Generation of human dopaminergic neurons from induced pluripotent stem cells to model Parkinson's Disease

Adriana Sánchez Danés

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DIRECTORS DE LA TESI :

Dra. Antonella Consiglio (Institut de Biomedicina de la UB)

Dr. Ángel Raya (Institut de Bioenginyeria de Catalunya)

DEPARTAMENT CIÈNCIES EXPERIMENTALS I DE LA SALUT (CEXS)

UNIVERSITAT POMPEU FABRA

"Quasi tot el que faig serà insignificant, però és molt important que ho faci."

Mahatma Gandhi

A la meva família,

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Abstract

Parkinson's disease (PD) is an incurable, chronically progressive neurodegenerative disease leading to premature invalidity and death. The locomotor disability of PD patients is mainly rooted in the gradual and insidious degeneration of dopaminergic (DA) neurons projecting from the midbrain substantia nigra (SN) to the basal ganglia striatum, a pathological process highlighted microscopically by the formation of insoluble cytosolic protein aggregates, known as Lewy bodies and Lewy neurites. The pathogenic mechanisms leading to PD remain poorly understood, arguably owing to the lack of suitable animal and cellular experimental models of the disease. Therefore, there is an urgent need for developing reliable experimental models that recapitulate the key features of PD. The recent development of induced pluripotent stem cell (iPSC) technology has enabled the generation of patient-specific iPSC and their use to model human diseases, although it is currently unclear whether this approach could be useful to successfully model age-related conditions. Importantly, disease modeling using iPSC largely relies on the existence of efficient protocols for the differentiation of disease-relevant cell types. Here, we first developed an efficient protocol for the differentiation of iPSC to authentic midbrain-specific DA neurons with SN properties by forced expression of LMX1A using a lentivirus-mediated gene delivery system. Next, we generated an iPSC-based cellular model of PD that recapitulates key phenotypic features of PD, such as DA neuron loss and α-synuclein accumulation in DA neurons from PD patients. Overall, our results demonstrate that we have developed a valuable tool for elucidating the pathogenic mechanisms leading to PD, as well as an experimental platform for screening new drugs that may prevent or rescue neurodegeneration in PD.

Resum

La malaltia de Parkinson (MP) és una malaltia neurodegenerativa incurable que causa invalidesa i mort prematura. Els pacients de la malaltia de Parkinson presenten alteracions motores degudes a una degeneració gradual de les neurones dopaminèrgiques que projecten des de la substància nigra fins a l'estriat. A nivell microscòpic s'observa la presència d'agregats proteics insolubles en el citosol de les neurones coneguts com cossos o neurites de Lewy. Els mecanismes patològics responsables de la MP no es coneixen bé, possiblement a causa de la manca de models animals i cel·lulars adequats. Per tant, existeix una gran necessitat de desenvolupar models experimentals fiables que recapitulin les característiques bàsiques de la MP. El recent desenvolupament de les cèl·lules mare pluripotents induïdes (iPSC) ha permès la generació de iPSC específiques de pacient i el seu ús per modelar malalties humanes, ara bé, no és clar si aquesta estratègia es pot utilitzar per modelar exitosament malalties d'origen tardà, com ara la MP. És important destacar que el modelatge de malalties utilitzant iPSC, es basa, en gran mesura en l'existència de protocols eficients per a la diferenciació de les iPSC cap al tipus cel·lular rellevant per a la malaltia. Durant aquest període, per primera vegada, s'ha desenvolupat un protocol per a l'eficient diferenciació de les iPSC cap a neurones dopaminèrgiques amb les propietats característiques de neurones dopaminèrgiques nigrostriatals, mitjançant l'expressió forçada de LMX1A utilitzant vectors lentivirals. A continuació, s'ha generat un model cel·lular usant iPSC derivades de pacients de MP que recapitula les principals característiques fenotípiques de la malaltia, com ara la pèrdua de neurones dopaminèrgiques i l'acumulació de α-sinucleïna en les neurones dopaminèrgiques. En general, els nostres resultats demostren que hem desenvolupat una eina valuosa per a l'estudi dels mecanismes patològics que condueixen a la MP, així com una nova plataforma pel descobriment de nous fàrmacs encaminats a prevenir o evitar la neurodegeneració.

CONTENTS

1- INTRODUCTION

1.1- Parkinson's Disease

In 1817, the British physician James Parkinson published his monograph *Essay on the Shaking Palsy* (Parkinson, 1817), in which he described the cardinal clinical features of the disease, which was named after him by the French neurologist Jean-Martin Charcot in 1888. Parkinson's disease (PD) is a progressive neurodegenerative disorder characterized mainly by motor clinical manifestations including bradykinesia, resting tremor, rigidity and postural instability. With progression of disease, patients may also experience sleep problems, olfactory deficits, depression and dementia, become dependent and bedridden. (Lees et al., 2009; Obeso et al., 2010; Shapira et al., 2010). Since the main clinical features of PD are due to the degeneration of specific dopaminergic (DA) neurons, dopamine agonists and anti-cholinergics or electrophysiological substitution after surgery (deep brain stimulation) have been used with success to reduce PD symptoms, but they do not cure or delay disease progression. Current symptomatic therapies compensate the movement deficit efficiently over a period of 5 years in average, but are ineffective to alleviate the loss of autonomic, olfactory, sleep, affective and cognitive abilities.

It is important to note that motor symptoms associated to PD are usually referred to as "parkinsonism". Therefore, this term is used to describe neurological disorders marked by muscular rigidity, tremor and impaired motor control with a specific or unknown cause. This specific cause could be ischemic injuries, exposure to toxins, and neuroleptic medications, or unknown causes such as in idiopathic PD. Remarkably, PD is the major cause of parkinsonism.

PD is the second most common degenerative disease after Alzheimer's disease, with a prevalence of 1% at age 65 and around 5% by age 85 (de Rijk et al., 2000). The mean age of PD diagnosis is in the seventh decade of life. It is a chronic and slowly progressive disease with a mean duration from disease diagnosis until death of 15 years, although in some cases patients can survive two decades or longer (Elbaz et al., 2003; Fahn et al., 2003). Given the increasing life expectancy of the population, the prevalence of PD is anticipated to augment dramatically, leading to an urgent need to find therapies to halt or delay DA neuron degeneration.

PD cases could be divided according to their family history in two categories: sporadic or familial PD. Sporadic PD accounts for 90-95% of the cases and is likely to result from complex interactions among gene susceptibility and environmental factors (Corti et al., 2011). In turn, 5-10% of PD patients have a family history of the disease and linkage to specific gene mutations has been established (see below).

1.1.1- Neuropathological features of Parkinson's Disease

The two main pathological hallmarks of PD include the loss of nigrostriatal DA neurons and the presence of intraneuronal cytoplasmic inclusions known as Lewy Bodies (LB) present mainly in the surviving neurons (reviewed in Martin et al., 2011)

The DA neurons of the nigrostriatal system have their cell bodies located in the substantia nigra pars compacta (SNpc) and project to the striatum, primarily to the putamen and secondly to the caudate (Figure 1A). These DA neurons are of A9 subtype and are responsible for the release of the neurotransmitter dopamine in the striatum to control the initiation and execution of movements. The loss of A9-subtype DA neurons in PD

patients' brains causes dysfunction in movement control and leads to motor manifestations (Rodriguez-Oroz et al., 2009) (Figure 1B). Indeed, by the time PD motor symptoms are clinically recognized, around 80% of striatal dopamine is depleted and more than 50% of DA neurons in the SNpc are lost. A correlation between the severity of the motor affectations and the reduced levels of striatum dopamine has been described. A9 DA neurons contain large amounts of neuromelanin, being SNpc pigmented in healthy conditions (Marsden et al., 1983) (Figure 1A), and depigmentated when A9 DA neurons are lost (Figure 1B).

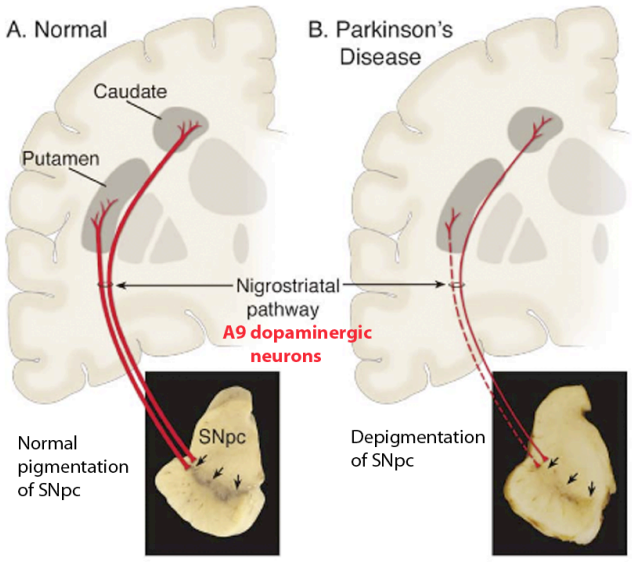

Figure 1. Nigrostriatal system in health and Parkinson's disease.
(A) Schematic representation of the nigrostriatal pathway (red) in a healthy person. It is composed of A9 dopaminergic neurons whose bodies are placed in the substantia nigra pars compacta (thick solid lines) and synapse in the striatum (putamen and caudate nucleus). The picture shows the normal pigmentation present in substantia nigra pars compacta due to neuromelanin produced within the dopaminergic neurons. (B) Schematic representation of the degeneration of nigrostriatal pathway (red) from a Parkinson's disease patient. There is a marked loss of dopaminergic neurons that project to the putamen (thin red solid line) and in less extent to the caudate. The image shows typical depigmentation of SNpc caused by the loss of pigmented A9 dopaminergic neurons (Modified from Dauer et al., 2003).

The second cardinal neurophatological feature of PD is the development of intraneuronal accumulations of insoluble proteins and protein fragments (Lewy bodies and Lewy neurites) in the surviving neurons. Specifically, LB are spherical eosinophilic cytoplasmic protein aggregates composed of numerous proteins (Figure 2), including α-synuclein, parkin, ubiquitin, and neurofilaments (Forno et al., 1996; Spillantini et al.,1998). LB are usually larger than 15μm in diameter and display an organized structure composed of two distinct areas: a dense hyaline core surrounded by a clear halo. LB are not specific of PD, being also observed in Alzheimer Disease, in a condition known as "dementia with LB disease", and in people of advanced age (Gibb and Lees., 1988).

The loss of DA neurons form the SNpc and subsequent striatal dopamine deficiency result in a dysfunction of the basal ganglia, which is ultimately responsible for the motor cardinal features of PD (Obeso et al., 2008). The basal ganglia is a cluster of deep nuclei that participate in the initiation and execution of the movements (Rodriguez-Oroz et al., 2009). It is composed of striatum, SNpc and substantia nigra pars reticulate (SNpr), globus pallidus pars interna (GPi) and pars externa (GPe), and subthalamic nucleus (STN). Components of the basal ganglia are in direct communication with the thalamus and the

cortex.

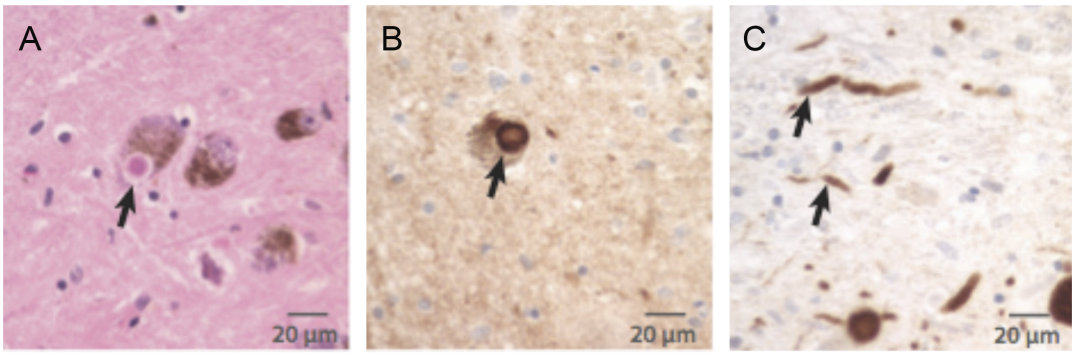

Figure 2. Presence of Lewy bodies and Lewy neurites in the brains of Parkinson's disease patients. **(A)** Hematoxylin and eosin staining of a Lewy Body (arrow) within a pigmented dopaminergic neuron. LB has a characteristic dense core surrounded by a paler halo. (B) Lewy body stained for α-synuclein (arrow). (C) Lewy neurites (arrows) stained for α-synuclein (From Shulman et al., 2011).

Cortical motor areas project glutamatergic axons to the putamen, which sends GABAergic (inhibitory) projections to the GPi and the SNr by two pathways: the monosynaptic GABAergic pathway also called the direct pathway (putamen-GPi) and the trisynaptic (putamen-GPe-STN-GPi/SNr) known as the indirect pathway (Figure 3). Dopamine from the SNpc activates putaminal neurons both in the direct and indirect pathways. Activation of the direct pathway leads to reduced neuronal firing in the GPi/SNr and movement facilitation, while activation of the indirect pathway suppresses movements (Figure 3A). The STN is also activated by an excitatory projection from the cortex, the hyperdirect pathway. In PD, dopamine deficit leads to a failure of the direct pathway activation, and to an increased activity in the indirect circuit (Figure 3B). Together, these actions result in increased GPi/SNr output inhibition of the ventrolateral nucleus of the thalamus and reduced activation of cortical motor regions, making precise movement selection and execution difficult.

