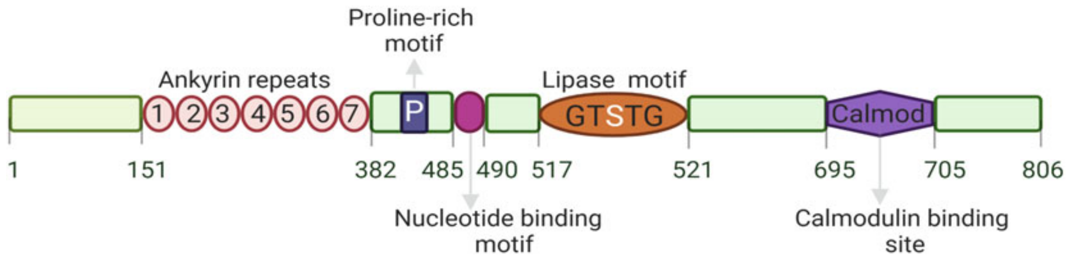

Fig. 1 Structure of PLA2G6 (iPLA2β) protein. The full-length protein is shown with seven ankyrin repeats (pink circles), a proline-rich motif (blue box), a glycine-rich nucleotide-binding motif (magenta), a lipase motif (orange with the active site highlighted), and a proposed C-terminal calcium-dependent calmodulin binding domain (purple). Numbers indicate amino acids

Table 2 List of PD-associated pathogenic mutations in *PLA2G6* (PARK14) gene loci

Mutation	Protein change
c.109 C > T	p.arg37-to-X (R37X)
c.216C > A	p.phe72-to-leu (F72L)
c.238 G > A	p.ala80-to-thr (A80T)
c.991G > T	p.asp331-to-tyr (D331Y)
c.1077 G > A	p.met358-llefsX6
c.1354C > T	p.gln452-to-X (Q452X)
c.1495G > A	p.ala499-to-thr (A499T)
c.1715C > T	p.thr572-to-ile (T572I)
c.1791delC	p.his597-fx69
c.1894C > T	p. arg632-to-trp (R632W)
c.1904G > A	p.arg635-to-gln (R635Q)
c.1976A > G	p.asn659-to-ser (N659S)
c.2215G > C	p.asp739-to-his (D739H)
c.2222G > A	p.argR741-to-gln (R741Q)
c.2239C > T	p.arg747-to-trp (R747W)

Mutations in the *PLA2G6* gene that are pathogenic (or likely pathogenic) and cause Parkinson's disease are documented

mimicking the human fibroblasts with R747W mutation (Kinghorn et al. 2015). Increased sensitivity to oxidative stress, progressive neurodegeneration and a severely reduced lifespan and impaired motor co-ordination is seen in *PLA2G6*-knockout flies (Iliadi et al. 2018). In yet another fly model, the loss of *PLA2G6* leads to shortening of phospholipid acyl chains, resulting in ER stress and impaired neuronal activity as well as formation of α-synuclein fibrils, demonstrating that phospholipid remodeling by *PLA2G6* is essential for DA neuron survival and function (Mori et al. 2019). Similarly, a rodent knockin model of D331Y *PLA2G6* mutation exhibited early degeneration of SNpc DA possibly via mitochondrial and ER stress, impaired autophagic mechanisms and gene expression changes (Chiu et al. 2019). Elevated levels of both α-synuclein and phosphorylated α-synuclein are seen in *PLA2G6* knockout mice models, facilitating the formation of LB and eventually death of affected DA neurons (Sumi-Akamaru et al. 2016). In an in vitro study examining the catalytic activity of PLA2G6 proteins, recombinant proteins containing the three mutations associated with dystonia-parkinsonism (R632W, R741Q and R747W) did not show any altered catalytic activity, whereas the mutations associated with INAD led to a significant loss of enzyme activity (Engel et al. 2010). In addition,

PLA2G6-PD mutants of SHSY5Y, a neuroblastoma cell line, failed to prevent rotenone-induced death of dopaminergic cells (Chiu et al. 2017). *PLA2G6*-PD does not present with a typical clinical scenario and continues to evolve with a wide phenotypic spectrum. It is safe to infer that the PD-relevant mutations do not significantly alter the catalytic activity of the enzyme but induce damage through parallel mechanisms like oxidative stress, mitochondrial dysfunction or even compromised lipid remodeling.

