From cell lines to pluripotent Stem Cells for Modelling Parkinson's Disease

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Highlights

- Available PD models range from classical cell lines to 3D midbrain organoids.
- A fully reliable *in vitro* model for the understanding of PD etiology is still lacking.
- Human-derived models open new routes for PD modeling and drug screening.

Abstract

Parkinson's disease (PD) is the second most common neurodegenerative disorder characterized by loss of dopaminergic (DAergic) neurons in the *substantia nigra* that contributes to the main motor symptoms of the disease. At present, even if several advancements have been done in the last decades, the molecular and cellular mechanisms involved in the pathogenesis are far to be fully understood. Accordingly, the establishment of reliable *in vitro* experimental models to investigate the early events of the pathogenesis represents a key issue in the field. However, to mimic and reproduce *in vitro* the complex neuronal circuitry involved in PD-associated degeneration of DAergic neurons still remains a highly challenging issue. Here we will review the *in vitro* PD models used in the last 25 years of research, ranging from cell lines, primary rat or mice neuronal cultures to the more recent use of human induced pluripotent stem cells (hiPSCs) and, finally, the development of 3D midbrain organoids.

Key words: cell cultures, striatum, in vitro models, dopamine, glutamate

1.1 Introduction

The use of *in vitro* models allows the analysis of molecular and cellular pathogenic events as well as drug screening in fully controlled environment conditions. However, taking into consideration that Parkinson's Disease (PD) is a very complex brain disorder that involves several neuronal circuitries and brain areas, it remains really challenging to reproduce *in vitro* in a cell culture the complexity of these aspects. In the last three decades a vast variety of cell culture systems, ranging from cell lines to the more recent 3D organoids, have been used as *in vitro* models to study PD pathogenesis as well as to test possible neuroprotective agents.

1.2 Cell lines

Established cell-lines (either differentiated or not) are immortalized cells derived from a multicellular organism. This type of cell lines has several advantages (Lopes et al., 2017):

1. highly proliferative;
2. high reproducibility,
3. can be easily genetically manipulated;
4. some of them, present all the DAergic machinery, for example enzymes for DA metabolism and synaptogenesis (Lopes et al., 2017);
5. many of them have human origins and therefore human genetic background.

However, the use of these models is limited by relevant disadvantages:
1. they often require further differentiation steps to have the morphological and/or physiological characteristics of DAergic neurons;
2. they have oncogenic origin; therefore, their main feature is the high proliferation which is in clear contrast with neuronal feature (Herrup and Young 2007; Lopes et al., 2017).

The pathological hallmarks of PD can be reproduced in these cultures either genetically, through the overexpression or silencing of PD-related genes (SNCA, Parkin, PINK1, LRKK2, GBA), or chemically, through the use of neurotoxins (such as 6-OHDA, MPP^+, rotenone), which alters many cellular processes. Therefore, enabling to highlight the different molecular pathways altered in PD complex pathophysiology, genetic models allow to reproduce a condition that is more similar to the

in vivo one. Overall, this type of cell lines should be used mainly for evaluating apoptosis, mitochondrial and gene dysfunction, oxidative stress, screening of potential drugs and their relative neuroprotective properties. In this review, we will focus on the following immortalized cell lines: SH-SY5Y, PC12, LUMHES, N2a, N27, MN9D.

1.2.1 SH-SY5Y cell line

SH-SY5Y are a subclone of the SK-N-SH neuroblastoma cells, obtained in 1978 starting from a 4-year-old girl bone marrow biopsy (Biedler et al., 1978). The original cell line SK-N-SH presents two phenotypes characterized by different morphological and biochemical features: one is defined as N-type (neuroblastic) and the other is called S-type (epithelial, adherent) (Encinas et al., 2000; Kovalevich and Langford, 2013); both can be differentiated in neurons (Ross et al., 1983). SH-SY5Y cells have a neuroblastoma-like morphology characterized by round cell bodies and short processes (Påhlman et al., 1984). As undifferentiated cells, they are not DAergic cells, having instead a catecholaminergic phenotype, as they can synthesize both DA and noradrenaline (NA) (Xicoy et al., 2017). SH-SY5Y cells present, when not differentiated, activity of DA-β-hydroxylase (Biedler et al., 1978) and of tyrosine hydroxylase (Ross and Biedler, 1985), a basal release of noradrenaline (NA) (Påhlman et al.,1984; Xicoy et al., 2017), and expression of DA transporter (DAT) (Cheung et al., 2009). Furthermore, alongside the good activity of the DAT, on the other hand, they are characterized by a low activity of the vesicular monoamine transporter type 2 (VMAT2). Accordingly, the concentration of endogenous DA (normally very low) can be regulated through external administration in the culture medium (Alberio et al., 2010, 2012). Additionally, although these cells present a complete DAergic apparatus, they do not exhibit neuronal features and are highly proliferative, as their oncogenic origin suggests. Moreover, these cells have a low expression of neuronal markers because they are in the early phases of neuronal differentiation (Lopes et al., 2017). To obtain cells with a neuronal phenotype, a differentiation protocol is needed. Several methods have been used: the most common is based on the use of retinoic acid (RA), with varying concentrations and incubation times (Xicoy et al., 2017). Another differentiation protocol involves the use of 12-O-Tetradecanoyllphorbol-13-acetate (TPA), with or without RA. This method is more effective to obtain a DAergic cell phenotype (Xicoy et al., 2017). In this regard, in 2004 Presgraves and colleagues, starting from the method used by Pennybacker et al., developed the differentiation protocol through RA and TPA, to obtain dopaminergic cells from SMS-KCNR neuroblastoma cells. In fact, RA/TPA treated cells have a dopaminergic phenotype with higher levels of expression of TH and DAT, as well as dopaminergic D2 and D3 receptors. Therefore, this cell-line could be a useful

tool in the study of the neuroprotection of dopamine agonists against MPP+ and DA-induced toxicity. [Pennypacker et al., 1989; Presgraves et al., 2004].