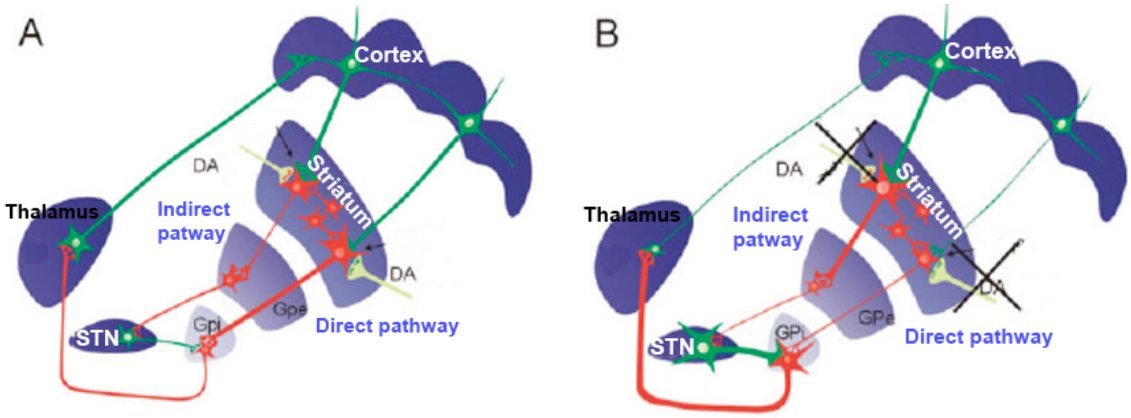

Figure 3. Pathophysiological model of the basal ganglia in (A) Normal state. Corticostriatal activity excites neurons within the striatum activating the "direct" striato-globus pallidus pars interna pathway and the indirect striatal-globus pallidus pars externa-subtalamic nucleus pathway. The former inhibits, whereas the latter activates GPi, providing inhibitory innervation to thalamocortical projections. Thus, the direct pathway serves to facilitate movement whereas the indirect to inhibit it. (B) Parkinsonian state, dopamine depletion leads to increased activity in the indirect pathway and reduced activity in the direct pathway. These changes lead to reduced movements (Modified from Rodriguez-Oroz et al., 2009)

PD symptoms include motor and non-motor manifestations. Motor manifestations stem for loss of DA neurons in the SNpc. Non-motor manifestations, including impaired olfaction, disordered sleep, constipation, autonomic dysfunction and neuropsychiatric manifestations, are thought to result from the spreading of the pathology beyond the basal ganglia to other brain systems (Langston et al., 2006; Chaudhuri et al., 2009). Indeed, neurodegeneration and LB formation are found in noradrenergic, serotonergic and cholinergic systems, cerebral cortex, olfactory bulb and autonomic nervous system in PD patients. Notably, Braak and colleagues have proposed that neuronal degeneration in PD might start in the enteric and olfactory nervous system, followed by alterations in the brainstem; after which, midbrain nigrostriatal DA neurons would start to deteriorate and finally the forebrain neurons would suffer degeneration (Braak et al., 2003). Indeed the concept of premotor PD is gaining support (Langston, 2006; Hawkes et al., 2008).

1.1.2-Treatments

Current treatments for PD are symptomatic, aimed to palliate motor affectations, and do not halt or retard DA neuron degeneration. Notably, existing only alleviate motor symptoms of PD, and are not effective with non-motor manifestations of the disease. The most widely used treatment for PD is pharmacological, specifically levodopa. However, other pharmacological and surgical treatments are recommended in specific conditions. On the other hand, new therapies that might be called "experimental" are being developed, including cell replacement therapies and gene therapy for PD. The efficacy and reliability of these therapies are currently being tested in animal models and small groups of PD patients.

Current treatments

Current treatments for PD could be classified into pharmacological and surgical treatments. Since late 1960s to the present day, the most widespread and efficacious treatment for PD has been levodopa oral administration (Cotzias et al., 1969). This strategy is based on the restoration of dopamine levels in the striatum through levodopa administration, leading in many cases to the improvement of motor symptoms. Levodopa is a precursor of dopamine that is able to cross the blood-brain barrier, whereas dopamine itself cannot. Once in the brain, the enzyme L-amino acid decarboxylase (AADC) converts levodopa to dopamine. Levodopa treatment is the most commonly used as it improves motor symptoms, and thus, patients' quality of life. However, as PD aggravates over time, increased doses should be administered to manage symptoms as they progress. Another remarkable limitation is the emergence of side effects after long-time treatments. Specifically, motor complications appear in about 50% of levodopa-treated patients after 5 years of treatment, in 80% of patients treated for 10 years, and in nearly all patients with young-onset disease (Fahn et al., 2000; Golbe et al., 1991; Forno et al., 1996). These motor complications include "wearing off" phenomenon (a gradual waning of the effect of dopaminergic treatment on motor symptoms before the next dose), "on-off" fluctuations (where symptoms can reappear and disappear randomly and suddenly), and even dyskinesia (involuntary movements and tics). Non-motor side effects such as hallucinations and illusions are also present in some patients.

Other pharmacological treatments used to treat PD patients include dopamine agonists and monoamine oxidase inhibitors (reviewed in Rascol et al., 2011). Dopamine agonists provide modest symptomatic improvement compared to levodopa. However, their effect lasts longer than that of levodopa and their use leads to less motor fluctuations (Stacy et al.,

2008, Hause et al., 2007). For this reason, they are used in combination with levodopa to smooth motor fluctuations ("wearing off" phenomenon, "on-off" fluctuations and dyskinesias) and, in many cases, they permit lowering levodopa doses. A therapy based on dopamine agonists is used for young-onset PD patients. Specifically, this treatment consists on administration of dopamine agonists in the early stages of the disease, followed by levodopa supplementation in later stages, when there is not a good control of the motor symptoms with the agonist alone.

Monoamine oxidase inhibitors prevent the breakdown of dopamine, both the endogenous and the one formed from levodopa. They accomplish their action inhibiting the activity of the enzyme monoamine oxidase B (MAO B), responsible for metabolizing dopamine to 3,4-dihydroxyphenylacetic acid (DOPAC) in the brain. They can be used alone or in combination with levodopa or dopamine agonists. When used alone, they confer small symptomatic benefits and side effects are rare, but may include confusion, headache and interactions with antidepressants, as they are prescribed for the treatment of depression.

The surgical treatment known as deep brain stimulation (DBS) is also used in specific PD cases. DBS can provide additional improvement of motor symptoms in selected patients whose symptoms are not sufficiently controlled by medication in advanced stages of PD (Deuschl et al., 2006, Krack et al., 2003). DBS is a surgical treatment involving the implantation of a medical device consisting on three components (implanted pulse generator, the lead and connective wires), which sends electrical impulses to the GPi, the STN, or the ventral medial nucleus of the thalamus. The target selection criteria for DBS in PD patients is based on the type of motor symptoms present. This strategy presents mainly two limitations. The first one is that it is only suitable for a small group of PD patients; specifically, those showing medically intractable motor fluctuations and absence of severe dementia, which account for less than 5% of PD patients (Morgante et al., 2007). DBS is not recommended for patients with dementia or severe psychosis, as there is high risk of these symptoms worsening (Limousin et al., 2008). The second limitation of DBS is the decrease in treatment efficacy with time, as gait, balance and speech often deteriorate (Limousin et al., 2008).

Experimental therapies

Cell replacement therapy (CRT) is aimed at the transplantation of dopamine-secreting cells in the striatum, thus restoring dopamine supply in the striatum and replacing the function of DA neurons lost during the course of PD. It was conceived as a long-lasting therapy to substitute pharmacological treatment. In the last 20 years, approximately 300-400 PD patients have been transplanted human fetal mesencephalic tissue and results have varied from success to failure. The encouraging and successful outcome has provided proof-of-principle that CRT could become a feasible therapy for PD (Piccini et al., 1999). In the most successful cases, patients were able to stop levodopa treatment for several years after transplantation. However, treatment failures have highlighted that more effort has to be done in order to establish CRT as a reliable and clinically competitive treatment for PD (Olanow et al., 2003).

CRT has to overcome some limitations before it becomes a sound clinical option for treating PD patients. The first issue to solve is the source of the donor tissue. Most transplants carried out so far have used human fetal mesencephalic tissue, as it contains DA progenitors that are committed to DA neuron fate and the risk of tumorigenicity seems to be low or absent. However, as this tissue is obtained from 6-9 week-old aborted

human fetuses, there are obvious ethical implications to this approach. Another constraint is the large amount of aborted fetuses needed paired with the limited available amount; specifically around 6 to 10 fetuses are needed per patient. Therefore, there is an urgent need to find alternative cell sources of A9 DA neurons in large supply, which could make transplantation a feasible procedure for treating PD. Human pluripotent stem cells (both human embryonic stem cells (hESC) and iPSC) meet these criteria, while fetal neural stem cells, adult brain-derived neural stem cells and adult multipotent stem cells all share a limited ability for *ex vivo* expansion. The second limitation of using fetal mesencephalic tissue grafting is the poor survival of DA cells after transplantation, in the best cases a 10% of the transplanted cells (Hagell et al., 2001). Finally, between 15% and 56% of patients receiving fetal tissue developed an unexpectedly high incidence of dyskinesias, which was found to be associated with the presence of severe of dyskinesias prior to grafting, or to the presence of more serotonergic than DA neurons within the graft (Carlsson et al., 2009).

Several studies have demonstrated that grafted DA neurons survive for as long as 16 years in the brain of PD patients after transplantation of human fetal mesencephalic tissue (Kordower et al., 2008; Li et al., 2008; Mendez et al., 2008). Interestingly, in one of these studies, Mendez and colleagues found disease-free DA neurons (free of LB) in post-mortem brains of PD patients that had been transplanted 9-14 years before. In contrast, two other studies found LB and α-synuclein aggregates in some cells in brains of patients grafted for 11-16 years (Kordower et al., 2008 and Li et al., 2008) and reduced dopamine transporter immunostaining (Kordower et al., 2008). These latter findings suggest host-to-graft disease propagation. Disease propagation was not found in patients surviving less than 10 years after transplantation (Kordower et al., 1998), indicating that the therapeutic window of CRT would include 10 years after intervention, and even longer in some cases.

A different approach that has been explored to treat PD is gene therapy using viral vectors to deliver genes encoding enzymes or neuroprotective proteins. Viral vectors offer some advantages over pharmacotherapy, such as direct delivery of the gene of interest in one specific anatomical loci, and requiring only one intervention to deliver-long term, stable, therapeutic action. In practice, however, diffusion of the viral vectors and low or transient expression of the gene of interest may limit the usefulness of gene therapy for PD. Several clinical trials using viral vectors have been pursued and some still are ongoing.

Enzyme replacement therapy aims at restoring the brain's ability to produce and release dopamine in the striatum. Shen and colleagues used a triple transduction of adenoviral-based vectors expressing the enzymes tyrosine hydroxylase (TH), AADC, and GTP cyclohydrolase 1 (GDC1), and showed sustained behavioral improvement in 6-hydroxydopamine (6-OHDA)-lesioned rats (Shen et al., 2000). TH is responsible for the conversion of L-tyrosine to L-dyhidroxyphenylalanine (L-DOPA), AADC catalyzes the decarboxylation of L-DOPA to dopamine, and GDC1 is responsible for converting GTP to 7,8-dihydroneopterin 3'-triphosphate, an essential cofactor required for AADC activity. Muramatsu and colleagues obtained similar results in monkeys treated with MPTP, and demonstrated greater than 90% transduction in the site of injection, the putamen, based on immunostaining (Muramatsu et al., 2002). More recently, and going one step further, efforts have been made to deliver all three functional genes in a single multicistronic lentiviral-based vector to achieve more efficient delivery and translational efficacy. Despite modest results in hemiparkinsonian rodents, a study in MPTP-treated primates showed significant motor benefit starting at 2 weeks after transfection, with sustained benefit up to 44 months (Jarraya et al., 2009).

An alternative strategy being investigated is the delivery of AADC to the striatum with the aim to increase the conversion efficiency of exogenously administered levodopa into active dopamine. This approach was first proven in MPTP-lesioned primates, which showed higher conversion rates of levodopa to dopamine following AADC gene transfer (Bankiewicz et al., 2000). Moreover, two recent phase I clinical trials have been carried out using this approach, both of them finding a significant improvement in "off" time at 6 months, with trends of improvement in the "on" time, and a reduction in the required dose of levodopa in the majority of patients. Importantly, sustained AADC activity was observed for as long as 96 weeks in the putamen (Eberling et al., 2008; Muramatsu et al., 2010).

Both enzyme replacement and AADC pro-drug strategies present some limitations. The main disadvantage is that the majority of the transduced cells are not DA neurons, thus they are not specialized in dopamine synthesis,storage and release and they lack the functional VMAT2 receptor. Therefore, the absence of this machinery leads to unregulated storage and release of dopamine. However, this problem could be overcome in the future through the addition VMAT2 gene in the vector.

Neuroprotection is a therapeutic strategy intended to slow or halt the progression of neuronal loss and, hence, alter the natural history of disease. Compared to symptomatic therapy, neuroprotective therapies intend to act on the pathogenic mechanisms underlying the clinical manifestations of the disease. Several neurotrophic factors have been described such as nerve growth factor, neurotrophins 3-6, brain derived neurotrophic factor or astrocyte-derived neurotrophic factor, however the ones that hold more expectations are glial-derived neurotrophic factor (GDNF) and neurturin (NTN), a naturally-occurring analogue of GDNF.

GDNF is a member of TGF-β superfamily and it has been described to be necessary for DA neuron survival, outgrowth and maturation during development and adulthood (Paratcha et al., 2008). Indeed, GDNF was shown to enhance survival and growth of DA neurons differentiated from hESC *in vitro* (Lin et al., 1993), and absolutely required for the survival of DA neurons throughout life (Pascual et al., 2008). Since GDNF is not able to cross the blood-brain barrier, intense research has focused on developing methods to deliver it to the brain. Vector-based delivery of GDNF was proposed to solve the issue of low penetration of GDNF protein after being delivered through an intraventricular cannula (Nutt et al., 2003; Kordower et al., 1999) and, moreover, it would allow a stable and long-lasting source of GDNF. Several studies have demonstrated the feasibility of adenoviral-based delivery of GDNF to rodent striatum. In 6-OHDA-treated rats, striatal delivery of GDNF was neuroprotective, preventing DA cell loss, (Kirik et al., 2000; Wang et al.,2002). Moreover, delivery of GDNF in a lentiviral-based vector resulted in protection of nigrostriatal loss and induction of cell regeneration in MPTP-treated primates (Kordower et al., 2000). Despite the neuroprotective effect of GDNF in toxin-induced experimental models of PD, it failed to work in a rat genetic model of PD, the mutant A30P human α-synuclein, via lentiviral delivery in the substantia nigra (Lo Bianco et al.,2004).

Neurturin (NTN) is a naturally-occurring analogue of GDNF and has been described to exert potent effects on survival of nigrostriatal neurons *in vitro* (Horger et al., 1998) and *in vivo* (Tseng et al., 1998). NTN effects on nigrostriatal system seem to be mediated by the same mechanism as GDNF, as NTN binds to the same receptor as GDNF, albeit with lower affinity (Creedon et al., 1997). Thus, to obtain the same signal comparatively more NTN is needed than GDNF. Adenoviral or lentiviral-mediated NTN delivery in toxin-

induced models of PD in rodents and nonhuman primates resulted in neuroprotection of nigrostriatal DA neurons (Fjord-Larsen et al., 2005; Kordower et al., 2006; Herzog et al., 2008). In a phase I clinical study, 12 PD patients received bilateral injections of AAV2-NTN in the putamen. The procedure was proven safe and there was significant improvement in motor symptoms (Marks et al.,2008).