2.1.1 *PLA2G6* and Store-Operated Calcium Entry (SOCE)

Store-operated calcium entry (SOCE) is an arm of calcium signaling activated by depletion of ER stores that triggers influx of calcium across the plasma membrane (PM) brought about by the calcium sensor *STIM* and the PM pore channel *Orai* (Feske et al. 2005, 2006; Vig et al. 2006). Interestingly, in a genetic screening of *Drosophila*, not only *STIM1* and *Orai1*, but also a fly orthologue of *PLA2G6* encoded by the *CG6718* gene, were identified as SOCE activators (Vig et al. 2006). Many groups have from this time identified *PLA2G6* as an endogenous activator of SOCE (Smani et al. 2016; Schäfer et al. 2012; Singaravelu 2006; Bolotina and Orai 2008). The physiological relevance of neuronal SOCE is disputed (Lu and Fivaz 2016) and its role in DA neurons, particularly, is not known. In PD, abnormal calcium homeostasis triggers a cascade of downstream events that eventually leads to cell death (Michel et al. 2016). Interestingly, primary skin fibroblasts from idiopathic and PLA2G6-PD (R747W) patients revealed a significant deficit in endogenous SOCE and similarly, MEFs from the exon2-knockout mice exhibited deficient store-operated *PLA2G6*-dependent calcium signaling (Zhou et al. 2016a). This was also mirrored in the iPSC-derived DA neurons along with low ER calcium levels and deficient autophagic flux. The knockout mice also showed age-dependent loss of DA neurons. This study for the first time arrived at a causal relationship between *PLA2G6*-dependent SOCE, depleted stores, dysfunctional autophagy in DA neurons and a PD-like phenotype (Zhou et al. 2016a). Recently, in a patient-derived (D331Y) DA neuron model, imbalance of calcium homeostasis, markedly deficient SOCE, increase of UPR proteins, mitochondrial dysfunction, increase of ROS, and apoptosis was reported. Interestingly, the UPR modulator, azoramide rescued the phenotype of the mutant DA neurons, possibly via CREB signaling (Ke et al. 2020). These recent developments have opened new exciting areas to study the significant contributions of *PLA2G6* and SOCE in PD, and may involve new undiscovered molecules providing a yet unexplored arena for PD-drug discovery.

2.2 *Parkin*, *PINK1* and *DJ-1*

The E3 ubiquitin ligase parkin (PARK2) and the serine/threonine kinase PINK1 (phosphatase and tensin homolog-induced putative kinase1, PARK6), act together in a mitochondrial quality control pathway and promote the selective autophagy of depolarized mitochondria (mitophagy) (Narendra et al. 2012). PINK1 levels are low in healthy cells as it is continually cleaved inside the mitochondria in a sequential manner by proteases (Yamano and Youle 2013). The import of PINK1 into mitochondria is stopped when the organelle loses its inner membrane electrochemical gradient (depolarization), which leads to the stabilization of the protein on the mitochondrial outer membrane (Lin and Kang 2008). This accumulation of PINK1 kinase on the mitochondria triggers parkin recruitment and activation resulting in ubiquitination of various outer mitochondrial membrane proteins (Taanman and Protasoni 2017; Matsuda et al. 2010). The damaged mitochondria are eventually eliminated by autophagy. Pathogenic PD-associated mutations in either *Parkin* or *PINK1* causes accumulation of impaired mitochondria, increased ROS and neuronal cell death (Seirafi et al. 2015). More than 100 different *Parkin* mutations have been reported from PD patients, including deletions, insertions, multiplications, missense and truncating mutations, and over 40 point mutations and, rarely, large deletions, have been detected in *PINK1* (Lesage and Brice 2009). Clinically,

both cause young-onset PD and show responsiveness to levodopa. The phenotype associated with the oncogene *DJ-1* mutations has been studied in a smaller number of patients but it is overall indistinguishable from that of the patients with *PINK1* or *Parkin* mutations (Bonifati et al. 2003). *DJ-1* is thought to be involved in the regulation of the integrity and calcium crosstalk between endoplasmic reticulum (ER) and mitochondria, and pathogenic mutations lead to impaired ER-mitochondria association in PD (Liu et al. 2019).