However, other studies show that this method leads to a formation of a heterogeneous neuronal population, with an increase of NA neurons (Xicoy et al., 2017, Påhlman et al., 1984). Another protocol, instead, promotes the use of brain-derived neurotrophic factor (BDNF) together with RA. This method consists in the induction of a low proliferative and homogeneous neuronal population (Encinas et al., 2000; Xicoy et al., 2017). The differentiation with RA and BDNF seems to induce a reduced degree of proliferation, a typically neuronal morphology with the development of neurites, an increase in the expression of neuronal (NeuN) and DAergic (TH) markers and greater sensitivity to 6-OHDA (Lopes et al., 2010, 2017).

As previously mentioned, SH-SY5Y can undergo both genetic and chemical modifications to mimic the pathological features of PD (Wu et al., 2018; Li N et al. 2019; Wang et al., 2019; Ferlazzo et al., 2019). Even if many cellular processes can be investigated by using this cell line (i.e. drug screening, oxidative stress, apoptosis, mitochondrial alteration, autophagy) (Li et al., 2019; Limboonreung et al., 2019; Lualdi et al., 2019; Md et al., 2019; Ramalingam et al. 2019; Ju et al., 2019), it cannot be used for electrophysiological studies or assessment of neurochemical alterations (Xicoy et al., 2017). Regarding their electrophysiological properties, different studies have shown variable responses between individual cells although exposed to the same experimental stimuli. These observations suggest that these cells are made up of non-uniform subpopulations, with a different expression and density of membrane channels (Tosetti et al., 1998; Santillo et al., 2014). SH-SY5Y cells have also been used in α-synuclein studies, in particular to better understand its role in the various cellular mechanisms altered in PD, such as autophagy-lysosomal pathways, mitochondrial functions and the role of dopamine agonists (Bellucci et al., 2008; Vogiatzi et al., 2008; Bellucci et al., 2011; Diogenes et al., 2012; Fares et al., 2014; Mahul-Mellier et al., 2014; Zaltieri et al., 2015).

1.2.2 LUHMES line

The LUHMES (Lund Human Mesencephalic cells) lines are a subclone of human mesencephalic cell lines MESC 2.10 that were isolated from the ventral mesencephalic brain region of an 8-week-old fetus and were immortalized by introducing a tetracycline-controlled, v-myc-gene (TET-off) (Lotharius et al., 2005; Zhang et al., 2014; Lopes et al., 2017). Unlike SH-SY5Y, this cell line shows specific DAergic markers and typical neuronal morphology with long neurites even if only after differentiation with tetracycline, cyclic AMP (cAMP) and glial derived neurotrophic factor (GDNF) (Lotharius et al., 2005; Scholz et al., 2011, Zhang et al., 2014). Once differentiated, these cells have spontaneous electrical properties, express DAergic factors such as TH, DAT, VMAT and D2

receptors, are able to release DA and acquire the morphology of primary neurons (Scholz et al., 2011; Zhang et al., 2014; Lopes et al., 2017). The pathophysiological characteristics of PD can be reproduced both through exposure to neurotoxins and genetic manipulation. In fact, the non-differentiated LUHMES can be easily transfected or infected with virus (Schildknecht et al., 2013; Stepkowski et al., 2015; Zhang et al., 2014; Höllerhage et al., 2017; Paiva et al., 2017). Several studies used these cell lines instead following treatment with neurotoxins, such as rotenone, 6-OHDA, MPP$^+$ (Schildknecht et al., 2009; Stepkowski et al., 2015; Harris et al., 2018).

The non-oncogenic human origin represents the main advantage of this cell line. However, these cells grow slowly, similarly to primary cells, and they need to have culture media supplemented with N2 and fibroblast growth factor (FGF) (Scholz et al., 2011; Lopes et al., 2017). Recently, this cell line was used in 3D culture thus bypassing the problem to culture these cells for long-time period (Smirnova et al., 2016). Finally, the 3D LUHMES culture permits to investigate neurotoxic effects of different compounds and neurodegenerative processes (Harris et al., 2018).