1.2- Etiology of Parkinson's disease

Approximately 90-95% of PD patients are idiopathic cases, of unknown origin, thought to be caused by a specific combination of environmental and gene susceptibility factors. In the remaining 5-10% of cases, the cause is known to be a genetic mutation (Corti et al., 2011). However, the mechanism by which a mutation in a gene associated with PD leads to the disease is not understood in the majority of the cases

1.2.1- Risk factors

Aging is the most prominent risk factor for developing PD (Bennett, et al. 1996; Post et al.,2007). Indeed, both the prevalence and incidence of PD increase with age. Aging has been described to lead to the accumulation of unrepaired cellular damage and to the failure of compensatory mechanisms such as mitochondrial activity, autophagy or degradation via proteasome (reviewed in Dauer et al.,2003). For example, with age there is a decrease of the activity of the proteasome leading to abnormal accumulation of proteins in the brain (Tai et al., 2008)

Interestingly, Collier and colleagues studied the effect of aging in DA neurons of non-human primates (Collier et al., 2011). Particularly, they studied the presence of neurochemical and morphological changes in DA neurons, typical traits of PD patients, in the brain of rhesus monkeys from different ages. These authors observed a decrease in TH staining, and an increase in α-synuclein staining, presence of ubiquitin-positive inclusions, and increased levels of oxidative stress in DA neurons in the substantia nigra in aged monkeys compared to young ones. From these observations, two important conclusions came out; the first one that aging could be responsible for PD-associated changes in the nigrostriatal system, although mild when compared to PD patients, suggesting that aging generates a pre-parkinsonian state. Second, that DA neurons in the substantia nigra are more vulnerable to age-associated degeneration than DA in the ventral tegmental area. Based on these results, Collier and colleagues hypothesized that this pre-parkinsonian state induced by aging in combination with genetic and environmental factors, may result in PD phenotypes in rhesus monkeys, and that this mechanism could be extended to humans.

Another risk factor for suffering PD, although much less important than aging, is gender. Indeed, incidence studies have reported a female-to-male ratio of approximately 1: 1.5-2 and that women present older age at onset (reviewed in Haaxama et al.,2007). A neuroprotective role of estrogen has been proposed, as the number of offspring, age at menopause, and duration of the fertile life correlated with older age at onset of PD in women (Haaxma et al., 2007). However, the protective role of estrogens on DA neurons is still an issue in controversy, since contrasting results have been obtained in different studies and the mechanisms involved in estrogen neuroprotection are still unclear. On the other hand, some dietary habits and lifestyles have been found associated with reduced risk of suffering PD, including cigarette smoking, coffee or tea drinking, diets high in uric acid (Gao et al., 2008), or high levels of physical activity in midlife (Thacker et al.,2008).

In around 90% of PD patients the disease is sporadic, with no family history of PD (Papapetropoulos et al., 2007). For this reason, environmental factors have been thought to be a predominant cause of PD, and intense efforts had been undertaken to identify the environmental factors contributing to PD (reviewed in Wirdefeldt et al., 2011). Many toxins and chemicals had been described to cause parkinsonian symptoms; the most relevant being MPTP, the herbicide paraquat, the pesticide rotenone and heavy metals including manganese and iron. Although these substances have been described to cause parkinsonism, no environmental substance has been associated with conclusive evidence to PD.

The perception that environmental factors are the main cause of PD is starting to change. Studies of rare large families showing classical Mendelian inherited PD has allowed the identification of genes involved in the pathology and recent genome-wide association studies (GWAS) have established new susceptibility loci (Table 1). Remarkably, many pathogenic mutations in different genes with diverse functions have been associated to PD (Table 1). This may represent the existence of different pathogenic mechanisms that converge on one or more common signaling pathways central to the loss of DA neurons, leading to the manifestation of clinical PD. Moreover, some dominant monogenic mutations show lack of family history due to reduced penetrance or a dominant *de novo* mutation (Corti et al., 2011).

1.2.2- The genetic contribution to Parkinson's disease

Mutations in the PD-associated genes account for small number of PD cases (5-10%), but there is evidence that these genes may also contribute in the sporadic forms of the disease. An example of this is the gene *Leucine rich repeat kinase-2* (*LRRK2*), mutations in which are not only found in genetic forms of autosomal dominant late-onset of PD (Paisan-Ruiz et al., 2004; Zimprich et al., 2004), but also in numerous cases of late-onset sporadic PD (Gilks et al., 2005 ; Lesage et al., 2007). Therefore, it has been postulated that PD may result from a genetic mutation, a genetic predisposition to an environmental factor, or a combination of susceptibility genes that each enhances the risk of the disease (Corti et al., 2011).

a) Autosomal dominant forms of PD

To date, two genes have been conclusively linked to autosomal dominant PD: *α-synuclein* (*SNCA*) and *leucine-rich repeat kinase 2* (*LRRK2*).

α-Synuclein (SNCA)

α-Synuclein is one of the three synuclein family members (a, b, g). Synucleins are exclusively present in vertebrates. α-Synuclein is a small protein of 140 aminoacids encoded by the *SNCA* gene. It has three differentiated domains: an amphipathic domain, which forms an amphipathic α-helical domain when it associates with lipid rafts; a central non-amyloid component, a region with high propensity to aggregate; and an acidic tail (Figure 4). This protein is characterized by the presence of seven imperfect repeats (KTKEGV) in the NH_2 terminus. α-Synuclein is predominantly localized in presynaptic nerve terminals, suggesting a role in synaptic transmission (Iwai et al., 1995; Maroteaux et al., 1988).

PARK Loci	Gene	Map Position	Inheritance	Disease Onset	Mutations
			PD-associated loci and genes with conclusive evidence		
PARK1/PARK4	*SNCA*	4q21	Dominant; rarely sporadic	Early onset	A30P, E46K, A53T genomic duplications/triplications
PARK8	*LRRK2*	12q12	Dominant; sporadic	Late onset	>80 Missense variants, >7 of them pathogenic, including the common G2019S
PARK2	*parkin*	6q25-q27	Recessive; sporadic	Juvenile; early onset	Approximately 170 mutations (point mutations, exonic rearrangements)
PARK6	*PINK1*	1p35-p36	Recessive	Early onset	Approximately 50 point mutations, rare large deletions
PARK7	*DJ-1*	1p36	Recessive	Early onset	Approximately 15 point mutations and large deletions
PARK9	*ATP13A2*	1p36	Recessive	Juvenile KRS, early-onset PD	>5 Point mutations
			PD-associated loci and genes with unknown relevance		
PARK3	Unknown	2p13	Dominant	Late onset	Not identified
PARK5	*UCHL1*	4p14	Dominant	Late onset	One mutation in a single PD sibling pair
PARK10	Unknown	1p32	Unclear	Late onset	Not identified
PARK11	*GIGYF2*	2q36-q37	Dominant	Late onset	7 Missense variants
PARK12	Unknown	Xq21-q25	Unclear	Late onset	Not identified
PARK13	*Omi/HTRA2*	2p13	Unclear	Late onset	2 Missense variants
PARK16	Unknown	1q32	Unclear	Unclear	Not identified
			Loci and genes associated with atypical parkinsonism		
PARK14	*PLA2G6*	22q12-q13	Recessive	Juvenile levodopa-responsive dystonia-parkinsonism	2 Missense mutations
PARK15	FBXO7	22q12-q13	Recessive	Early-onset parkinsonian-pyramidal syndrome	3 Point mutations

Table 1. Summary of PD-associated loci and genes. (From Corti et al., 2011).
SNP: single-nucleotide polymorphism

Two types of disease-causing mutations in the *SNCA* gene have been described: point mutations causing missense variations of the protein, and multiplications of the entire locus, mostly duplications and triplications that result in pathogenic overexpression of the wild-type protein. Only three point mutations have been identified: A53T, A30P and E46K. The most common is the A53T mutation. Patients carrying this mutation present from a mild to a more severe phenotype, indicating the existence of genetic and/or non-genetic factors that modulate the phenotype (Michell et al., 2005; Puschmann et al., 2009). The clinical manifestations showed by carriers of *SNCA* A30P mutations are similar to idiopathic PD, presenting late-age onset and a mild phenotype (Kruger et al., 2001). Patients carrying the E46K mutation present severe parkinsonism, with early-age onset and diffuse Lewy body dementia (Zarranz et al., 2004).

Multiplications of the *SNCA* gene are more common than point mutations. Gene triplications are associated to more severe phenotypes, earlier age onset, and faster progression, than duplications (Fuchs et al., 2007). Therefore, there is a remarkable correlation between *SNCA* copy number and severity of PD phenotype.

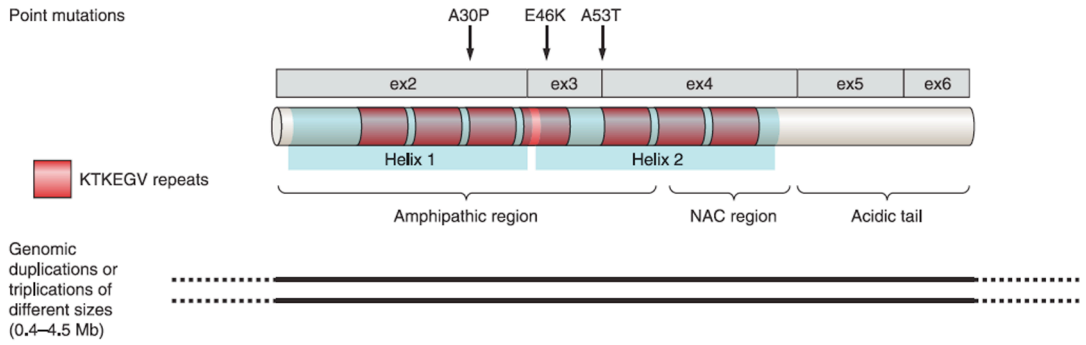

Figure 4. Schematic representation of the domains present within α-synuclein protein. Three point mutations are shown above the protein organization and genomic multiplications below. (Modified from Corti et al., 2011).

In healthy conditions, α-synuclein associates with lipids. In contrast, in the brain of PD patients, α-synuclein is found as the major fibrillar component of LB and Lewy neurites (Spillantini et al., 1997, Spillantini et al., 1998). Within cells, α-synuclein can adopt different conformations. It shows high propensity to aggregate forming initially an intermediate annular oligomeric structure known as protofibrils. Protofibrils, in turn, can ensemble and give rise to fibrils, which are insoluble aggregates (Expanded in section 1.3)

SNCA mutations, in particular the A30P mutation, has been shown to reduce the affinity for lipids (Bussell et al., 2004), thus increasing the intracellular protein pool and potentiating its natural tendency to form oligomers and fibrils. Moreover, duplications and triplications would result in higher amounts of intracellular α-synuclein, leading to the formation of oligomers first and fibrillar aggregates later.

Leucine-rich repeat kinase 2 (LRRK2)

LRRK2 or dardarin is a protein ubiquitously expressed in many organs and tissues (Westerlund et al., 2008; Biskup et al., 2007). Remarkably, its expression is not particularly high in A9 DA neurons. On a subcellular level in the brain, LRRK2 protein is localized in the soma and also in the dendrites and axons of neurons and a substantial fraction of this protein appears to be membrane-associated (Biskup et al., 2006; Higashi et al., 2007).

The *LRRK2* gene comprises 144kb, contains 51 exons, and encodes a large 2,527-amino acid multidomain protein. LRRK2 has several potential protein-protein interaction regions surrounding a central catalytic core (Figure 5). The potential protein-protein interaction regions include 4 conserved domains: armadillo repeat folds (ARM), ankyrin repeats (ANK), leucine-rich repeats (LRR) and WD40 repeats, the presence of these domains suggests that LRRK2 might serve as a scaffold for assembly of multiprotein signaling complexes (Mata et al., 2006). The central catalytic core is composed of the Ras of complex (Roc) GTPase domain, the carboxy-terminal of Roc (COR) domain, and the kinase domain of the tyrosine kinase-like (TKL) subfamily, being the latter homologous to other mitogen-activated kinase kinase kinase (MAPKKK)(Figure 5).

LRRK2 is a particularly interesting protein, as it presents two distinct enzymes, kinase and GTPase in the same molecule. The presence of both domains in the same protein has led to the hypothesis that LRRK2 may be a cell signaling protein. The intracellular functions of

LRRK2 are not fully understood, indeed they are subject of intensive research. MacLeod and colleagues pointed out that mammalian LRRK2 regulates neurite maintenance and neuronal survival, showing that neurons expressing mutant forms of LRRK2 display reduced neurite process length and complexity, and apoptotic cell death, in studies performed using primary neuronal cultures and intact rodent CNS (MacLeod et al., 2006). Recent evidence suggests that LRRK2 regulates protein translation through two different mechanisms. First, LRRK2 phosphorylates the eukaryotic translation initiation factor 4E-binding protein (4E-BP), thus disrupting its binding to eukaryotic translation initiation factor 4E (Imai et al., 2008). Second, LRRK2 may interact with the microRNA pathway to regulate translation (Gehrke et al., 2010).

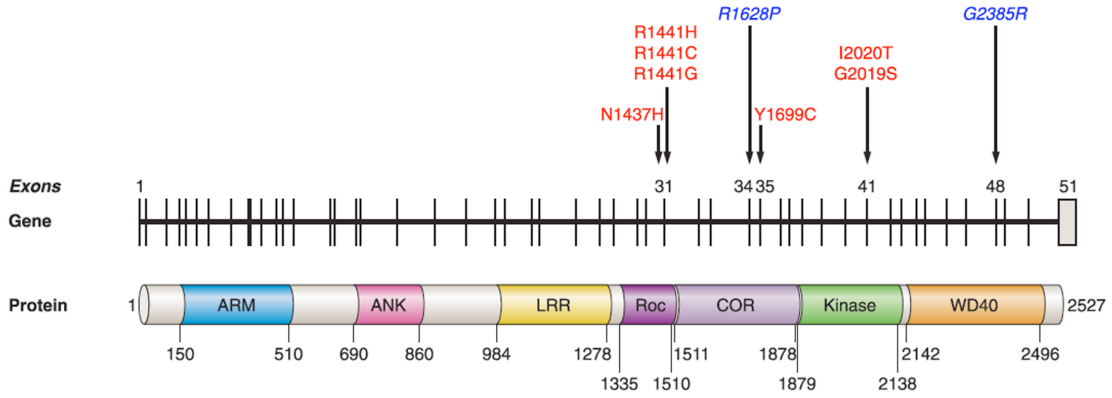

Figure 5. Schematic representation of *LRRK2* gene and protein. The seven mutations considered to be pathogenic are shown in red and Asian risk factors in blue. (Modified from Corti et al., 2011).