2.3 *ATP13A2, FBXO7, SPG11* and *POLG*

Mutations in *ATP13A2, FBXO7, spatacsin* and *POLG* cause juvenile-onset ARPD along with *PLA2G6* (Bonifati 2012). Mutations in *ATP13A2* or PARK9, were first identified in 2006 in a Chilean family and are associated with a juvenile-onset, levodopa-responsive type of parkinsonism called Kufor–Rakeb syndrome (KRS). KRS involves pyramidal degeneration, supranuclear palsy, and cognitive impairment (Ramirez et al. 2006). The *ATP13A2* gene encodes a large lysosomal protein, belonging to the P5-type ATPase family of transmembrane active transporters. Its substrate specificity remains unknown. It is suggested that *ATP13A2* recruits HDAC6 to lysosomes to promote autophagosome–lysosome fusion and maintain normal autophagic flux (Wang et al. 2019). This, in turn, is required for preventing *α*-synuclein aggregation in neurons. FBXO7, in turn is an adaptor protein in SCF^{FBXO7} ubiquitin E3 ligase complex that mediates degradative or non-degradative ubiquitination of substrates. *FBXO7* mutations aggravate protein aggregation in mitochondria and inhibit mitophagy (Zhou et al. 2018). *Parkin*- and *FBXO7*-linked PD have overlapping pathophysiologic mechanisms and clinical features. Wild-type FBXO7, but not PD-linked FBXO7 mutants, has been shown to rescue DA neuron degeneration in *Parkin* null *Drosophila* (Zhou et al. 2016b). Loss of activity of FBXO7 in seen in patients with PARK 15-PD and is therefore crucial for the maintenance of neurons (Zhao et al. 2011). *SPG11* or *spatacsin* mutations present with bilateral symmetric parkinsonism at an early age with rapid deterioration and development of spastic paraplegia and thinning of the corpus callosum on MRI, typical of spastic paraplegia 11 (Guidubaldi et al. 2011; Cao et al. 2013). An involvement of *POLG*, the mitochondrial DNA polymerase that is responsible for replication of the mitochondrial genome is considered in early-onset PD especially in the presence of additional symptoms, such as ophthalmoparesis, non-vascular white matter lesions and psychiatric comorbidity (Anett et al. 2020). A role of mitochondrial DNA defects in the pathogenesis of neurodegenerative parkinsonism with *POLG* mutations is speculated (Miguel et al. 2014).

3 Common Pathways in ARPD

There are many converging features seen at the molecular and clinical levels in ARPD that are discussed in the following section. Understanding these causal molecular mechanisms is crucial to identify common targets and devise therapeutic approaches. However, some fundamental underlying processes still remain unclear. The contributions of the intracellular organelle ER in ARPD pathology via the calcium signaling pathway, SOCE is poorly understood. The mitochondrion, which is the star player in PD pathogenesis, regulates SOCE activity possibly via sub-plasmalemmal calcium buffering, the generation of mediators, local ATP modulation and regulation of STIM1 (Malli and Graier 2017). In turn, SOCE-derived calcium significantly affects mitochondrial metabolism. Hence, the communication between SOCE and mitochondria is hypothesized to be interdependent and complex leading to fine-tuning of both SOCE and mitochondrial function (Spät and Szanda 2017). Though PD-relevant mitochondrial processes are studied extensively, SOCE and its role in PD are largely unexplored. The microbiota–gut–brain axis and its imbalance by alterations in the human microbiome also represent a risk factor

for PD (Sampson et al. 2016). Such pathways that are not well-proven are omitted from this section, for ease of understanding.

3.1 Mitochondrial Pathways

The most compelling evidence for loss of mitochondrial fidelity comes from the genes *PINK1* and *Parkin*. As mentioned earlier, PINK1 accumulates on the outer membrane of damaged mitochondria and activates Parkin's E3 ubiquitin ligase activity. Parkin recruited to the damaged mitochondrion ubiquitinates the outer mitochondrial membrane proteins to trigger selective autophagy. In the late 1970s when accidental exposure to 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine (MPTP) was found to cause PD and neurodegeneration, the first causal mechanism speculated was mitochondrial dysfunction (William Langston et al. 1983). A specific defect of Complex I activity is also seen in the substantia nigra of patients with PD (Schapira et al. 1990). We now understand that the pathways included in mitochondrial quality control system are fission/fusion, mitochondrial transport, mitophagy and mitochondrial biogenesis (Scott et al. 2017). The precise mechanisms by which Parkin and PINK1 regulate fission and fusion is debated, but studies from *Drosophila* and mammalian culture systems, though contradictory, indicate unbalanced mitochondrial fission and fusion in PINK1 mutants (Scott et al. 2017; Chen and Chan 2009; Pryde et al. 2016; Scarffe et al. 2014; Yu et al. 2011). *DJ-1* (Irrcher et al. 2010) and *ATP13A2* (Park et al. 2014) mutants also show fragmented mitochondria. The combined effects of Parkin and PGC-1α in the maintenance of mitochondrial homeostasis in dopaminergic neurons is demonstrated (Zheng et al. 2017). PINK1 is also involved in mitochondrial motility along axons and dendrites of neurons. PINK1 interacts with Miro, a component of the motor/adaptor complex binding mitochondria to microtubules and allowing their movement to and from cellular processes (Brunelli et al. 2020). Miro is phosphorylated by PINK1 and ubiquitinated by parkin, leading to its degradation and halting mitochondrial transport promoting clearance of damaged mitochondria (Liu et al. 2012). Parkin/PINK1 is hence involved in mitochondrial trafficking (Scott et al. 2017; Weihofen et al. 2009).