1.2.3 PC12 line

PC12 is a cell line derived from a transplantable rat pheochromocytoma of the adrenal medulla. It can easily differentiate into neuronal-like cells through NGF induction when cultured on type IV collagen coated-coverslips (Greene and Tischler, 1976; Grau et al., 2012). In this way, cells become able to release neurotransmitters from the vesicles, to develop axons and acquire electrical properties. PC12 contains DA and other catecholamines and can release NA following treatment with ascorbic acid. The treatment with NGF in combination with dexamethasone leads to increased vesicles and neurotransmitter release. Due to these features, PC12 cells have been used for the study of endocytosis through amperometry experiments (as reviewed by Westerink and Ewing, 2008). As described-above for the SH-SY5Y and the LUHMES cell lines, also the PC12 cells can be used as an *in vitro* model for PD through treatment with neurotoxins such as MPP$^+$, 6-OHDA, and rotenone (Sai et al., 2008; Xu et al., 2017; Lee et al., 2018). They are also used for evaluation of α-synuclein pathological properties (Wang et al., 2017). Together with SH-SY5Y cell line, they represent the most used PD cellular model, but results obtained with these oncogenic cellular models must be taken with caution and evaluated in other cellular and animal models.

1.2.4 MN9D line

The MN9D cell line was obtained from the somatic fusion of mouse embryonic mesencephalic cells with neuroblastoma cells (N18TG2). One of the obtained clones, the MN9D, has the ability to synthesize catecholamines, express TH and generate sodium currents (Choi et al., 1991). In the

undifferentiated state, cells have a round cellular soma and have no process extension (Rick et al., 2006). Treatment with GDNF and/or other factors (retinoic acid, Nurr1) leads to the arrest of the cell cycle, the formation of neurites and DA synthesis (Heller et al., 1996; Castro et al., 2001; Hermanson et al., 2003). Moreover, only through differentiation with GDNF followed by butyric acid (Rick et al., 2006), MN9D cells acquire electrophysiological properties comparable to the mature DA neurons. Similar to the other above-mentioned cell lines, MN9Ds are also used to investigate the role of α-synuclein and undergo treatment with neurotoxins (Hussain et al., 1999; Wu et al., 2011; Spittau et al., 2012; Shao et al., 2015; Li et al., 2017).

1.2.5 N27 line

N27 cells were obtained from immortalized rat mesencephalic cells. These cells express low levels of TH and DA transporter (DAT), but are sensitive to 6-OHDA and to H_2O_2 (Clarkson et al., 1998). This cell-line has been used mainly in neurotoxicity, neurodegeneration studies and other cellular processes (Cantu et al., 2011; Dranka et al., 2012; Lopert et al., 2012; Cristòvao et al., 2013; Thomas et al., 2013; Harischandra et al., 2015). Recently, Gao and collaborators have recloned N27 cell lines, identifying a new clone with higher TH and DAT levels. Furthermore, these cells express VMAT and other transcription factors (NURR1, EN1, Fox42, PITX3) and release DA in normal and depolarizing conditions (Gao et al., 2016).

1.2.6 Neuro 2a line

Neuro 2a cells derive from mouse neural crest (Klebe et al., 1969) and present a neuronal and amoeboid morphology. Due to their characteristics these cells have been used in studies aimed at evaluating neuronal differentiation, axonal growth and signaling pathway neurotoxicity (Le Page et al., 2005; Salto et al., 2015). Moreover, they have been used to study Alzheimer's Disease (Kim et al., 2014). When differentiated, Neuro 2a cells express neuronal properties, such as the presence of neurofilaments, large amounts of microtubular proteins (Olmsted et al., 1970) and are easily transfected. Their peculiarity is the capability to differentiate into neurons in a very short time, through serum deprivation and/or modification of various culture medium factors (Lee and Nikodem, 2004; Evangelopoulos et al., 2005; Wasilewska-Sampaio et al., 2005; Tremblay et al., 2010). Tremblay and collaborators (2010) showed that the use of dibutyryl cyclic adenosine monophosphate (dbcAMP) promotes Neuro 2a differentiation into DAergic cells, enhancing Nurr-related factor 1 (Nurr1), TH and DA expression levels and thus being useful as PD model (Tremblay et al., 2010).

1.3 Primary cultures

1.3.1 Primary DAergic cultures

Even though different neurotransmitter systems are involved in PD, the hallmark of the disease is the degeneration of DAergic neurons of the *substantia nigra pars compacta* (SNpc) with a consequent reduction of DA levels in the striatum (Surmeier et al., 2017; Obeso et al., 2017). This leads to the appearance of the classical motor symptoms of the disease, namely resting tremors, bradykinesia and rigidity (Obeso et al., 2017). Accordingly, the setting up of reliable cell cultures of

DAergic neurons provide a very useful *in vitro* model for the study of the molecular and cellular mechanisms involved in the neurodegeneration and for the understanding of the causes of selective neuronal vulnerability for this disorder (Studer, 2001).

Early studies that used dissociated primary neuronal cultures obtained from rat SNpc showed the presence of about 40% DAergic neurons characterized by thick and straight primary processes dividing into several branches (Masuko et al., 1992). Physiological properties of these cultured DAergic neurons were similar to those reported for DAergic neurons in brain slices suggesting that it is possible to obtain dissociated cultures of the SN (Masuko et al., 1992). Results obtained by Kim and coworkers (1997) indicated that SN neurons of neonatal rats, after being dissociated, produce the same transmitters and the same receptors in culture as those in vivo.