Mutations in *LRRK2* are the most prevalent genetic cause of PD, and found in patients with typical, late-onset familial and sporadic PD. To date, nearly 50 different mutations in *LRRK2* have been associated with PD, most of them representing missense mutations. From the 50 described mutations, only seven are considered pathogenic affecting GTPase (N1437H, R1441G/C/H), COR domain (Y1699C), and the kinase domain (G2019S and I2020T); remarkably they are all clustered around the catalytic core. Moreover, two common mutations in Asian population have been described to be risk variants (G2385R and R1628P).

The G2019S mutation is the most common cause of dominant familial PD and accounts for up to 2% of sporadic PD cases (Coorson et al., 2010). This mutation is also found in unaffected people, suggesting an incomplete penetrance that is age-dependent and varies between 25-100% by age 80 (Latourelle et al., 2011). Therefore, it would be interesting to identify the genetic and environmental factors that modulate penetrance of the G2019S mutation. The clinical phenotype of patients carrying the G2019S mutation is remarkably uniform and resembles that of patients with idiopathic PD, i.e. a mild phenotype with good response to treatment and slow progression.

It has been reported that both the kinase and GTPase activities of LRRK2 are required for inducing cell death (Xiong et al., 2010; Greggio et al., 2009). Specifically, the G2019S mutation has been shown to result in increased kinase activity in a number of assays, suggesting a gain-of-function pathogenic mechanism (West et al., 2005 ; Luzon-Toro et al., 2007). Mutant G2019S expression is associated with progressive neurite shortening that leads to gradual cell death *in vivo* and *in vitro* (MacLeod et al., 2006). Studies from Plowey and colleagues found that the G2019S mutation was responsible for activation of

autophagy resulting in neurite shortening, thus establishing a link between autophagy and neurite retraction in differentiated SH-SY5Y cells transfected with G2019S (Plowey et al., 2008). However, additional research is needed to identify the *in vivo* LRRK2 substrates relevant to PD.

b) Autosomal recessive forms of parkinsonism

Nowadays, homozygous or compound heterozygous mutations in three genes: *PARKIN*, *PTEN induced putative kinase 1 (PINK1)* and *DJ-1* have been associated with parkinsonism with early-age at onset and no atypical signs. A fourth gene, ATP13A2 may also be responsible for rare cases with early-onset.

Parkin

Parkin is a E3 ubiquitin ligase and it is localized primarily to the cytoplasm. It tags proteins for proteasomal degradation and it has been reported to activate mitophagy (Dawson et al., 2010). Parkin is a 465-aminoacid protein that contains an N-terminal ubiquitin-like (UBL) domain followed by two RING (really interesting new gene) finger domains separated by a 51-residue IBR (in-between-ring) domain and a third RING finger domain in the C-terminal part (Figure 6).

To date, more than 170 different mutations have been described throughout the large sequence of this gene (1.35 Mb), including point mutations, large deletions, multiplications, and small insertions (Nuytemans et al., 2010). Mutations in the *PARKIN* gene are the most frequent described cause of early-onset PD (<40-50 years) accounting for 10-20% of the cases worldwide and 50% of recessive familial forms (Lucking et al., 2000; Periquet et al., 2003).

Pathologically, *PARKIN* mutations are associated with loss of A9 DA neurons and half of the patients show LB formation (Mori et al., 1998). Most mutations in *PARKIN* disrupt its E3 ligase activity or its interaction with E2 enzymes, leading to insufficient substrate clearance (Shimura et al., 2000). Moreover, Parkin could be inactivated by nitrosive, dopaminergic and oxidative stress in sporadic PD (Dawson et al., 2010), indicating that Parkin dysfunction may be causative of both sporadic and genetic PD.

Recently, the identification of a Parkin-interacting substrate, PARIS, has pointed out a novel molecular mechanism for neurodegeneration due to Parkin inactivation (Shin et al., 2011). Parkin controls PARIS levels via the ubiquitin proteasome system. PARIS, when overexpressed, initiates a signaling cascade that leads to decreased mitochondrial biogenesis and cell death (Shin et al., 2011) (See section 1.3).

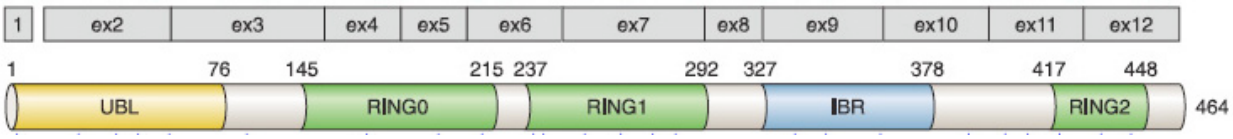

Figure 6. Shematic representation of the domains present within Parkin protein. (Modified from Corti et al., 2011) .

PTEN induced putative kinase 1 (PINK1)

PINK1 is a protein kinase associated with the mitochondrial outer membrane, in which the kinase domain is placed facing the cytosol (Zhou et al., 2008). PINK is a 581-aminoacid protein with a mitochondrial targeting signal (MTS) motif, a putative transmembrane (TM) region and a serine-threonine kinase domain (Figure 7). It has been described to promote autophagy of damaged mitochondria in cooperation with Parkin (Vives-Bauza et al., 2010).

PINK1 mutations are the second most frequent cause of autosomal recessive early-onset PD. The most frequent mutations in this gene include point mutations, small deletions or small insertions, however, large genomic deletions and rearrangements have also been described (Cazeneuve et al., 2009; Marongiu et al., 2007). Mutations in *PINK1* could mainly produce two effects: destabilization of the protein or decreased kinase activity (Beilina et al., 2005), resulting in altered mitochondrial turnover in either case.

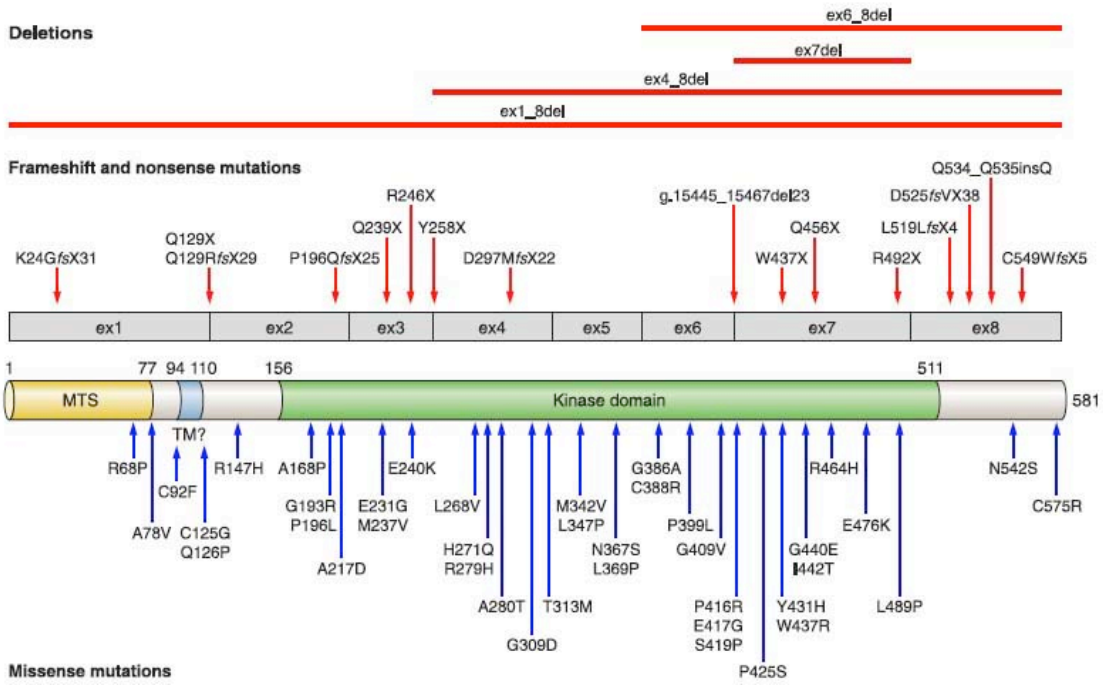

Figure 7. Schematic representation of the domains present within PINK1 protein with indication of known pathogenic mutations (From Corti et al., 2011).

DJ-1

DJ-1 is a molecular chaperone, member of ThiJ/Pfp1 family of molecular chaperones. It is a protein of 189 aminoacids with a single protein domain (Figure 8). Under conditions of oxidative stress, DJ-1 translocates from the cytoplasm to mitochondria, and it is proposed to play a role in neuroprotection under these conditions (Canet-Aviles et al., 2004). Specifically, it has been described that DJ-1 plays an important role in the maintenance and function of the mitochondrial pool (Thomas et al., 2010). This chaperone protects mitochondria and mitigates cell death induced by oxidative stress via scavenging of H_2O_2 (Andres–Mateos et al., 2007), regulating redox-dependent kinase signaling pathways (Kahle et al., 2009) and upregulating the synthesis of the antioxidant glutathione (Zhou et al., 2005).

Mutations in *DJ-1* account for only 1% of early-onset PD. Mutations in this gene include point mutations in the promoter and coding regions, frame-shift and splice-site mutations and, to a lesser extent, exon deletions (Figure 8) (Corti et al., 2010). Mutations in the *DJ-1* gene cause early-onset parkinsonism that closely resembles that associated to *PARKIN* and *PINK1* mutations. The small number of DJ-1 patients does not allow establishing accurate genotype/ phenotype correlations.

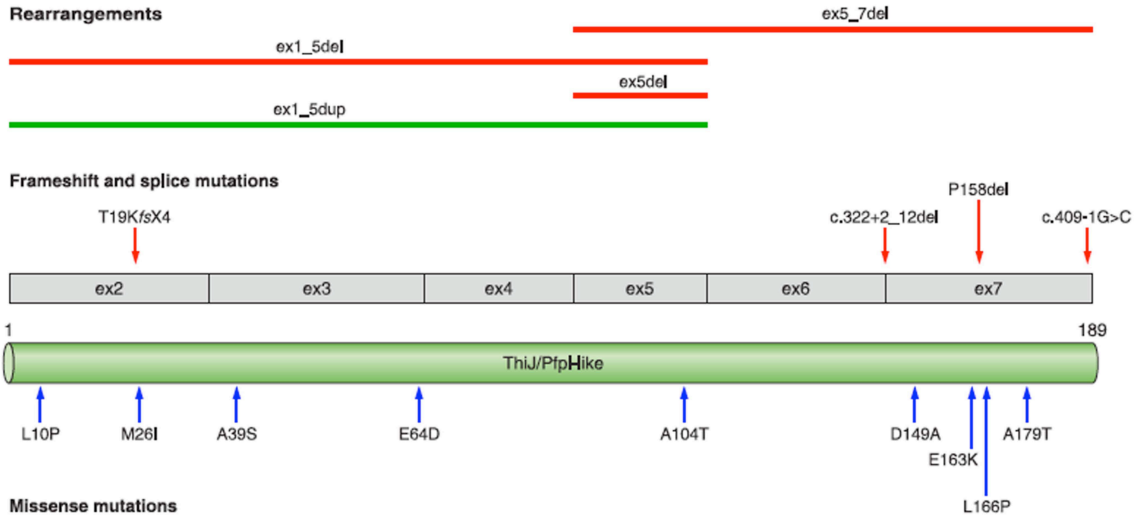

Figure 8. Shematic representation of DJ-1 on transcript and protein levels. Pathogenic homozygous and compound heterozygous mutations are shown (From Corti et al., 2011).

Lysosomal type 5 P-type ATPase (ATP13A2)

ATP13A2 is a large transmembrane protein with putative ATPase activity. It localizes to the lysosomal and mitochondrial membrane. Its function is unknown. Mutations in ATP13A2 are associated with Kufor-Rakeb syndrome (KRS), a recessively inherited atypical parkinsonism (Najim al-Din et al.,1994; Williams et al., 2005). Seven missense mutations have been identified (DiFonzo et al., 2007). Transient transfection of wild-type ATP13A2 in cells showed lysosome membrane localization and transfection of truncated mutants lead to retention in the endoplasmic reticulum prior to proteasomal degradation (Ramirez et al., 2006).

1.3-Mechansisms involved in PD pathogenesis

The exact mechanisms leading to A9 DA neuron death in PD are poorly understood, although several mechanisms have been proposed to contribute to this process.

1.3.1-Pathogenic protein aggregation

One of the pathological hallmarks of PD is the presence of LB, composed of protein aggregates. The presence of ubiquitin-positive proteins in LB lead to the hypothesis that the ubiquitin and proteosomal system (UPS) could be involved in the formation of LB. The important role of protein aggregation and UPS in the pathogenesis of PD was confirmed by the description of two genes responsible for genetic PD directly linked to protein aggregation (*SNCA* gene) and UPS (*PARKIN* gene).

α-Synuclein is the major component of LB. The precise physiological function of α-synuclein is not fully understood, however, it appears to be involved in elementary synaptic functions, e.g. plasticity and neurotransmitter release (Norris et al., 2004). The molecular mechanism involved in α-synuclein aggregation and how aggregates can cause selective DA neuron loss is also not completely understood (Figure 9). *In vitro*, wild-type α-synuclein shows a spontaneous tendency to form amyloid fibrils, a property that is enhanced in the A53T, A30P and E46K mutant variants of α-synuclein (Choi et al., 2004). Cytotoxicity of α-synuclein can on the one hand, be associated with a dosage effect by protein overexpression, due to supernumerary copies of the *SNCA* gene, as shown in a screening study of 119 individuals from families with familial PD (Ibanez et al., 2004). On the other hand, cellular dysfunction might be related to a change in the conformation of α-synuclein. Some studies have suggested that oligomeric intermediates are the cause of cellular dysfunction and cell death (Caughey and Lansbury, 2003), whereas several others have pointed to the direct correlation between fibrillar inclusion bodies and neurodegeneration (Li et al., 2004; Dawson et al., 2002). Remarkably, Winner and colleagues developed recombinant α-synuclein variants able to form oligomers and not fibrils, as well as accelerating fibril-promoting variants. Lentivirus encoding these α-synuclein variants were injected to the substantia nigra of adult rats. Interestingly, they observed high loss of DA neurons (50%) in oligomer-prone variants and no significant loss in fibril-promoting variants. Moreover, oligomer-prone variants interacted more strongly with membranes than fibril-promoting variants (Winner et al., 2011). Therefore, this study demonstrated a direct link between membrane-associated α-synuclein oligomers and DA neurodegeneration in a murine model, thus supporting the idea that oligomeric prefibrillar α-synuclein, rather than α-synuclein fibrils, may represent the pathogenic species of α-synuclein in PD.