The clearance of damaged mitochondria or mitophagy is a pathway common to mostly all ARPD-related genes. The role of Pink1-Parkin in mitophagy is well-established (Deas et al. 2011; Yamano and Youle 2020). Fbxo7 is also shown to induce mitophagy in response to mitochondrial depolarization in a common pathway with Parkin and PINK1, and PD-associated mutations interfere in this mechanism (Burchell et al. 2013). Parkin (Kuroda et al. 2006) and PINK1 (Pirooznia et al. 2020) is also linked to mitochondrial biogenesis, therefore probably being a part of mitochondrial transcription/replication.

3.2 Autophagy–Lysosomal Pathways

In addition to impaired mitophagy, protein degradation pathways, especially the autophagy–lysosomal pathway, are affected in PD. ATP13A2 is suggested as a regulator of the autophagy–lysosome pathway. ATP13A2 acts in concert with another PD-protein SYT11 and its loss of function results in dysfunctional autophagy–lysosomal pathway as seen in PD (Bento et al. 2016). α-synuclein-independent neurotoxicity due to endolysosomal dysfunction has also been demonstrated in ATP13A2 null mice (Kett et al. 2015). *Parkin* knockout neurons too show perturbed lysosomal morphology and mitochondrial stress (Okarmus et al. 2020). DJ-1 is associated to chaperone-mediated autophagy (CMA) and its deficiency aggravates α-synuclein aggregation by inhibiting CMA activation (Xu et al. 2017). Loss of DJ-1 could also lead to impaired autophagy and accumulation of dysfunctional mitochondria (Krebiehl et al. 2010). Autophagic defects are a mainstay in *PLA2G6*-PD. Genetic or molecular impairment of PLA2G6-dependent calcium signaling is a trigger for autophagic dysfunction, progressive loss of DA neurons and age-dependent L-DOPA-

sensitive motor dysfunction in a mouse knockout model (Zhou et al. 2016a).

3.3 Cell Death and Oxidative Stress

Oxidative stress and apoptosis are frequently involved in ARPD pathogenesis. ROS accumulation plays a key role in the initiation and acceleration of cell death, compromising neuronal function and structural integrity (Schieber and Chandel 2014). The protein products of Parkin, PINK1 and DJ-1 are associated with disrupted oxidoreductive homeostasis in DA neurons. Impaired cell survival in part due to defective oxidative stress response is implicated in *PARK2* knockout neurons (Bogetofte et al. 2019). Further, transgenic overexpression of the parkin substrate, aminoacyl-tRNA synthetase complex interacting multifunctional protein-2 (AIMP2) leads to a selective, age-dependent progressive loss of dopaminergic neurons via activation of poly(ADP-ribose) polymerase-1 (PARP1) (Lee et al. 2013). Similarly, PINK1 is also shown to exert a neuroprotective effect by inhibiting ROS formation and maintaining normal mitochondrial membrane potential and morphology in cultured SN dopaminergic neurons (Wang et al. 2011). The profiles of oxidative damage in the whole brain and neurochemical metabolites in the striatum of *PINK1* knockout rats at different ages and genders were studied and oxidative damage revealed as a crucial factor for PD (Ren et al. 2019). Loss of PINK1 inhibits the mitochondrial Na(+)/Ca(2+) exchanger (NCLX), resulting in impaired mitochondrial calcium extrusion, which was, however, fully rescued by activation of the protein kinase A (PKA) pathway (Kostic et al. 2015). DJ-1 also has a role in cell death and combating oxidative stress. It suppresses PTEN activity, thereby promoting cell growth and promoting cellular defence against ROS through PI3K/Akt signaling (Chan and Chan 2015). Reduced anti-oxidative stress mechanisms have been reported in PD patients with mutant DJ-1 protein (Takahashi-Niki et al. 2004). It is also described that Daxx, the death-associated protein, translocated to the cytosol selectively in SNpc neurons due to MPTP-mediated down-regulation of DJ-1 after treatment with the neurotoxin in mouse models (Karunakaran et al. 2007). DJ-1 is also hypothesized to regulate the expression of UCP4 by oxidation and partially via NF-κB pathway in its protective response to oxidative stress (Xu et al. 2018). DJ-1, particularly in its oxidized form, is documented as a biomarker for many diseases including PD. DJ-1 may also work by increasing microRNA-221expression through the MAPK/ERK pathway, subsequently leading to repression of apoptotic molecules (Oh et al. 2018). Additionally, cell-permeable Tat-DJ-1 protein exerts neuroprotective effects in cell lines and mouse models of PD (Jeong et al. 2012). Data from the field indicate that DJ-1 may become activated in the presence of ROS or oxidative stress, but also as part of physiological receptor-mediated signal transduction and acts as a transcriptional regulator of antioxidative gene batteries (Kahle et al. 2009). ATP13A2, on the contrary, is thought to protect against hypoxia-induced oxidative stress (Xu et al. 2012). A recent study revealed a conserved neuroprotective mechanism that counters mitochondrial oxidative stress via ATP13A2-mediated lysosomal spermine export (Vrijsen et al. 2020). PLA2G6 protein is also indicated in oxidative stress-related pathways (Kinghorn et al. 2015; Ke et al. 2020).