The method for culturing midbrain DAergic neurons from rat and mouse embryos has been optimized in several more recent studies (Weinert et al., 2015; Lopes et al., 2017) showing that this neuronal population undergoes to a rapid maturation and differentiation with the acquisition of synaptic, functional and morphological properties suitable for the analysis of molecular and cell biology events. Primary DAergic neurons have been widely used for evaluation of cell survival after treatment with neurotoxic compound possibly involved in PD pathogenesis or putative neuroprotective agents. Using cultured mouse mesencephalic neurons, an early study compared the toxicity of sequential exposure to rotenone (an inhibitor of mitochondrial complex I) and glutamate on GABAergic and DAergic neurons (Marey-Semper et al., 1995). This neuronal subtype was confirmed to be more vulnerable than inhibitory neurons to the toxic clues through a mechanism involving both NMDA receptor activation and a defective energy metabolism (Marey-Semper et al., 1995). More recently, the involvement of complex I inhibition in DAergic neuronal loss has been examined using a genetic mouse strain lacking *Ndufs4* gene, encoding for a complex I essential subunit. Mesencephalic neurons in culture, however, did not show compromised survival despite lacking functionality of the complex (Choi et al., 2008). These results support the existence of other intrinsic properties that confer to DAergic neurons higher vulnerability to toxins like rotenone, paraquat and MPP+. Substance P, Neurokinins A/B, and Synthetic Tachykinin were shown to exert a selective neuroprotective action on mesencephalic DAergic neurons, highlighting the importance of excitatory inputs for survival of

this neuronal subtype (Salthun-Lassalle et al., 2005). A similar culture model in which DAergic neurons progressively die upon maturation was used to assess the mechanism of nicotine-mediated protection in PD. Interestingly, the beneficial effect occurred only in presence of simultaneous depolarizing stimuli able to elevate cytosolic calcium levels, through a mechanism involving a particular subtype of nicotinic acetylcholine receptors (Toulorge et al., 2011).

The more recent development of PD-related transgenic mice has brought back a more extensive use of primary DAergic neurons prepared from these animal models. For instance, a work from Ramonet and collaborators in 2011 investigated the effects of two familiar PD mutations of LRRK2, R1441C and G2019S, in transgenic mouse models. Interestingly, cultures of midbrain DAergic neurons derived from G2019S-LRKK2 mice, which exhibit degeneration of the nigrostriatal pathway *in vivo*, were characterized by a considerable reduction of neurite length and complexity. (Ramonet et al., 2011). A different approach, a binary tetracycline-dependent inducible gene expression system, was exploited to generate a transgenic mouse model expressing the mutated A53T α-syn in mDAergic neurons. Several neuronal abnormalities were found *in vivo*, including decreased DA release, Golgi-apparatus fragmentation and impairment of the autophagy lysosomal pathway. Moreover, using midbrain cultures derived from transgenic mice, they reported an amelioration of α-syn-induced degeneration of mDAergic neurons by preventing proteasomal degradation of the transcription factor Nurr1 (Lin et al., 2012).

1.3.2 Primary striatal neuronal cultures

A well-known aspect of striatal anatomy and function is the presence of more than 95% spiny projection neurons (SPNs) in absence of a structured local excitation system. At dendritic spines of striatal SPNs, DAergic terminals from the *substantia nigra pars compacta* converge with glutamatergic terminals from the cerebral cortex. Molecular and functional interactions between these two neurotransmitter systems have been widely described (Gardoni and Bellone, 2015). The degeneration of the nigrostriatal DAergic pathway in PD leads to significant morphological and functional changes in the striatal neuronal circuitry, including modifications of the corticostriatal glutamatergic synaptic architecture with consequent loss of striatal synaptic plasticity (Calabresi et al., 2007). Overall, an integrated cross-talk between DA and glutamate systems plays an essential role in the regulation of a physiological motor behavior. Accordingly, early studies showed that when striatal neurons are grown alone in culture, they do not show a physiological spontaneous network activity and develop only a very low number of dendritic spines. However, primary neuronal striatal cultures have been widely used to investigate molecular mechanisms regulating DA and glutamate receptors and their signaling pathways at SPNs. Immunocytochemical and electrophysiological

characterization of cultured rat striatal neurons indicate that these cells have both DA D1 and D2 receptors and NMDA-type glutamate receptors, abundant expression of main striatal markers such as calbindin, calretinin and the cannabinoid-1 receptor and voltage-gated K+ channel subunits characteristic of adult tissue (Falk et al., 2006; Hallett et al., 2006). In dissociated striatal neurons, DA or D1 receptor agonists potentiate NMDA receptor currents (Flores-Hernandez et al., 2002), with a mechanism involving the phosphorylation of the transcription factor Ca^{2+} and cyclic AMP response element binding protein (CREB) in the nucleus by means of NMDA receptor-mediated Ca^{2+} signaling (Dudman et al., 2003). Moreover, Hallett and colleagues (2006) observed a selective effect of DA D1 receptor activation on the localization of NMDA subunits in striatal neurons.