Several studies have shown that the presence within the cytosol of nitric species and dopamine could lead to nitration and dopamine-induced modification of α-synuclein, modifications shown to increase α-synuclein tendency to form different types of oligomers (Mazzulli et al., 2006;). Moreover, it has been shown that phosphorylation of α-synuclein at Ser 129 promotes fibril formation (Fujiwara et al., 2002; Takahashi et al., 2003). Interestingly, another mechanism for α-synuclein aggregation has been postulated after the observation of post-mortem brains of individuals that received transplantation of fetal DA neurons (Kordower et al., 2008; Li et al., 2008; Mendez et al., 2008). Specifically, three out of eight brains transplanted for more than 9 years contained LB within the graft, suggesting that misfolded α-synuclein can be transmitted between cells and initiate aggregation in neighboring neurons, in a prion-like fashion (Figure 9).

Remarkably, the accumulation of α-synuclein in cells depends on the imbalance between production and clearance. An increase in the synthesis through gene triplication has been show to cause PD, supporting the hypothesis that high protein levels contribute to PD (Singleton ,2003). The maintenance of normal α-synuclein levels depends on the proper function of the metabolic pathways that degrade α-synuclein. The causative link of UPS dysfunction in the pathogenesis of PD came from the observation that ubiquitin-positive LBs were observed in post-mortem analysis of PD patients (Dawson et al., 2003). This hypothesis was confirmed by the identification of mutations in *PARKIN*, encoding an E3 ligase, responsible for recessive familial PD. Moreover, it has been found that proteasomal activity is decreased in different brain areas in patients with idiopathic PD (McNaught et al., 2003). Several studies support the notion that α-synuclein degradation is accomplished through UPS (Bennett et al., 1999) and chaperone-mediated autophagy (CMA) (Mak et al.,

2010). Remarkably, both α-synuclein mutants and dopamine-modified α-synuclein have been shown to impair the CMA pathway, thus suggesting a potential mechanism of accumulation of abnormal α-synuclein and activation of macroautophagy as a compensatory mechanism to degrade aggregates (Cuervo et al., 2004; Martinez-Vicente et al., 2008) (Figure 14, see section 1.3.4)

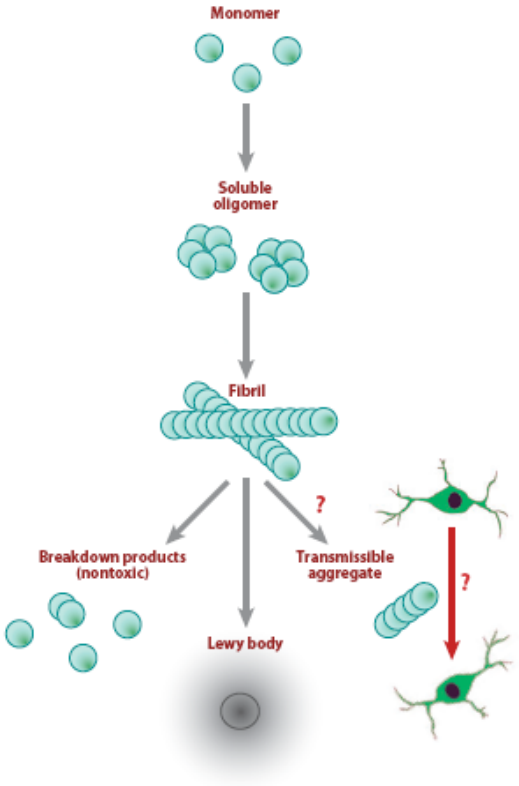

Figure 9. α-Synuclein aggregation in PD. α-Synuclein monomers assemble to form soluble oligomers, which aggregate to generate insoluble fibrils. Fibrils can break down to generate non-toxic soluble monomers, or can accumulate in Lewy bodies or might break down via incomplete aggregates that can be transmitted from one dopaminergic neuron to another in a prion-like manner. (Modified from Martin et al., 2011).

1.3.2- Mitochondrial dysfunction

The first link between mitochondrial dysfunction and PD pathogenesis was described some decades ago when people intoxicated with inhibitors of the mitochondrial complex I (MPTP) developed parkinsonism (Langston et al., 1983). Indeed, some toxin-induced models of parkinsonism are based on the use of mitochondrial inhibitors to generate PD-related phenotypes (extended in section 1.4). Moreover, mutations in some genes involved in mitochondrial function and homeostasis have been associated with parkinsonism such as *PARKIN*, *PINK1* or *DJ-1*, and the products of other PD-associated genes have been shown to be localized to mitochondria, such as α-synuclein and LRRK2, confirming the link between mitochondria alterations and PD.

In addition, a reduced activity of the mitochondrial complex I was found in the substantia nigra of PD sporadic patients (Schapira et al., 1990). Moreover, several catalytic subunits of complex I presented increased levels of carbonyls (oxidative modification) in PD brains, which correlated with reduced electron transfer rates, suggesting that excessive oxidation of complex I subunits may lead to complex I dysfunction (Keeney et al., 2006). Additionally it has been described in mice that the mitochondria mass in A9 DA neurons is lower, compared to A10 DA neurons or to non-DA cells (Liang et al., 2007). Indeed, A9 DA neurons seem to be more vulnerable to impairments of complex I activity, when compared to other brain areas, probably due to lower mitochondria content and increased production

of reactive oxygen species resulting from dopamine metabolism (Chinta and Andersen, 2008).

Among the PD-associated genes, three have been identified as "guardians" of mitochondrial homeostasis: those encoding the E3 ubiquitin ligase Parkin, the mitochondrial serine-theronine PINK1 and the chaperone DJ-1. Mutations in these three genes are associated to familial PD.

Parkin is an essential player in mitochondrial homeostasis, as it stimulates biogenesis of mitochondria via PARIS interaction (Figure 10) and promotes mitophagy of damaged mitochondria via interaction with PINK1 (Figure 11). Specifically, Parkin is present in mitochondria of proliferating cells and enhances transcription and replication of mitochondrial DNA (Kuroda et al., 2006), meaning that Parkin is recruited to mitochondria during mitosis. In normal conditions, PINK1 is localized in the outer membrane of the mitochondria where it is cleaved in a voltage-dependent manner to a smaller fragment. However, when mitochondria are depolarized, no cleavage occurs, resulting in the retention of full-length PINK1 in the outer mitochondrial membrane (OMM), which in turn leads to the recruitment of Parkin that ubiquitinates OMM proteins (VDAC-1 and mitofusin), finally triggering mitophagy (Figure 10). Therefore, mutations in *PARKIN* lead to decreased mitochondrial biogenesis and reduction of mitophagy.

A B

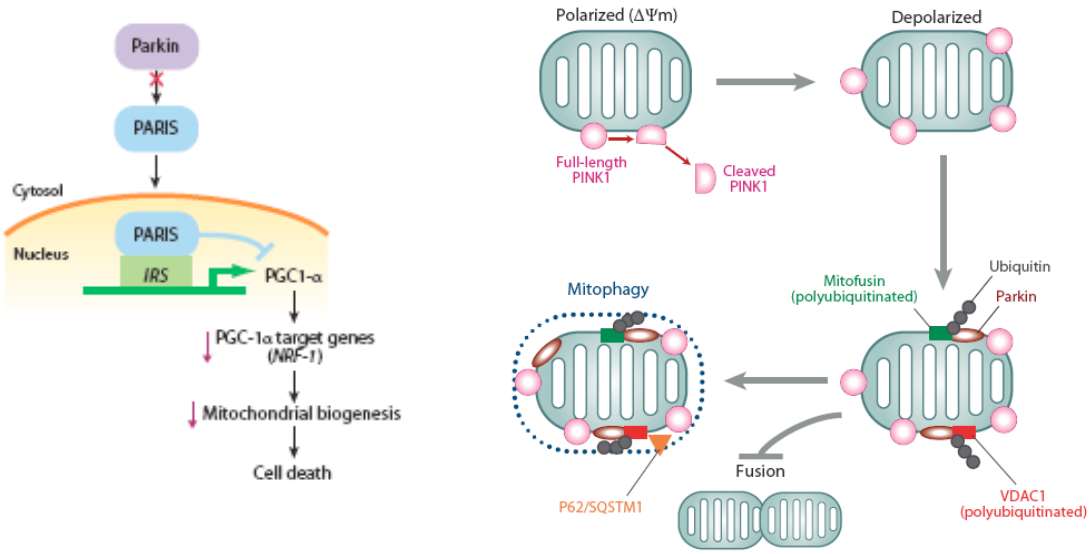

Figure 10. Parkin interacts with PARIS and PINK1. (A) Parkin is responsible for the ubiquitination of PARIS in normal conditions leading to mitochondrial biogenesis. When Parkin is mutated it does not ubiquitinate PARIS, a transcriptional repressor. In this situation, PARIS internalizes to the nucleus and inhibits PGC1-α leading to a decrease in mitochondrial biogenesis and consequent cell death. (B) In a polarized mitochondria PINK1 is cleaved and a soluble fragment is released form the outer membrane of the mitochondria. However, when the mitochondria is depolarized PINK1 is not cleaved and Parkin is recruited to the OMM, where it ubiquitinates two proteins VDAC1 and mitofusin. This ubiquitination recruits the autophagic adapter protein P62/SQSTM1 and mitophagy is activated, thus, preventing fusion of the damaged mitochondria with other healthy mitochondria. (Modified from Martin et al., 2011)

DJ-1 is a redox-regulated chaperone that translocates to the mitochondria during conditions of oxidative stress. It is thought that one of its possible substrates is α-synuclein,

so that DJ-1 would inhibit α-synuclein oligomerization avoiding mitochondrial membrane and synaptic vesicles permeabilitzation through the generation of pores by α-synuclein oligomers. Moreover it has been described that DJ-1 can prevent misfolding of different subunits of mitochondrial respiratory complexes, specifically complex I, controlling mitochondrial homeostasis (Figure 11).

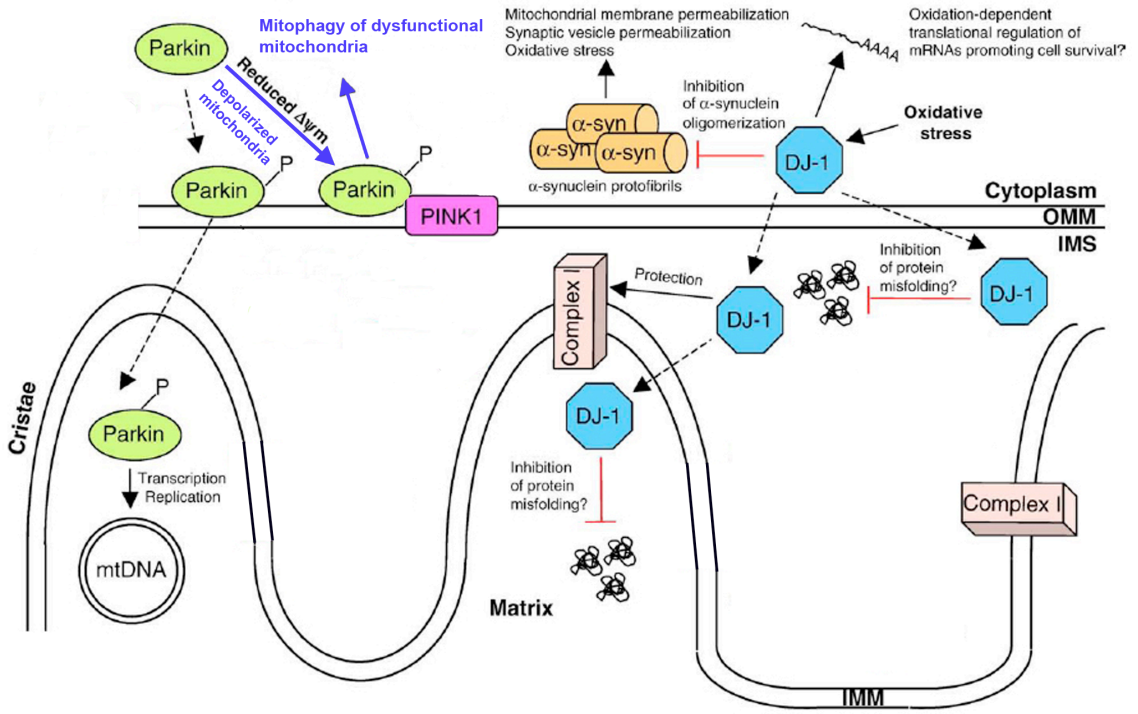

Figure 11. Schematic representation of the dynamic regulation of mitochondrial homeostasis by α-synuclein, Parkin, PINK1 and DJ-1. Parkin interacts with PINK1 to activate mitophagy of dysfunctional mitochondria. Moreover, Parkin translocates to the matrix of the mitochondria contributing to the transcription and replication of mitochondrial DNA. DJ-1 a chaperone, translocates to the mitochondria to prevent misfolding of mitochondrial proteins and in the cytoplasm inhibits α-synuclein oligomerization. In oxidative stress conditions, DJ-1 promotes cell survival through regulation of mRNAs encoding for survival factors. (Modified from Brüeler, 2009).

α-Synuclein and LRRK2 proteins are associated to mitochondria and it has been described that their wild-type forms and/or their mutated variants could affect mitochondrial homeostasis. α-Synuclein is a predominantly cytosolic protein, but a fraction is associated with or present in mitochondria (Nakamura et al., 2008). Remarkably, hippocampus, striatum and substantia nigra contain higher amounts of mitochondrial α-synuclein compared to other areas, and α-synuclein levels in the mitochondria of PD patients are several-fold higher compared to healthy individuals (Devi et al., 2008). Indeed, α-synuclein located in or associated to the mitochondria has been linked to increased levels of nitric oxide and oxidation of mitochondrial proteins resulting in mitochondrial dysfunction and neuronal death (Parihar et al.,2008). α-Synuclein exerts another deleterious effect on mitochondria, as α-synuclein protofibrils are known to form annular pores in membranes, resulting in permeabilitzation of intracellular membranes, including those of mitochondria (Figure 11 and 12) (Lashuel et al., 2002). It is important to note that transgenic mice lacking α-synuclein present alterations in membrane lipids and reduced activities of the electron complexes I and II (Ellis et al., 2005), suggesting a physiological role of α-synuclein in the mitochondria.