4 Induced Pluripotent Stem Cells (iPSC) in Parkinson's Disease Research

Yamanaka's discovery in 2007 where key transcriptional factors (*Oct4*, *Sox2*, *Klf4* and *c-Myc*) were used to reprogramme adult cells to a de-differentiated, poised cell type called Induced pluripotent stem cells (iPSCs) revolutionized the field of human disease modeling (Takahashi et al. 2007). Reprogrammed iPSCs are similar to embryonic stem cells or ESCs, are pluripotent and can differentiate to multiple lineages. iPSCs when derived from a PD patient has the patient's complete genetic background and provides a valuable platform to study the impact of genetic mutations. Within a year of the discovery of

iPSCs, PD-patient-derived iPSCs (Park et al. 2008) and DA neuron differentiation from iPSCs were reported (Soldner et al. 2009). iPSC models have successively been established from various sporadic and familial PD patients. To date, iPSCs are the most robust cellular system to understand PD and generate disease-relevant cell types for PD (Playne and Connor 2017).

iPSC studies typically involve few participants and random selection of cases and controls, which results in heterogeneous models in vitro. Consequently, a large sample size is required to increase statistical power and sample sizes of 10–30 individuals per iPSC study may be required to achieve a statistical power of 80% (Hoekstra et al. 2017; Tran et al. 2020). These are, in turn, labour-intensive and expensive; hence, it is unlikely that these requirements are met. A smaller number may be used if clinically and genotypically homogeneous subjects are used to reduce the variance in the cellular phenotypes. Hence, a preponderance of familial PD is seen in these studies. A recent report elegantly summarizes a meta-analysis of 385 iPSC-derived neuronal lines modeling mutations/deletions/ triplications in *LRRK2, PRKN, PINK1, GBA* and *SNCA* (Tran et al. 2020). The authors discuss the importance of using the right controls in such studies. When healthy subjects are used as controls, differences in genetic background may give rise to variance in neuronal phenotypes studies that are not caused by disease mutations. Gene-editing techniques (TALEN, ZFN, CRISPR/Cas9) aid in the generation of isogenic lines that differ in only one single mutant gene, and this circumvents the issue of variance due to genetic background. CRISPR/Cas9 system, an RNA-based endonuclease, is the most common and effective tool used in the iPSC model for introducing the genetic changes seen in PD, including but not limited to knockout, knockin and gene correction (Arias-Fuenzalida et al. 2017; Qing et al. 2017; Soldner et al. 2016; Vermilyea et al. 2020). Disease-causing mutations, therefore, can be inserted in healthy ESC or iPSC lines or gene-correction of a single mutation can be performed in PD-lines to include comparative isogenic control lines (Tran et al. 2020).

Differentiation protocols for DA neurons mimic embryologic development in utero. Unlike cortical neurons, midbrain DA neurons are derived from the ventral floor plate of the neural tube (Ono et al. 2007). The molecular mechanisms that regulate the development of midbrain DA neurons in vivo, and how taking cues from this, one can generate in vitro human midbrain DA neurons from iPSCs was systematically reviewed previously (Arenas et al. 2015). Dual-SMAD inhibition along with modulation of sonic hedgehog (SHH) and WNT signaling by CHIR99021 (GSK3β inhibitor), and addition of FGF8 is routinely used to generate midbrain floor-plate precursors (Kirkeby et al. 2012; Kriks et al. 2011; Reinhardt et al. 2013). BDNF, GDNF, TGFβ3, dbcAMP, ascorbic acid, DAPT and ActivinA are used to enhance the purity and maturity of DA neurons which express the key marker TH (tyrosine hydroxylase) (Monzel et al. 2017a; Smits and Schwamborn 2020; Smits et al. 2019). A schematic and generalized diagram outlining the midbrain DA differentiation protocol from iPSCs is shown in Fig. 2 (the starting population can also be ESCs). It is important to note that, irrespective of the protocols used a heterogeneous cell population is attained, with neurons, glia and NSCs. To attain a high percentage of TH^+ DA neuron population, several strategies have been employed. Sorting of DA progenitors which are $CD184^{high}/CD44^-$(Suzuki et al. 2017) or $CORIN^+$(Kikuchi et al. 2017) is shown to increase the TH^+ DA neuron yield. CRISPR/ Cas9-based knockin of a fluorescent reporter to visually identify and purify TH^+ DA neurons has also been attempted (Calatayud et al. 2019). A monolayer-based neural differentiation protocol was described recently that reproducibly generates ~70–80% midbrain DA neurons (Stathakos et al. 2020; Stathakos et al. 2019). A higher concentration of 300 ng/ml SHH (100–200 ng/ml is used normally) in combination with a lower concentration of 0.6 μM CHIR99021 (0.8 μM–3 μM is used normally) and passaging and replating in the early differentiation and patterning stages maximized the yield of midbrain DA neurons as early as day 30. This monolayer platform is amenable to imaging and functional