Growing of striatal neurons in presence of GFP-expressing cortical neurons leads to the appearance of spontaneous and evoked excitatory synaptic currents and a remarkable increase in the density of dendritic spines (Segal et al., 2003). Importantly, this event was strictly modulated by the application of tetrodotoxin (Segal et al., 2003). Fagni and collaborators further demonstrated the need of a cortical innervation for the correct development of striatal neurons in culture (Burguiere et al., 2013). They developed an *in vitro* model of mouse corticostriatal primary co-cultures, in which cortical neurons were isolated from embryonic wild-type mice and striatal neurons from embryonic mice expressing GFP. This allows the study of dendritic spine development and patch-clamp recording of the striatal neurons visualized by GFP fluorescence (Burguiere et al., 2013). Consistent with previous studies (Segal et al., 2003), striatal cells remained almost aspiny when cultured in the absence of cortical neurons. When cultured with cortical neurons, striatal neurons developed high density of dendritic spines (Burguiere et al., 2013). However, many of the above-mentioned approaches used for the study of cortical influences on striatal neurons have serious pitfalls. In particular, co-cultures of cortical and striatal neurons, although spontaneously active, are almost absent of cholinergic interneurons and develop connections from striatal cells to cortical cells that are not present in vivo, such as inhibitory striatocortical connections.

To overcome some of these issues, more recently Garcia-Munoz and co-workers (2015) developed a method for growing cortical and striatal neurons in separated compartments that allows cortical neurons to innervate striatal cells in culture. The activity of both areas can be recorded in multielectrode arrays or individual patch recordings from pairs of cells.

1.4 Human induced pluripotent stem cells

The introduction of human induced pluripotent stem cells (iPSCs) technology, discovered by Yamanaka and Takahashi in 2006, revolutionized the modelling of human diseases. The possibility to reprogram fully differentiated cells from patients to the stemness and then redirecting them towards

the desired cell type brought a great power to biomedical research. This is particularly true for the *in vitro* modelling of physiology and pathophysiology of the central nervous system including the field of neurodegenerative disorders, previously restrained by the impossibility to obtain affected tissues and cells directly from patients. Therefore, iPSCs allow to overcome some of the obstacles, given either by the use of animal models, such as species-specificity of cellular pathways, ethical issues and the limited availability of post-mortem human brain tissue. More in detail, iPSCs give the opportunity to have disease-relevant neuronal subtypes that retain the specific genetic background of the patient. Taking into account the specific genetic settings of an individual, the development of a personalized drug treatment is a fundamental requirement and a highly relevant topic in high throughput drug screening (Haston and Finkbeiner, 2016).

In this framework, the combination of iPSC technology and very recent genome editing techniques (Mali et al., 2013) allows to generate isogenic control cells that maintain the same genetic background of the patient leading to a great advantage in the modelling of complex brain disorders such as PD.

1.4.1 Modelling genetic PD with iPSCs

Aberrations in *SNCA* gene, coding for the protein α-synuclein, are one of the most common defect causing familiar autosomal dominant Parkinson's disease. Devine and collaborators (2011) successfully differentiated a subset of iPSCs lines from patients bearing multiplication of *SNCA* locus into midbrain DAergic neurons (mDA). Genomic DNA analyses confirmed the alteration of SNCA to be still present in iPSC lines. In addition, mDA neurons differentiated from mutated iPSCs demonstrated to have a double quantity of the protein with respect to healthy relative-derived neurons (Devine et al., 2011).

The work of Byers and collaborators in the same year (2011) also reported accumulation of α-synuclein in DA neurons from a PD patient bearing SNCA triplication. Aberrant levels of α-synuclein were not the only disease feature exhibited by PD neurons, which also showed increased sensitivity to oxidative stress, caused by exposure to hydrogen peroxide, together with augmented oxidative stress markers (Byers et al., 2011).

Soldner and co-workers (2011) used for the first time iPSCs technology combined to genome editing in the field of PD research. Exploiting Zinc-finger nuclease (ZFN)-mediated genome editing they generated a set of isogenic disease and control human iPSCs by correcting two point mutations in the α-synuclein gene. Specifically, they either derived hiPSCs from a patient carrying the A53T (G209) α-synuclein mutation followed by the correction of this mutation or, alternatively, by generating either the A53T (G209A) or E46K (G188A) mutation in the genome of wild-type hESCs. They showed that patient-derived hiPSCs upon targeted gene correction of the A53T mutation were still

able to differentiate into tyrosine hydroxilase-expressing DA neurons, lacking the expression of the mutated A53T/G209A transcript.

In the same year, Nguyen and collaborators (2011) reported the generation of iPSC lines from PD patients harbouring the LRRK2 G2019S mutation. Midbrain DAergic neurons (mDA) derived from iPSC lines exhibited cardinal PD features as accumulation of α-synuclein, upregulation of oxidative stress response genes and increased susceptibility to neurotoxins (Nguyen et al., 2011). Increased α-synuclein levels accompanied by alterations of autophagy were also reported by another study in mDA neurons differentiated from iPSCs lines harbouring the same LRRK2 G2019S mutation (Sánchez-Danés et al., 2012). Interestingly, they found the same pathogenic features also in mDA neurons from idiopathic PD patients (Sánchez-Danés et al., 2012). Moreover, besides accumulation of autophagic vacuoles, both sporadic PD and LRRK2-mutated mDA neurons were characterized by decreased number of neurites and aberrant neurite arborization with respect to neurons derived from age and sex matched healthy controls. However, polymorphisms in other genes involved in PD pathogenesis, as *SNCA* or *MAPT*, give a large contribution in affecting phenotypes of individuals with familiar LRRK2-PD. This genetic variability makes the study of the effect of the specific mutation more complex (Botta-Orfila et al., 2012; Golub et al., 2009). However, the use of isogenic gene correction in iPSCs from patients can help to overcome this issue. Reinhardt and collaborators (2013a) used this tool to analyse the molecular alterations conferred by the G2019S LRRK2 lesion in mDA neurons differentiated from isogenic iPSC lines. The targeted gene correction was shown to rescue PD-associated phenotypes, including neurite shortening and basal autophagy defects.