LRRK2 is a protein with protein kinase and GTPase activities, which is found associated to intracellular membranes, including lysosomes, transport vesicles and mitochondria (Biskup et al., 2006). It has been described that transient overexpression of G2019S, Y1699C and R1441C mutant forms of LRRK2 induces neuronal death that can be blocked using caspase inhibitors and requires Apaf1, suggesting that mutant LRRK2 induces mitochondrial-dependent apoptosis (Iaccarino et al., 2007). Interestingly, overexpression of G2019S mutant LRRK2 in differentiated neuroblastoma cells leads to neurite retraction that correlated with increased autophagy (Plowey et al., 2008). It has been hypothesized that increased autophagy could be responsible for pathogenic degradation of mitochondria in neurites (Büeler et al., 2009)(Figure 12).

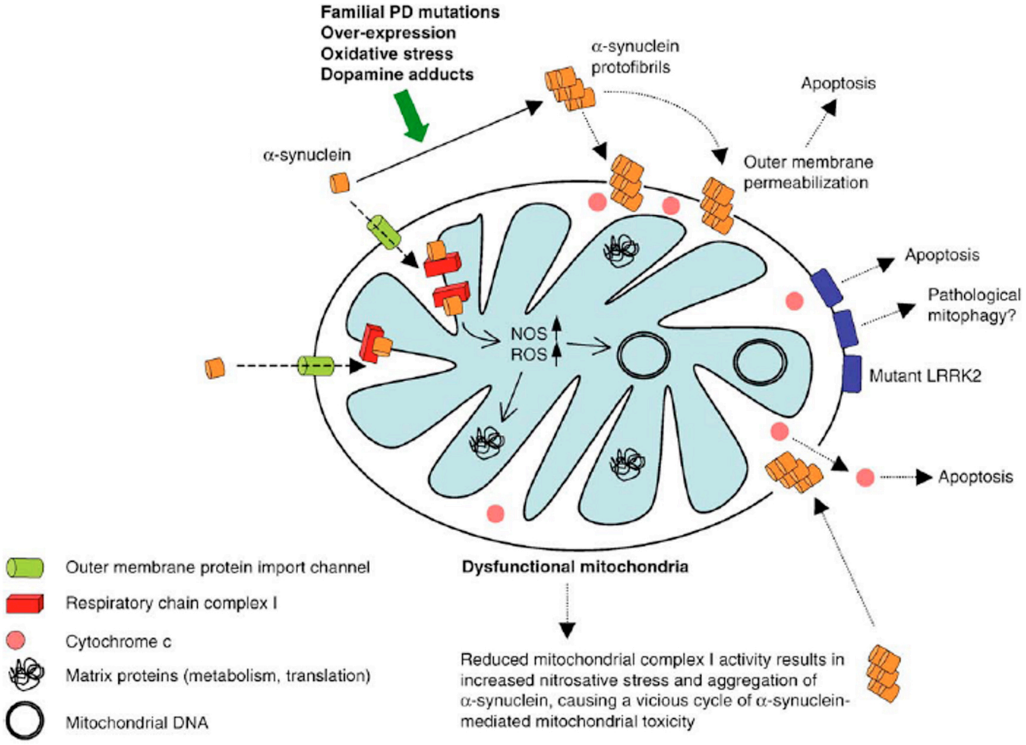

Figure 12. α-synuclein and LRRK2 in mitochondrial pathology. (Modified from Brüeler , 2009)

1.3.3- Oxidative and nitrosative stress

Oxidative and nitrosative stresses have been shown to be increased in PD, suggesting that they play an important role in the pathogenesis of PD (Figure 13).

Oxidative stress can be generated mainly by three mechanisms. Generation of reactive oxygen species (ROS) is the result of oxygen-dependent respiration that takes place in mitochondria. Specifically, ROS result when electrons leak from the electron transport chain, mainly from complex I, and lead to the partial reduction of molecular oxygen to superoxide ($\cdot O_2$), which can then be converted to hydrogen peroxide (H_2O_2). Mitochondria are protected from this by superoxide dismutase, which converts hydrogen peroxide to non-toxic derivatives H_20+O_2 (Swerdlow et al., 1996). The conversion of $\cdot O_2$ and H_2O_2 in non-toxic species is crucial for survival as they are extremely toxic to the cell. A second process responsible for ROS generation is inflammation as a defense mechanism against small pathogens (Winterbourn, 2008). Specifically, enzymes within the phagocytes generate ROS to kill invading pathogens in the process of inflammation. However, excessive

production of ROS during chronic inflammation may have deleterious effects. Third, the metabolism of dopamine itself generates hydrogen peroxide as byproduct, and dopamine oxidation can generate quinones, toxic reactive species that can modify proteins such as α-synuclein (Chinta et al., 2003; Stokes et al., 1999).

Furthermore, another essential contributor to oxidative stress is the metabolism of nitric oxide (NO). Overstimulation of NMDAR by glutamate results in exocitotoxicity (Calabrese et al., 2007). This overstimulation leads to the activation of neuronal nitric oxide synthase (nNOS) that produces NO. NO is an important signaling molecule, but it can react with other ROS to form highly toxic reactive nitrogen species (RNS) (Szabo et al., 2007). Moreover, NO can S-nitrosylate many proteins, a reversible modification of cysteine residues (Stamler et al., 1992). Remarkably, some neuroprotective proteins have shown to be modulated by S-nitrosylation, thus nitrosative stress could abnormally regulate their activity contributing to neurodegeneration (Chung et al. ,2007).

The generation of ROS and RNS may contribute to the pathogenesis of PD through two main mechanisms (Figure 13). The first one would be by inducing protein modifications that enhance protein misfolding, leading to protein aggregation and triggering the generation of oligomers and fibrils that are toxic and block degradation systems (chaperone-mediated autophagy or ubiquitin proteasome system). Second, mitochondria are a site of ROS generation but they are also highly affected by these species. Specifically, ROS can modify the subunits of the electron transport chain, resulting in disorganization and inhibition of the respiratory complexes. Inhibition of complex I can induce leakage of electrons, leading to ROS generation, a feedback loop that would result in reduced ATP biosythesis and neuronal death (Figure 13) (Swerdlow et al., 1996; Schapira et al.,1989).

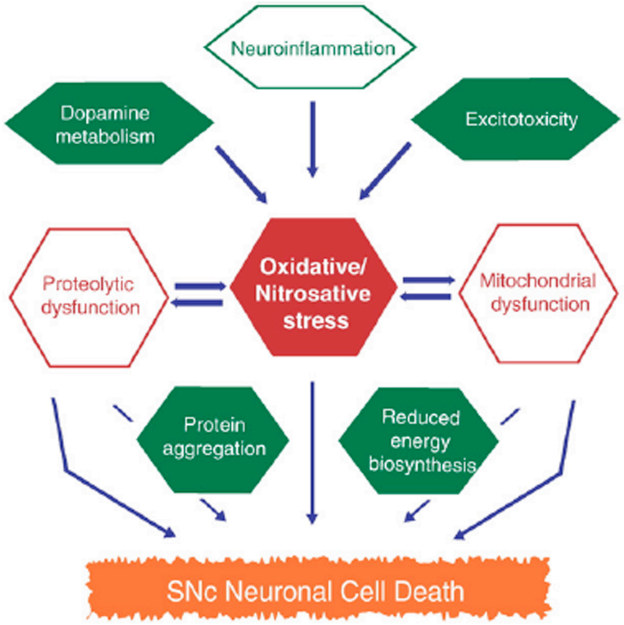

Figure 13: Oxidative and nitrosative stress activates mechanisms that contribute to A9 dopaminergic death. Oxidative and nitrosative stresses result from dopamine metabolism, neuroinflammation, excitoxicity, mitochondrial, and proteolytic dysfunction. In addition, oxidative and nitrosative stresses lead to proteolytic and mitochondrial dysfunction, closing a loop that leads to protein aggregation and reduced energy resulting in dopaminergic neuron cell death. (From Tsang et al., 2009).

1.3.4- Autophagy

Autophagy is a process by which lysosomes degrade intracellular components. Three different forms of autophagy have been identified: macroautophagy, microautohagy and chaperone-mediated autophagy (CMA). Whether autophagy has a protective role or contributes to cell death in PD is still controversial.

Autophagy plays an important role in PD as it is responsible for the degradation of α-synuclein by CMA and macroautophagy (Webb et al., 2003; Cuervo et al., 2004). Specifically, soluble α-synuclein is degraded by the proteasome and CMA (Webb et al., 2003). Remarkably, A53T and A30P α-synuclein mutants inhibit CMA (Cuervo et al., 2004); this inhibition leads to the activation of a compensatory mechanism resulting in degradation of mutated forms by macroautophagy. Macroautophagy is also responsible for degradation of insoluble α-synuclein aggregates (Figure 14).

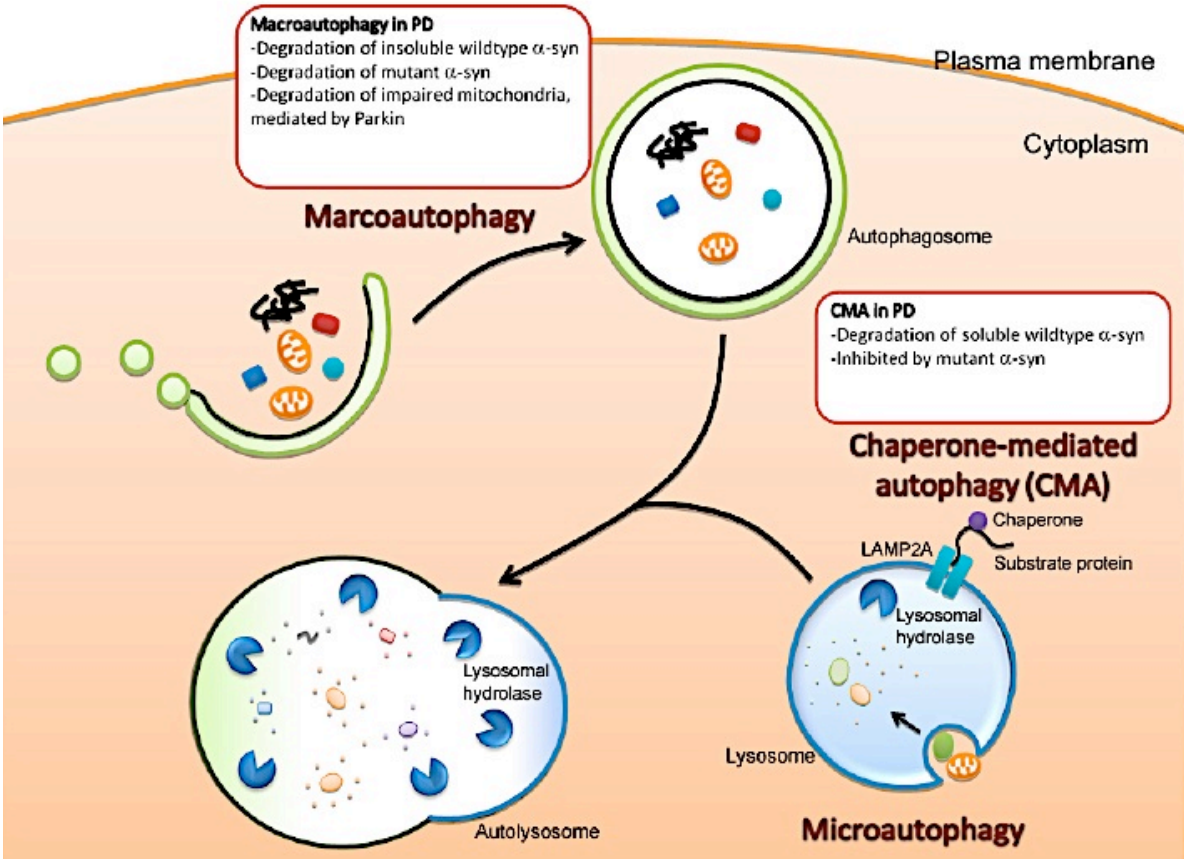

Figure 14. Deregulation of autophagy in PD. Wild-type α-synuclein is degraded by CMA. However, mutant α-synuclein inhibits CMA. In this scenario, macroautophagy is activated as a compensatory mechanism leading to the degradation of mutant α-synuclein by macroautophagy. Macroautophagy is also responsible for the elimination of oligomeric α-synuclein and impaired mitochondria mediated by Parkin and PINK1. (Modified from Cheung et al., 2009).

The autophagic pathway is also involved in the turnover of mitochondria. Parkin and PINK1 are responsible to activate mitophagy of damaged mitochondria, thus controlling the turnover of dysfunctional mitochondria (Martin et al., 2011). Failure to activate mitophagy may act as a PD pathogenic mechanism. Previous observations support a protective role of autophagy, as it eliminates toxic protein aggregates as well as damaged

mitochondria (Figure 14).

However, high/ abnormal presence of autophagic vacuoles is observed in brains of PD patients, in comparison to rare detection of autophagosomes in healthy brains, due to rapid clearance of these vesicles in the central nervous system (Anglade et al., 1997). The presence of autophagosomes in the brain of PD patients could be explained by an abnormal activation of macroautophagy, a defective clearance of autophagic vacuoles, or the combination of both. Remarkably, overexpression of G2019S mutant LRRK2 results in neurite retraction that correlates with increased autophagy in differentiated neuroblastoma cells (Plowey et al., 2008). It has been proposed that increased autophagy could be responsible for pathogenic degradation of mitochondria in the neurites (Büeler et al., 2009). Moreover, excessive activation of autophagy is associated with neuronal loss (Bredesen et al., 2006).

1.4- Modeling PD

Nowadays, PD research needs to overcome two main obstacles. First, we need a much better understanding of the pathogenic mechanisms leading to PD. Second, there is an urgent need to develop treatments aimed at halting the progression of the disease. Therefore, cellular and animal models for PD constitute an essential tool to elucidate the unknown mechanism/s underlying PD and as drug screening and testing platforms to find new treatments that not only palliate the symptoms.

1.4.1- Animal models of PD

It is important to remark that the ideal animal model should recapitulate most, if not all, the features of PD, including loss of A9 DA neurons and LB formation in a progressive, age-dependent fashion, and ideally, also, the non-motor features of the disease.