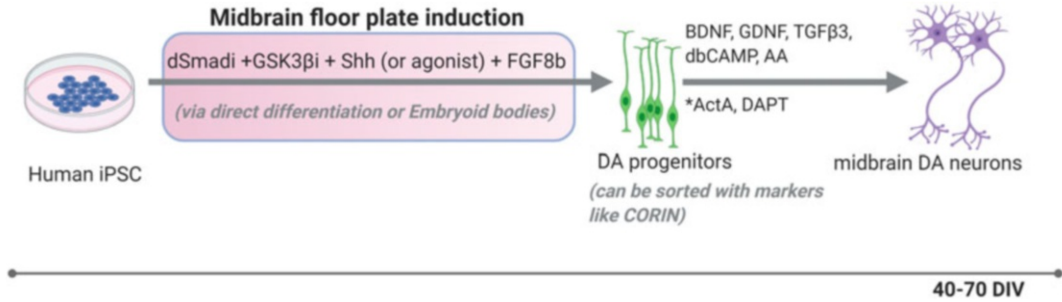

Fig. 2 A generalized schematic protocol for differentiation of human induced pluripotent stem cells (iPSCs) into midbrain dopaminergic (DA) neurons. Human iPSCs are treated with small molecules, such as GSK3β inhibitors (GSK3i) and SMAD inhibitors, along with Shh/FGF8b, to induce midbrain floor-plate formation and subsequent midbrain DA specification. This is done either by means of direct differentiation from iPSCs or through embryoid bodies (EBs). The DA progenitors can be sorted with a midbrain cell surface marker like CORIN to achieve higher purity of DA neurons via elimination of unwanted contaminant cells. Mature midbrain DA neurons are generated from these progenitors by addition of mentioned factors at the end of 40–70 DIV (days in vitro) in total. * indicates factors that may be used in the final differentiation step, but not compulsory. ActA, activin A; AA, ascorbic acid; BDNF, brain-derived neurotrophic factor; DAPT, γ-secretase inhibitor; dbcAMP, dibutyryl cyclic adenosine monophosphate; FGF8, fibroblast growth factor 8; GDNF, glial-cell-derived neurotrophic factor; Shh, sonic hedgehog; TGFβ3, transforming growth factor beta-3

assessments of autophagy/mitophagy (Stathakos et al. 2020). In an interesting study, autophagic dysfunction and premature aging was shown by PD-patient-derived NSCs (Zhu et al. 2019). One of these patients had early-onset PD with a novel mutation in *PLA2G6* gene. The authors hypothesize that developmental defects, and the subsequent depletion of NSC pool size could lead to lower DA neuron number and this impacts the onset and severity of the disease progression (Zhu et al. 2019). Hence, not only iPSC-derived DA neurons, but also the developmentally upstream NSCs could be a disease-relevant phenotype for prediction analyses and design of intervention therapies.

4.1 iPSC-Derived Two-Dimensional (2D) and Three-Dimensional (3D) Culture Models of ARPD

Mutations in the *PARK2* gene, encoding the protein parkin, have been identified as the most common cause of ARPD. Unsurprisingly, the limited in vitro iPSC-derived ARPD models primarily examine the cellular pathologies of this gene. Human iPSC-derived neurons with *PARK2* knockout is known to demonstrate severe mitochondrial dysfunction even in the absence of external stressors. *PARK2* patient iPSC-derived neurons showed increased oxidative stress, higher α-synuclein accumulation and enhanced activity of the nuclear factor erythroid 2-related factor 2 (Nrf2) pathway (Imaizumi et al. 2012). Interestingly, iPSC-derived neurons, but not fibroblasts or iPSCs, exhibited abnormal mitochondrial morphology and impaired mitochondrial homeostasis in their study. In a similar study, the loss of parkin significantly increased the spontaneous DA release independent of extracellular calcium and showed decreased dopamine uptake by reducing the total amount of correctly folded DAT along with DA-dependent oxidative stress. All these phenotypes could be rescued by overexpression of *parkin*, but not its PD-linked T240R mutant or GFP (Jiang et al. 2012). Mitochondrial dysfunction, elevated α-synuclein, synaptic dysfunction, DA accumulation, and increased oxidative stress and ROS have been reported in *PARK2*- and *PINK1*-patient-derived neurons in a floor-plate-based but not a neural-rosette-based directed differentiation strategy (Chung et al. 2016). Impairment of mitophagy via formation of S-nitrosylated PINK1 (SNO-PINK1) has also