Experimental modelling of *PINK1* and *PARK2*-dependent PD has always been complex. *In vivo*, knockout of the two genes in mouse models do not lead to degeneration of nigral DA neurons (Dawson et al., 2010). Issues in reproducing disease phenotype are not shown solely by *in vivo* models. Recent studies on iPSC derived DA neurons reported that loss of function mutations in both the two genes are not sufficient to induce cell death in culture if not exposed to external clues (Miller et al., 2013; Chang et al., 2016). To note, the majority of studies examining *PINK1*-associated PD has been usually performed by means of knockdown strategies and models with limited relevance for human pathophysiology. A contribution to dissect the role of *PINK1* mutations using iPSC technology was given by a work from Seibler (2011). Skin fibroblasts taken from three patients harbouring different missense and nonsense PINK1 mutations - nonsense (c.1366C>T; p.Q456X) and missense (c.509T>G; p.V170G) - were reprogrammed to iPSCs and then directed to DA neurons. Several defects became apparent after mitochondrial depolarization, most importantly the defective recruitment of the exogenously expressed Parkin to mitochondria. Moreover, neurons were characterized by increased mitochondrial copy number coupled to upregulation of the mitochondrial

biogenesis regulator PGC-1α, all defects rescued by wild-type PINK1 expression (Seibler et al., 2011).

iPSC technology has proven to be useful also to dissect convergent disease mechanisms shared between different familiar PD forms. Cooper and co-workers (2012) generated neural cells from iPSCs of PD patients or pre-symptomatic subjects mutated in PINK1 and LRRK2 genes. Compared to neurons derived from healthy individuals, these cells had several alterations in the mitochondrial response that could be rescued by coenzyme Q10, rapamycin or by an inhibitor of LRRK2 kinase activity.

The G2019S LRRK2 mutation was shown to delay the arrest of damaged mitochondria and thus to slow mitophagy initiation in iPSC derived neurons (Hsieh et al., 2016). The analysis revealed that the pathways of LRRK2 and PINK1/Parkin works in parallel, converging on the mitochondrial protein Miro. To note, dysregulation of the same process and aberrant mitochondrial motility were found also in sporadic PD patients-derived cell lines (Hsieh et al., 2016).

A very recent study by di Domenico and coworkers (2019) contributed to analyse astrocyte-neuron interplay in PD pathogenesis, already described to be involved in mechanisms of α-synuclein spreading and degradation. (Braak 2007; Lee et al., 2010; Loria et al., 2017). Co-cultures experiments were set up deriving ventral mDA (vmDA) neurons and astrocytes from iPSC of PD patients carrying the LRRK2 G2019S mutation and healthy controls. Co-culturing healthy vmDA neurons with PD patients-derived astrocytes was sufficient to reduce neuron number and increase α-synuclein levels. On the other hand, neurodegenerative signs of PD vmDA neurons were ameliorated by co-culturing them with healthy astrocytes. More in detail, PD derived astrocytes were characterized by a progressive α-synuclein accumulation along days in culture, probably due to altered proteostasis of this protein and alteration of the autophagy flux (di Domenico et al., 2019).

1.4.2 Modelling sporadic PD with iPSCs

iPSC neurons derived from familiar PD patients with known genetic lesions have provided clues about the molecular aspects of DAergic degeneration. However, up to now data on iPSCs neurons derived from sporadic PD patients are less available with respect to hereditary forms, mostly due to the difficulty of deriving neurons from large cohort of idiopathic PD patients.

Epigenomic alterations are common events in both sporadic PD patients and those with a monogenic hereditary form of the disease. The work of Santiago and collaborators (2015) reported for the first time that iPSC-derived DAergic neurons from sporadic and LRRK2-associated PD patients share the same epigenomic changes compared to healthy individuals. Interestingly, these methylation

abnormalities become apparent only upon differentiation into DAergic neurons but not in other neuronal types, as well as they are lacking in somatic parental cells or undifferentiated iPSCs.

A recent work conducted an extensive transcriptomic and epigenomic study on fibroblast, iPSCs and sporadic PD-derived neuronal cells. In the latter, pathways involved in PD pathogenesis, such as CREB and PGC1α-regulating pathway, showed alterations in gene expression. Moreover, they also found a differential regulation of miRNA and piRNA molecules between control and PD-patients, both in cells and post-mortem tissue samples. The derivation of neurons from idiopathic PD patients can thus provide relevant data about the epigenetic signature of sporadic PD, to identify possible novel pathogenetic pathways (Schulze et al., 2018).

Genome editing in hiPSC can provide insights into novel associated risk variants in complex neurodegenerative disorders, functionally connecting these variations to a phenotype. A novel strategy was used by Soldner and collaborators in 2016 to unravel the transcriptional effects of variants in non-coding regulatory elements, by specifically tuning the genetic settings of the experimental system. A novel common risk variant in a distal enhancer that regulates *SNCA* expression was found to be associated to PD development. Moreover, as a genetic mechanism responsible for the altered *SNCA* expression, it was identified an aberrant binding to their target sequences of *EMX2* and *NKX6-1*, two transcription factors specific of the brain (Soldner et al., 2016). Increasing evidence reports a relevant role of non-cell autonomous component in PD, which seems to modulate disease progression and spreading.