Animal models of PD have been widely used in the past four decades to investigate the pathogenesis and pathophysiology of this neurodegenerative disorder. These models have been classically based on the systemic or local administration of neurotoxins that are able to replicate the neurodegeneration of DA neurons in the SNpc, typical of PD, in mammals (rodents or primates) (reviewed in Bové et al ., 2011). In the last decade, the identification of PD-associated genes has opened the door to the generation of genetic models of PD, specifically, the development of various mammalian (mice and, more recently, rats) and non-mammalian transgenic models that replicate most of the disease-causing mutations identified for monogenic forms of familial PD. Both toxic and transgenic PD animal models have their own advantages and limitations, which must be taken into consideration when choosing the model to be used.

Neurotoxic animal models of PD

Neurotoxic models represent the classical and earliest experimental PD animal models. They aim to reproduce the pathological and behavioral changes of the human disease in rodents or primates by using neurotoxins, pharmacological agents that cause the selective degeneration of nigrostriatal neurons. These neurotoxins can be administered either systemically or locally, depending on the type of toxin and animal species used. The most commonly used neurotoxic animal models are summarized in Table 2.

Toxic model	Behavioral alterations	Nigro-striatal damage	Lewy bodies/α-synuclein inclusions	Uses of the model
Unilateral 6-OHDA injection into rodent MFB	Quantifiable turning behavior after systemic administration of a dopaminergic agonist. Bradykinesia and impaired paw use on the contralateral side.	Massive loss of dopaminergic neurons (>90%). Dose-dependent loss of striatal dopamine innervation.	No intracellular inclusions.	Test symptomatic therapies. Used in l-dopa-induced dyskinesia and motor fluctuation models. Study of the consequences of dopaminergic denervation on the basal ganglia circuitry.
Unilateral 6-OHDA injection into rodent striatum	Quantifiable turning behavior after systemic administration of a dopaminergic agonist. Bradykinesia and impaired paw use on the contralateral side after multiple 6-OHDA injections.	Partial and less acute loss of dopaminergic neurons (30–75%). Circumscribed loss of striatal dopamine innervation at injection site.	No intracellular inclusions.	Test of symptomatic therapies. Study of mechanisms of cell death.
Acute MPTP mouse model	Coordination and motor impairments patent in challenging situations.	Massive loss of dopaminergic neurons (about 70%). Reduced dopamine levels in the striatum.	No intracellular inclusions.	Test neuroprotective strategies. Study of mechanisms of neuroinflammation.
Chronic MPTP mouse model	Less obvious motor and coordination impairments that are in some cases only detectable in aged mice.	Partial loss of dopaminergic neurons (30–50%). Reduced dopamine innervation in the striatum.	Increased α-synudein immunoreactivity in the substantia nigra. No intracellular inclusions.	Test neuroprotective strategies. Study of cell death mechanisms.
Nonhuman primate MPTP model	Motor impairments resembling PD symptoms. Cognitive impairments.	Loss of dopaminergic neurons depending on the route of administration and regimen (60–90%). Reduced dopamine innervation in the striatum.	Inclusions resembling Lewy bodies in the locus coeruleus of older squirrel monkeys.	Test of symptomatic therapies. Used in l-dopa-induced dyskinesia and motor fluctuation models. Study of the consequences of dopaminergic denervation on the basal ganglia circuitry.
Rotenone in rodents	Reduced motor activity (rats) and reduction in the endurance time in rotarod (mice).	Loss of dopaminergic neurons (20–75%). Reduced dopamine innervation in a circumscribed area of the striatum (rat). Reduced TH levels in the striatum (mouse).	α-synuclein aggregation in dopaminergic neurons and other neuronal populations.	Test neuroprotective strategies. Study of mechanisms of cell death.
Paraquat in mice	No clear motor impairments detectable.	Little or no cell loss of dopaminergic neurons (<25%). Little or no measurable changes in striatal dopaminergic denervation.	Increased α-synudein immunoreactivity in the substantia nigra. No intracellular inclusions.	Test neuroprotective strategies. Study of cell death mechanisms.

Table 2: Summary of the neurotoxin-based animal models of PD commonly used. (From Bové et al., 2011).

The first PD animal model was generated by the intracerebral injection of 6-hydroxydopamine (6-OHDA) (Ungerstedt et al., 1968). 6-OHDA is the neurotoxin *per excellence* to model PD in rats. 6-OHDA is a hydoxylated analogue of dopamine with high affinity for the dopamine transporter (DAT), which is responsible for the transport of the toxin inside DA neurons. The toxin needs to be delivered by local injection, as it is not able to cross the blood-brain barrier. The injection is commonly carried out unilaterally, with the contralateral hemisphere serving as control; moreover, high mortality rates have been associated with bilateral injections (Ungerstedt et al., 1971). Injection of 6-OHDA in to the SNpc or, preferably, into the medial forebrain bundle (MFB) that conveys the efferent fibers from nigral cell bodies to the striatum, causes massive degeneration of the nigrostriatal pathway. This procedure produces the highest level of nigral cell loss and striatal DA depletion obtainable in PD animal models (90–100%) (Full and Laverty, 1969). One variant of this procedure is the injection of 6-OHDA in the striatum (Sauer et al., 1994), which produces degeneration of 30-75% of DA neurons, a degree of SNpc damage less marked compared to MFB. Importantly, striatal injection provides a progressive model of nigrostriatal degeneration, being more similar to the gradual neurodegeneration that occurs in human PD. One major limitation of 6-OHDA models is that DA neurodegeneration is not accompanied by the formation of LB-like cytoplasmic inclusions. Therefore, these models do not recapitulate the two neuropathological hallmarks of PD. Nevertheless, 6-OHDA models have been used to test symptomatic therapies and, in the case of striatal lesion, to study the mechanism of cell death (Table 2).

One classical systemic model is based on the administration of 1-Methyl-4-phenyl-1,2,3,6-tetrahydropyridine (MPTP), an agent with a selective toxicity for DA neurons. The selective toxicity of MPTP for the nigrostriatal system was discovered in the mid 1980s in young drug users from Northern California, who showed marked parkinsonism caused by the intravenous injection of a street preparation of an analogue of the narcotic meperidine containing MPTP (Langston et al., 1983). MPTP crosses the blood-brain barrier and, once in the brain, it is transformed to its active metabolite 1-methyl-4-phenylpyridinium ion (MPP+). MPP+ is internalized by A9 DA neurons through the dopamine transporter DAT and it has been described to block mitochondrial complex I activity. When administered to primates, MPTP causes a levodopa-responsive parkinsonian syndrome, characterized by all of the cardinal symptoms of PD, representing the best PD-like clinical picture obtainable in experimental animals (reviewed in Bezard, 2011). To date, the MPTP-monkey model remains the gold standard for preclinical testing of therapeutic strategies for PD. MPTP can also be administered to rodents, although rats are highly resistant to MPTP (Giovanni et al., 1994). Indeed, rats are sensitive to MPP+ only if injected directly into the SNpc. In mice we should distinguish between the acute and chronic MPTP model. The acute model causes essentially the same phenotype observed in MPTP-treated monkeys (Jackson-Lewis et al., 1995), but lacking a neuropathological hallmark of PD: LB formation. Remarkably, Fornai and collegues developed a chronic mice model of MPTP administration, via osmotic minipumps that resulted in DA degeneration accompanied by formation of nigral inclusions immunoreactive for α-synuclein (Fornai et al., 2005). However, many groups have not been able to reproduce Fornai's results (Alvarez-Fisher et al., 2008).

Two pesticides, rotenone and paraquat, have also been used to generate neurotoxin PD models. Rotenone is a flavonoid extracted from *Leguminosa* plants that has been widely used to kill insects and nuisance fish in lakes. It easily crosses the blood-brain barrier and, once in neurons, it inhibits mitochondrial complex I. In rats, rotenone induced the selective degeneration of nigrostriatal DA neurons and LB-like cytoplasmic inclusions containing ubiquitin and α-synuclein (Betarbet et al., 2000). The rotenone model has also been extended to mice, resulting in outcomes similar to those observed in rats. Despite its positive features, the rotenone model has not been widely used, as a number of critical issues have been highlighted. The first concern is related to the high variability in the response to the toxin and the inability to reproduce the parkinsonian neurophatology and phenotype of this model in different laboratories (Hoglinger et al., 2003; Lapointe et al., 2004). Second, rotenone is highly toxic to various organs leading to high mortality. The third concern is related to the specificity of the rotenone-induced lesions, it has to be clarified if other areas of the brain such as the striatum or locus coeruleus become affected after rotenone injections.

Paraquat is an herbicide that has been shown to be highly selective for nigral DA neurons. When injected into mice, it has been reported to induce DA neuron loss (Brooks et al., 1999), and increased expression and aggregation of α-synuclein in the SNpc (Manning-Bog et al., 2002). However, it is not an extended model as damage induced by paraquat in DA neurons has not been consistently reproduced (Thiruchelvam et al., 2000; Cicchetti et al., 2005)

Neurotoxin-based models have been essential for the development of treatment aimed to palliate motor symptoms and to identify key players in PD such as apoptosis, oxidative stress, or mitochondrial dysfunction. However, the lack of an age-dependent, slowly progressive lesion and the fact that LBs are typically not observed in these models represent major drawbacks. Reproducibility issues are also an important concern.

Genetic animal models of PD

The identification of monogenic forms of PD has provided considerable insights into the pathogenic mechanisms that underlie PD initiation and progression, and recent genome-wide association studies have provided evidence that familial and sporadic forms of PD may share common genetic backgrounds. These discoveries have enabled the development of new animal models, in particular mice, expressing transgenic counterparts of known PD-associated mutations. Importantly, apart from mice and rats, other alternative models in non-mammalian organisms, such as *Drosophila* and *C. elegans* have been developed.

In the case of genetic models for PD, two main strategies have been pursued. First, ectopic overexpression of wild-type or mutated forms of a protein is usually performed for autosomal dominant genes such as *SNCA* or *LRRK2*. In a second strategy, knockout or knockdown approaches are used in the case of autosomal recessive genes such as *PARKIN, PINK1* o *DJ-1*. Moreover, the development of genetic models of PD is not limited to research on familial PD. Indeed, it has been described an intimate link between familial and sporadic PD, and analyses of genes mutated in familial PD cases have provided valuable insights into sporadic PD.

Mouse genetic models

α-Synuclein

SNCA was the first gene to be linked to familial PD. Three PD-associated point mutations (A30P, A53T and E46K) have been described. Duplications and triplications have been identified to cause PD, suggesting that the level of expression of the protein is a causal factor for developing the disease. Several α-synuclein transgenic mice have been generated using a variety of promoters and variants of the α-synuclein protein (reveiwed in Chesselet et al., 2008). It has been described that the degree of overexpression achieved in different transgenic mice heavily depends on the promoters used to drive the expression of the transgene. However, none of the α-synuclein mice models generated so far faithfully recapitulates PD pathology, as they do not show progressive selective loss of DA neurons. From all of the reported models, the prion promoter:A53T-α-synuclein transgenic mice showed the most complete range of α-synuclein-related phenotypes observed in humans, including α-synuclein aggregation, fibril formation and progressive age-dependent (albeit not DA-specific) neurodegeneration (Chesselet et al., 2008; Dawson et al., 2002).

LRRK2

Mutations in the *LRRK2* gene represent the most prevalent cause of autosomal dominant PD and to date, seven disease-causing mutations have been identified (R1441G, R1441C, R1441H, N1437H, Y1699C, G2019S, and I2020T). Deletion of the *Lrrk2* gene in mice does not result in obvious developmental phenotypes, and mutant mice do not show neuropathological abnormalities or motor dysfunctions up to 12 months of age (Wang et al., 2006) (Table 2). Moreover, transgenic overexpression of wild-type Lrrk2 did not induce PD-relevant phenotypes (Li et al., 2010; Ramonet et al., 2011). These results indicate that mutated LRRK2 protein may be necessary to drive pathogenic toxicity, and suggest that LRRK2 pathogenesis is likely to be caused by a gain-of-function of the mutant LRRK2 protein.

Current LRRK2 transgenic mouse models are not very useful to model PD as they do not recapitulate key pathological hallmarks of PD (Table 3). Bacterial artificial chromosome (BAC) transgenic mice expressing wild-type LRRK2, as well as the R1441G and G2019S mutant variants, have been developed (Melrose et al., 2010; Li et al., 2009; Li et al., 2010; Ramonet et al., 2011), and show (with the exception of the study by Ramonet and collegues), a reduction of striatal dopamine neurotransmission when the mutated protein was overexpressed, suggesting a role of LRRK2 in neurotransmission. Importantly, motor symptoms were identified in mice overexpressing R1441G LRRK2 (Li et al., 2009; Ramonet et al., 2011). Loss of nigrostriatal DA neurons was identified in mice overexpressing the G2019S variant at 19 months of age (Ramonet et al., 2011), and after intrastriatal injection of LRRK2 G2019S through HSV amplicon-mediated delivery (Lee et al., 2011). Additionally, conditional expression of wild-type and G2019S LRRK2 failed to induce degeneration of DA neurons, although this could be explained by the low levels of LRRK2 overexpression achieved using the calcium/calmodulin-dependent protein kinase IIa promoter (Lin et al., 2009; Wang et al., 2008).

The reasons why mouse LRRK2 transgenic models do not exhibit clear PD features are currently unclear. It could be due to the fact that LRRK2 mutations are partially penetrant and that the presence of other genetic and/or environmental factors are required for PD-related neurodegeneration. Another possible explanation could be the activation of compensatory mechanisms during development that may prevent loss of DA neurons.

Parkin, PINK1 and DJ-1

PARKIN mutations are the most common cause of autosomal recessive parkinsonism. Remarkably, none of the *Parkin* knockout mice exhibit substantial DA neurodegeneration or behavioral alterations (Goldberg, 2005; Itier et al., 2003; Perez and Palmiter, 2005; Von Coelln et al., 2004). However, these mice show higher susceptibility to neurotoxins, suggesting that Parkin mutations may sensitize DA neurons to cellular insults. Interestingly, overexpression of mutant human Parkin resulted in progressive motor deficits and age-dependent degeneration of DA neurons, suggesting that some Parkin mutations might act in a dominant-negative fashion (Lu et al., 2009; Sang et al., 2007; Wang et al., 2007).

Mutations in the *PINK1* gene are the second most common cause of autosomal-recessive parkinsonism. *Pink1* knockout mice do not exhibit any major abnormality in the number of DA neurons, levels of dopamine or DA receptors, whereas mild mitochondrial deficits have been found (Gautier et al., 2008; Gispert et al., 2009; Kitada et al., 2007). Both *Parkin* and *Pink1* transgenic animal models exhibit similar phenotypes, including mitochondrial defects (Gautier et al., 2008; Palacino et al., 2004) suggesting interplay between these two genes.