been shown in iPSC-derived parkin-mutant neurons (Oh et al. 2017). In a recent study, *PARK2* knockout neurons from isogenic lines exhibited lysosomal impairments and autophagic perturbations, suggesting an impairment of the autophagy–lysosomal pathway in parkin-deficient cells (Okarmus et al. 2020). The same group had earlier shown disturbances in oxidative stress defence, mitochondrial respiration and morphology, cell cycle control and cell viability of parkin-deficient neurons (Bogetofte et al. 2019).

Midbrain-specific 3D cultures are at present a powerful tool for modeling PD in vitro. The use of microwells by Tieng et al. was the very first attempt in this direction to generate embryoid bodies, which were then placed on an orbital shaker before being seeded and grown at air–liquid interface (Tieng et al. 2014). DA progenitor cells expressed FOXA2 and LMX1A as well as TH within a short span of 3 weeks. Subsequently, a number of reports have been published for midbrain organoids with neuromelanin expression seen in long-term cultures (Smits and Schwamborn 2020; Kim et al. 2019; Jo et al. 2016; Monzel et al. 2017b; Qian et al. 2016). However, PD modeling with midbrain organoids is largely focused on dominant mutations like *LRRK2* (Smits et al. 2019; Kim et al. 2019), *SNCA* (Jan et al. 2018) and also an only report on sporadic PD (Chlebanowska et al. 2020). A very recent study used CRISPR-Cas9 genome editing to develop isogenic loss-of-function 3D models of early-onset autosomal recessive PD (PARKIN$^{-/-}$, DJ-1$^{-/-}$, and ATP13A2$^{-/-}$) to identify common pathways (Ahfeldt et al. 2020). The DA neuronal population was markedly reduced in *PRKN*$^{-/-}$ organoids but no significant differences were observed in the other two cell lines. The death of newly differentiated TH^{+} neurons and higher expression of VTA marker CALB1 in the *PRKN*$^{-/-}$ organoids were indicative of A9-like neurons being more severely affected than others. A dysregulation of the autophagy–lysosomal pathway and upregulated ROS in all cell lines and an upregulation of pathways associated with oxidative phosphorylation, mitochondrial dysfunction, and Sirtuin signaling, as well as a significant depletion of mitochondrial proteins were seen in the *PRKN−/−* DA neurons (Ahfeldt et al. 2020). Astrocytic pathologies in human *PRKN*-mutated iPSC-derived midbrain organoids were revealed for the first time, suggesting a non-autonomous cell death mechanism for dopaminergic neurons in brains of *PRKN*-mutated patients (Kano et al. 2020). Mutations in *PINK1* have also been reported to generate reduced TH^{+} counts in midbrain organoids (Jarazo et al. 2019). Human midbrain organoid/spheroid cultures are a scalable and reproducible system to obtain DA neurons expressing markers of terminal differentiation along with neuromelanin production in a 3D environment that replicates the neuronal and glial cytoarchitecture of the human midbrain (Galet et al. 2020). They can hence provide a crucial platform to explore the molecular basis of ARPD, and also to delineate the associated cellular pathologies.

Cell Replacement Therapy with iPSC-Based DA Derivatives The various challenges pertaining to the safety and efficacy of stem-cell-based cell transplantations in PD have been elegantly reviewed and described (Fan et al. 2020). The right neural cell type for transplantation is of utmost importance. FGF8b inclusion in the differentiation protocols helps in acquisition of a caudal midbrain fate and promotes high dopaminergic graft volume, density and yield as evidenced by deep sequencing of more than 30 human ESC-derived midbrain tissues (Kirkeby et al. 2017). Dopaminergic precursors beyond the floor-plate progenitor stage but before formation of TH^{+} dopaminergic neurons are found to be most efficient for graft survival, integration and function in animal models (Kikuchi et al. 2017; Nolbrant et al. 2017). Grafting of these precursors into the putamen area, where SNpc dopaminergic neurons innervate, is an approach most likely to succeed (Fan et al. 2020). The number of cells to be transplanted is still debated. Takahashi's group reported a minimum of 16,000 TH^{+} cells in a primate model to see improvements in PD score and motor function (Kikuchi et al. 2017). The generation and

implantation of iPSC-derived autologous dopaminergic progenitor cells in a patient with idiopathic PD is reported with clinical and imaging results suggesting possible benefit over a period of 24 months (Schweitzer et al. 2020). A global consortium, GForce-PD (http://www.gforce-pd.com), was set up in 2014, with major academic networks in Europe, the United States and Japan working on developing stem-cell-derived neural cell therapies for PD (Barker et al. 2015). The clinical trials using human ESCs are ongoing in Australia (NCT02452723) and China (NCT03119636), with their pre-clinical data published (Garitaonandia et al. 2016; Wang et al. 2018). A clinical trial (JMA-IIA00384, UMIN000033564) in Japan to treat PD patients by using iPSC-derived DA progenitors (DAPs) was started in 2018 by Takahashi and colleagues (Barker et al. 2017; Doi et al. 2020). The results of these trials are eagerly anticipated.