1.4.3 iPSCs in drug screening

Besides the new possibilities iPSCs introduced for human disease modelling, their intrinsic features could also improve drug screening and identification of new therapeutic targets. Stem cell technology combined to development of high through-put screening (HTS) and high-content screening platforms (HCS) can circumvent the issues intrinsic to these assays. More specifically, these platforms screen billions of compounds measuring parametric outputs from an incredibly high number of wells, making the reproducibility of the assay one of the fundamental features to get consistent results. However, statistical significance in these types of assays is usually not so easy to obtain, considering the high variability of stem cell cultures. Reinhardt and collaborators (2013b) described the derivation and propagation of human neural progenitor cells (smNPCs) by means of only small molecules for self-renewal and expansion, thus significantly reducing time-consuming steps of selection, manual handling and the related costs. smNPCs demonstrates their ability to be efficiently directed towards central nervous system and neural crest lineages, as well as a robust capability of immortal expansion. Moreover, after two weeks of maturation mDA neurons derived from smNPCs resulted to be

electrophysiologically active and able to form integrate synaptic connections. smNPCs-derived neurons demonstrated to recapitulate neurodegenerative signs of the LRKK2 G2019S mutation. Upon exposure to oxidative cues, mDA neurons from patients exhibited a disease phenotype overlapping to the one already described by a previous study (Nguyen et al., 2011). Compared to the other cell types used in HTS analyses, smNPCs- derived neurons represent a more affordable tool to be used in screening of neuroprotective compounds. (Reinhardt et al., 2013b).

Concerning phenotypic-based drug screening, iPSCs derived neurons were used to assess the efficacy of small molecules inhibiting α-synuclein toxicity previously identified through a yeast screening. In particular, cortical neurons were derived from familiar PD patients bearing the A53T mutation in *SNCA*, known to have a high probability to develop cortical α-synuclein pathology and PD dementia. Two compounds identified to suppress α-synuclein toxicity in yeast were then tested in human cortical neurons. The N-arylbenzimidazole NAB2, acting through the Rsp5-Nedd4 pathway, was demonstrated to revert some of the pathologic phenotypes of PD neurons (as increased NO levels and immature forms of glucocerebrosidase and nicastrin proetins), found also in A53T-mutated patient cortices (Chung et al., 2013).

One of the drawbacks of iPSCs' disease modelling is represented by a heterogenous and not always consistent appearance of the disease phenotypes from line to line. Taking advantage of this issue, a recent study applied both bulk and high-resolution single cell transcriptomics in order to decipher gene expression changes driven by the N370S variant in glucocerebrosidase gene (*GBA)*. Heterozygous mutations in *GBA* were demonstrated to be the most common genetic risk factor for PD (Sidransky et al., 2009), carried by about 5-10% of patients. Individuals harbouring the most common GBA mutation (N370S) have a clinical presentation typical of idiopathic PD forms (Beavan and Schapira, 2013). Transcriptomic analyses of DA neurons differentiated from PD-GBA patients revealed a deregulated axis, triggered by nuclear mislocalization of HDAC4, that progressively leads to endoplasmic reticulum stress. DA neurons were then used to test different pharmacological compounds able to modulate localization and activity of HDAC4. Repurposed drugs, already in clinical trial setting for various types of cancer, were tested in diseased neurons for their ability to modulate localization and activity of HDAC4. This regulation proved to be efficacious in rescuing most of the cellular abnormalities of GBA-PD neurons, highlighting HDAC4 as a possible novel target in PD treatment. Moreover, since single cell RNA-sequencing can harness inter-neuronal variability, transcriptomic analyses built a stratification of patients harbouring GBA N370S mutation consistent with the clinical one. (Lang et al., 2019).

1.5 Organoids

A key progress in the last five years has not only been the generation of DAergic neurons from human induced pluripotent stem cells (Grealish et al., 2014) but also the development of a valid method for their differentiation into large multicellular organoid-like structures expressing characteristic markers of human midbrain. Lancaster and collaborators (2013) reported for the first time the generation of a three-dimensional cerebral organoid derived from pluripotent stem cells. The key step of their protocol, which was modified from neural rosettes derivation technique, is the neural induction of embryoid bodies kept in suspension and in agitating conditions. Without the use of patterning growth factors, cerebral organoids are allowed to self-organize in discrete identities, resembling human brain early development (Lancaster et al., 2013, Lancaster and Knoblich, 2014). In the last few years, different modifications have been implemented to the protocol, mostly to reach an increased neuronal survival, oxygen supply and axon outgrowth. The overall improvement of these systems allows a more accurate modelling of neuronal circuitry and physiological brain organization. An interesting approach derived from organotypic slice culture method has been applied to the technique. Air–liquid interface cerebral organoids are characterized by a more physiological morphology and significantly improved survival, together with an axon outgrowth dynamic similar to the one found *in vivo* (Giandomenico et al., 2019). Jo and coworkers (2016) produced human 3D midbrain-like organoids containing electrically active and functionally mature midbrain DAergic neurons and with detectable DA production. These midbrain-like organoids also produced neuromelanin-like granules that were comparable to those characterized from human *substantia nigra*. Notably, few years ago different groups published the use of 3D brain organoids as model for pathological human phenotype, including neurodegenerative diseases such as Alzheimer's disease (Raja et al., 2016; Pavoni et al., 2018) Accordingly, the rapid advancement of this approach offers the possibility of a key progress in the evaluation of therapeutic approaches on more physiologically relevant models opening new perspectives also for future approaches in drug discovery.