5 Limitations to iPSC-Based Disease Modeling of PD

Although iPSCs and their derivatives are currently in the forefront as PD models, there are many challenges which remain unaddressed. The most significant drawback of in vitro models is the absence of LB formation in PD iPSC-derived DA neurons. Increased levels of phosphorylated pS129 α-synuclein, however, have been observed (Lin et al. 2016). Additionally, the efficiency of generating DA neurons varies significantly between different methods and approximately 20–30% of the final cells are identified as DA neurons even with the most robust method such as the floor-plate induction protocol (Cui et al. 2016). Sorting of DA progenitors with markers such as CORIN seems to aid in a better yield of mature and functional DA neurons (Doi et al. 2020; Paik et al. 2018). Knocking in a reporter gene in the endogenous TH locus has been attempted to quantify the final yield of DA neurons (Cui et al. 2016) to understand the efficiency of different published protocols. However, no significant progress has been made to analyze if the DA neurons derived in vitro are similar to the SNpc neurons impacted in a PD patient. A TH^+ DA neuron is necessarily not a representation of the A9 or SNpc nuclei of the brain, though GIRK2/TH positivity and low Calbindin is considered as an A9 signature (Hartfield et al. 2014; Sánchez-Danés et al. 2012; Woodard et al. 2014). A reliable strategy would be multiplexing markers for reliable subtype identification (Kim et al. 2020). Another difficulty in modeling PD with iPSCs is the induction of ‘aging’ in a culture dish. Pharmacological inhibition of telomerase by the inhibitor BIBR1532 demonstrates moderate disease-relevant phenotypes in *PINK1* and *PARKIN* DA neurons (Vera et al. 2016). Progerin (the protein associated with premature aging) overexpression as a strategy to induce aging is also reported (Miller et al. 2013) but interpretation is complex as disease-relevant phenotypes and progerin-phenotypes are indistinguishable (Sison et al. 2018). Moreover, contrary to what is seen in PD pathology, an exogenous stressor is always necessary to observe disease phenotypes in an iPSC–DA system. In a PD-patient-derived iPSC model, DA neurons exhibit apoptosis only after exposure to stressors including hydrogen peroxidase, MG132 and 6-OHDA (Cooper et al. 2012). Lastly, 2D culture systems that are normally used to differentiate DA neurons do not mimic the complex in vivo environment. 3D organoids fill this gap by representing a more physiologically relevant model system. However, the tremendous progress seen in the field is largely limited to cortical or cerebral organoids. A few midbrain spheroid or organoid culture systems are nonetheless reported (Smits et al. 2019; Kim et al. 2019; Jo et al. 2016; Monzel et al. 2017b). Results from these studies indicate that 3D midbrain cultures are definitely an improvement over 2D cultures to model PD. A recent study describes the robust generation of midbrain organoids with homogeneous distribution of midbrain DA neurons along with other neuronal subtypes as well as functional glial cells, including astrocytes and oligodendrocytes (Kwak et al. 2020). Nevertheless, an overall low efficiency of generation and heterogeneity within the midbrain

organoids along with its ethical considerations raises contentious questions towards a bench-to-bedside approach.

6 Conclusions

Human pluripotent stem cells, iPSCs in particular, are an invaluable tool to help us better understand PD pathology by generating functional DA neurons with A9-like identity and also reproducing the midbrain cell composition. Improvement in differentiation protocols and 3D culturing techniques combined with genome-editing technologies aids in better PD modeling studies. Additionally, these cultures exhibit key features of PD, such as α-syn accumulation, autophagic defects, oxidative stress and impairment of mitochondrial function. However, it may be advantageous to include other cell types like microglia in PD studies rather than focusing on midbrain-specific organoids to understand the disease pathology in a relevant way. The blood-brain barrier (BBB) and its dysfunction in PD should also be emphasized. Further, the future direction in investigating PD should make use of the organ-on-chip or organoids-on-chip model with a multi-organ configuration to study different cell types and involvement of various organs in PD progression and pathology. Lastly, ARPD genes other than *PRKN* and *PINK1*, though rare, may provide insights into the common molecular pathways of the monogenic disease forms and should be included in such detailed studies.

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