A very recent study described the generation of isogenic iPSC-derived midbrain organoids containing a G2019S mutation in LRRK2 (Kim et al., 2019). 3D midbrain organoids show in a differentiation-dependent manner the expression of several DAergic neuronal markers such as VMAT2, TH, NURR1 and DAT as well as the expression of additional midbrain markers suggesting that these organoids from day 45 closely resemble the mature DAergic midbrain (Kim et al., 2019). Of relevance, the study showed that LRRK2-mutant midbrain organoids are characterized by *i.* pathological signatures detected in LRRK2 PD patients, such as augmented aggregation and aberrant clearance of α-synuclein and *ii.* gene expression profiles mimicking those observed in mutant LRRK2-associated sporadic PD patients (Kim et al., 2019). Overall, this study suggests that midbrain 3D organoids can recapitulate LRRK2-associated PD better than traditional 2D cultures.

1.6 Conclusions

PD is mainly characterized by the degeneration of nigrostriatal DAergic neurons leading to the onset of motor symptoms of the disorder in patients. The use of a large number of *in vitro* experimental models provided the identification of several cellular and molecular mechanisms involved in PD pathogenesis. Overall, these models gave a great contribution to PD research in the last decades. However, as described also above, all available *in vitro* models have many disadvantages and currently, a reliable and optimal *in vitro* model for the understanding of all aspects of PD etiology is still lacking (see table 1).

Of key relevance, the recent development of iPSC and organoids technology has allowed the setting up of *in vitro* models of midbrain DAergic neurons using cells derived from PD patients. In particular, novel differentiation protocols for both iPSC and organoids have clearly demonstrated that these *in vitro* models have all molecular, functional, maturation and morphological features to be used for the study of neurodegenerative diseases such as PD. Accordingly, these human-derived models open new routes for PD modeling and drug screening that can be further developed and optimized in the next few years.

Declaration of interest: The authors declare that they have no competing interests

Table 1 - List of the main advantages and disadvantages of the *in vitro* models used in the field of PD.

Model	Advantages	Disadvantages

Cell lines	- high reproducibility - easy transfection and genetic manipulation - expression of the DAergic machinery upon differentiation - human genetic background	- oncogenic (presence of chromosomal and genomic aberrations) - absence of several key neuronal functional properties - simplified cell system
Primary nigral cultures	- physiological properties similar to *in vivo* DAergic neurons - rapid maturation and differentiation - possibility to derive neurons from genetic animal models	- heterogeneous system, low reproducibility - high percentage of glial cells - no human genetic background
Primary striatal cultures	- high expression of DA receptors, NMDA-type glutamate receptors and of the main striatal markers - possibility of co-colturing with cortical neurons	- heterogeneous system, low reproducibility - absence of spontaneous network activity and almost aspiny
iPSCs	- mature neuronal physiology and morphology - human genetic background - possibility to derive cells from PD patients and to generate isogenic controls	- technical difficulties, time consuming - issues regarding loss of epigenetic influence during reprogramming - difficulties in modelling age-related events
Midbrain organoids	- overcoming of simplified 2D models - presence of glia-neurons interactions - possibility to recapitulate cell autonomous and non-cell autonomous aspects - exhibit key features of mature midbrain DAergic neurons - patients' specific model	- rise of ethical issues - highly expensive systems - time consuming and several technical challenges

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Model	Advantages	Disadvantages
Cell lines	- high reproducibility - easy transfection and genetic manipulation - expression of the DAergic machinery upon differentiation - human genetic background	- oncogenic (presence of chromosomal and genomic aberrations) - absence of several key neuronal functional properties - simplified cell system
Primary nigral cultures	- physiological properties similar to *in vivo* DAergic neurons - rapid maturation and differentiation - possibility to derive neurons from genetic animal models	- heterogeneous system, low reproducibility - high percentage of glial cells - no human genetic background
Primary striatal cultures	- high expression of DA receptors, NMDA-type glutamate receptors and of the main striatal markers - possibility of co-colturing with cortical neurons	- heterogeneous system, low reproducibility - absence of spontaneous network activity and almost aspiny
iPSCs	- mature neuronal physiology and morphology - human genetic background - possibility to derive cells from PD patients and to generate isogenic controls	- technical difficulties, time consuming - issues regarding loss of epigenetic influence during reprogramming - difficulties in modelling age-related events
Midbrain organoids	- overcoming of simplified 2D models - presence of glia-neurons interactions - possibility to recapitulate cell autonomous and non-cell autonomous aspects - exhibit key features of mature midbrain DAergic neurons - patients' specific model	- rise of ethical issues - highly expensive systems - time consuming and several technical challenges

Table 1: List of the main advantages and disadvantages of the in vitro models used in the field of PD.