Point mutations in the *DJ-1* gene have been associated to rare autosomal-recessive parkinsonism. Similar to the cases of *Parkin* and *Pink1*, mice knockout for *DJ-1* do not show abnormalities in DA neuron number, level of striatal dopamine, or levels of receptors. However, abnormalities in neurotransmission in the nigrostriatal circuit and mitochondrial alterations were observed in some animals (Andres-Mateos et al., 2007; Chen et al., 2005; Goldberg et al., 2005; Kim et al., 2005). Importantly, *DJ-1* mutant mice appeared more sensitive to toxins and oxidative stress. To the best of our knowledge, no transgenic mouse lines overexpressing wild-type or mutant DJ-1 have been developed. Interestingly, DJ-1 overexpression in nigrostriatal neurons of mice has been accomplished using adeno-associated vectors (Paterna et al., 2007), or by administration of the histone

Table 3: Transgenic mouse models for LRRK2-associated Parkinson's disease (From Xu et al., 2012)

Group	LRRK2 genetic construction	Motor activities and age onset	Loss of TH positive neurons in substantia nigra	Striatal dopamine neurotransmission	Phospho-tau species
1. H.L. Melrose, M.J. Farrer [18,19]	Human BAC Wt and G2019S with vector in FVB	Wt: not changed at 7 mo G2019S: not changed at 7 mo	Wt: not changed at 2 y G2019S: not changed at 2 y	Wt: ↓ without pharmacological manipulation at 8 mo G2019S: ↓ without pharmacological manipulation at 8 mo	Wt: ND G2019S: ↑ aged
2. Y. Li, C.J. Li [20]	Human BAC without pBACe3.6 vector in FVB: a. Wt, b. R1441G	Wt: not changed R1441G: ↓ at 6 mo	Wt: not changed R1441G: not changed, but had signs of ↓ neurite density and cell size at 9 mo	Wt: ND R1441G: ↓ at 12 mo	Wt: not changed R1441G: ↑ at 9 mo
3. X. Li, Z. Yue [21]	Mouse BAC FLAG-tagged LRRK2 in C57BL6: 1. Wt, 2. G2019S	Wt: hyperactive at 6 mo G2019S: not changed at 12 mo	Wt: not changed at 18 mo G2019S: not changed at 12 mo	Wt: not changed at 12 mo G2019S: ↓ at 12 mo	Wt: ↓ at 18 mo G2019S: not changed at 18 mo
4. D. Ramonet, D. Moore [22]	CMVE-PDGFβ promoter driven, Myc-tagged human LRRK2 in C57BL6J+C3H/HeJ: 1. Wt, 2. G2019S, 3. R1441C	Wt: ND G2019S: not changed at 15 mo R1441C: ↓ at 15 mo	Wt: not changed as lack of expression G2019S: ↓ at 19 mo, ↓ neurite density R1441C: not changed as lack of expression	Wt: not changed G2019S: not changed at 15 mo R1441C: not changed in striatum. ↓ in cerebral cortex at 15 mo	Wt: ND G2019S: not changed R1441C: ND
5. Y. Tong, J. Shen [23]	R1441C knockin in C57BL6+129	↓ in response to AMPH	not changed	not changed	not changed
6. Y. Tong, J. Shen [24]	Knockout promoter and exon 1 (KO1), or exon 29–30 (KO2) in C57BL6/129.	not changed at 2 y	not changed	not changed	ND, but α-Synuclein aggregation in kidney
7. E. Andres-Mateos, V.L. Dawson [25]	Knockout exon 39–40 in C57BL6.	not changed at 2 y	not changed	not changed	ND
8. L. Wang, H. Cai [26]	Human HA-tagged LRRK2 G2109S expression was suppressed by doxycycline to >90% after 4 weeks in tetO-G2109S/CaMKII-tTA in forebrain in C57BL6.	ND	ND	ND	ND
9. X. Lin, H. Cai [27]	Human HA-tagged LRRK2 in forebrain in 3 sets of lines: 1. tetO-WT/CaMKII-tTA, 2. tetO-G2019S/CaMKII-tTA, 3. tetO-KD/CaMKII-tTA Knockout exon2 in C57BL6+129.	2. tetO-G2019S/CaMKII-tTA: ↑ at 12 mo, Others: ND KO: not changed	ND	ND	ND
10. B.D. Lee, T. Dawson [28]	Herpes simplex virus (HSV) amplicon-based untagged constructs applied in C57BL6: 1. WT, 2. G2019S, 3. G2019S/D1994A (KD)	ND	At 3 weeks after HSV: WT: not changed G2019S: ↓ G2019S/D1994A (KD): not changed	ND	ND

↑, increase; ↓, decrease; AMPH, amphetamine; BAC, bacterial artificial chromosome; HA, hemagglutinin; KD in #9 group, kinase-dead by deleting kinase domain 1887–2102 aa; ND, not determined; Wt, wild type.

deacetylase inhibitor phenylbutyrate (Zhou et al., 2011). In both cases, DJ-1 overexpression was associated with increased protection against toxin-induced neurodegeneration, suggesting a protective role of this protein against toxic insults.

Parkin, *Pink1* and *DJ-1* mutant mice failed to show nigrostriatal degeneration and LB pathology. However, knockout mice for these three genes exhibited mild alterations in the nigrostriatal circuit, thus suggesting that these could be good models for early dysfunction of nigrostriatal DA system, but not to model later stages of PD.

Unfortunatelly, none of the genetic mouse models developed to date represents a good system to model PD, since they do not recapitulate key neuropathological features of this disease. One possible explanation for this failure could be that mouse DA neurons are particularly resistant to the effect of these genetic modifications. This could be due to the expression of intrinsic protective factors in the mouse strains used to generate transgenic mice. Moreover, there could be compensatory mechanisms activated during embryo development that prevent degeneration of DA neurons during the lifespan of the mouse.

It is generally accepted that PD results from the combination of genetic factors with environmental stressors. Indeed, investigation of PD-related environmental stressors in the context of genetic PD mouse models could provide evidence for the interrelation between genetic and environmental factors, and ultimately provide new, useful models of PD.

Rat genetic models

Recently, transgenic rat models reproducing monogenic PD mutations have been developed. Rat models present two main advantages when compared to mice genetic models. First, compared to mice, the rat neuronal circuitry resembles more closely that of humans. Second, rats are less prone to anxiety than mice, providing a major advantage for behavioral evaluation. Transgenic rats expressing human A53T and A30P mutated α-synuclein under the TH promoter have been developed, showing no major motor impairment, up to 25 months of age, although significant olfactory deficits were observed, reminiscent of those that can be detected in the early phases of human PD (Lelan, et al., 2011). Rats conditionally expressing G21019S LRRK2 have also been generated (Zhou et al., 2011); specifically, transgene expression was turned on in 5-month- old rats, and impaired DA uptake by DAT was observed. No signs of DA cell loss were detected in these animals. Remarkably, progressive degeneration of nigrostriatal neurons was found when neuron-specific expression of G2019S mutant LRRK2 was driven in adult rats by adenoviral vectors (Dursonchet et al., 2011). It is important to mention that constitutive overexpression of G2019S LRRK2 has no effect, suggesting the presence of compensatory mechanisms.

Drosophila and *C. elegans*

Drosophila and *C. elegans* are very popular experimental animal models for which potent genetic tools are available. Indeed, these organisms have been used to screen for drugs and genetic factors or mutations that may modify neurodegeneration. Moreover, they also offer the possibility to identify evolutionary conserved pathways. However, their main limitation is that being invertebrates, they may lack some pathways and genes present in humans, such as α-synuclein.

Overexpression of wild-type, A53T and A30P α-synuclein in *Drosophila* recapitulated many features of PD, including age-dependent selective loss of DA neurons, LB-like filamentous inclusions, and DA-responsive locomotor deficits (Feany and Bender et al., 2000). *C.elegans* overexpressing α-synuclein, in turn, exhibited loss of DA neurons, although this phenotype was not progressive, and no α-synuclein inclusions were found. It is important to note that the results from these studies should be interpreted with caution, since these two animal species do not naturally express α-synuclein.

Overexpression of *LRRK2* in *Drosophila* resulted in loss of DA neurons and age-dependent DA-responsive reductions in locomotor activity (Liu et al., 2008; Venderova et al., 2009). Similarly, overexpression of *LRRK2* in *C. elegans* induced neurodegeneration (Saha et al., 2009). Knockout of the *Parkin* gene in *Drosophila* resulted in flies with reduced lifespan, male sterility, and severe defects in both flight and climbing activities. Moreover, mutant flies exhibited muscle and sperm mitochondrial defects leading to apoptosis and cell death (Greene et al., 2003; Whitworth et al., 2005). In the case of the *PINK1* gene, *Drosophila* lacking *PINK1* showed male sterility, inability to fly, slower climbing speed, and mitochondrial degeneration leading to apoptosis in muscles (Clark et al., 2006; Park et al., 2006). Therefore, both *Parkin* and *PINK1* transgenic animal models exhibit similar phenotypes suggesting interplay between these two genes. In support of this view, it has been described that transgenic expression of Parkin ameliorated PINK1 loss-of-function phenotypes, while transgenic expression of PINK1 did not have any effect on Parkin loss-of-function animals (Clark et al., 2006; Park et al., 2006), again indicating that Parkin and PINK1 may function in a common pathway.

In *Drosophila*, several DJ-1 mutants have been generated to study the contribution of this gene to PD pathogenesis. Interestingly, *Drosophila* possesses two homologues of DJ-1: DJ-1a and DJ-1b. Double null flies for both homologues have normal number of DA neurons and lifespan, but acute knockdown of DJ-1a by RNAi led to DA and photoreceptor neurons degeneration (Yang et al., 2005).

1.4.2- Cellular models of PD

Several cell-based models of PD have been generated by a number of research groups, with a particular interest in the development of cellular models of human origin. These models have been used to investigate the pathogenic mechanisms underlying PD progression and to develop or discover new drugs for treating PD. In general terms, cell-based models allow dissecting the molecular function of genes and proteins implicated in PD and lend themselves to genetic modification, thus representing a valuable tool for elucidating the mechanisms leading to PD. Similarly, cell-based PD models have emerged as good candidates for drug discovery, as they are amenable to parallelization and enable the implementation of high-throughput screens for candidate drugs, while at the same time reducing the number of experimental animals used for such studies.

The most widely used cell line for studying neurodegeneration and neurotoxicity in the context of PD research is the human neuroblastoma cell line SH-SY5Y. This cell line was derived in 1970 from a metastatic neuroblastoma. Cells from this line show moderate levels of dopamine beta hydroxylase activity and can be differentiated to cells expressing DA markers such as TH, dopamine transporter (DAT) and VMAT2, when treated with retinoic acid or tetradecanoyl-phorbol-13-acetate (TPA) (Pahlman et al., 1990; Presgraves et al., 2004). Both neurotoxin-induced neurodegeneration and genetic modification of SH-SY5Y cells have been used in attempts to model PD. Specifically, it has been reported that

rotenone, 6-OHDA and MPP+ cause degeneration of SH-SY5Y cells, and toxin-induced models have been used to identify drugs that may prevent neurodegeneration (Watabe et al., 2004). Dopamine has been shown to enhance α-synuclein aggregation in SH-SY5Y cells transfected with α-synuclein (Yamakawa et al., 2009). Moreover, transfection of mutant G2019S LRRK2 in SH-SY5Y cells allowed identifying, for the first time, a link between autophagy and neurite retraction (Plowey et al., 2009). However, the use of SH-SY5Y cells to model PD has two important limitations, as these cells are not authentic DA neurons, and they are of cancerous origin.

Immortalized human embryonic mesencephalic cell lines have been developed as an alternative towards the goal of obtaining faithful cellular model for PD. The cell line MESC2.10 is the most widely used among several that have been generated, and was derived from ventral mesencephalic cells of an 8-week-old human embryo immortalized by ectopic v-myc overexpression in a tetracycline-dependent fashion (Lotharius et al., 2002). MESC2.10 cells can be differentiated towards a DA phenotype when treated with cyclic adenosine monophosphate (cAMP) and glial cell-derived neurotrophic factor (GDNF). Differentiated cells express DA markers such as TH and DAT (Lotharius et al., 2002). However, the differentiated cells do not retain the DA phenotype after transplantation into the 6-OHDA rat model (Paul et al., 2007), their use being therefore restricted to *in vitro* studies.

It is important to mention another immortalized human embryonic mesenchephalic cell line immortalized with v-myc (ReNcell VM NSCs) (Donato et al.,2007). This cell line could be differentiated towards TH-expressing cells of DA phenotype (Hoffrogge et al., 2006). Remarkably, this cell line was successfully used to study the function of the *PINK1* gene (Wood-Kaczmar et al., 2008).

Cybrids are hybrid cell lines generated by transferring mitochondria from platelets obtained from PD patients into neuron-based cells that lack mitochondrial DNA (mtDNA) (Ghosh et al., 1999). Since mitochondrial function was shown to be altered in PD, the generation of cybrids constituted the first attempt to model aspects of PD at the cellular level. Importantly, cybrids have been reported to spontaneously generate fibrillar and vesicular inclusions after 3-4 months in culture that showed some biochemical features of LB, such as staining for eosin, α-synuclein, and ubiquitin (Trimmer et al., 2004). Remarkably, cybrids have also been shown to suffer mitochondrial alterations, including enlarged and swollen mitochondria (Trimmer et al., 2000). Cybrids have provided important insights into the mitochondrial alterations in PD, and how they are linked to the neurodegenerative process underlying PD. Cybrids represent one of the few examples of cell-based models of PD that recapitulate key pathological features of the disease at the cellular level. Probably, their use has not been more widespread due to the technical challenges of developing and maintaining these cell lines.

1.5-Induced pluripotent stem cells

The development in 2007 of human induced pluripotent stem cells (iPSC) enabled a new generation of disease modeling strategies. Yamanaka's group described the process of induced cell reprogramming, by which human fibroblasts could be reprogrammed to a pluripotent state after ectopic expression of just four transcription factors (OCT4, SOX2, KLF4 and c-MYC) (Takahashi et al., 2007). This conversion from a somatic cell to a pluripotent state is accomplished by the transient overexpression of a few transcription factors through a process known as reprogramming (Figure 15). Subsequent studies have

demonstrated that iPSC can be generated, albeit with lower efficiency, using only three factors (*OCT4, SOX2,* and *KLF4*) (Nakagawa et al., 2008).

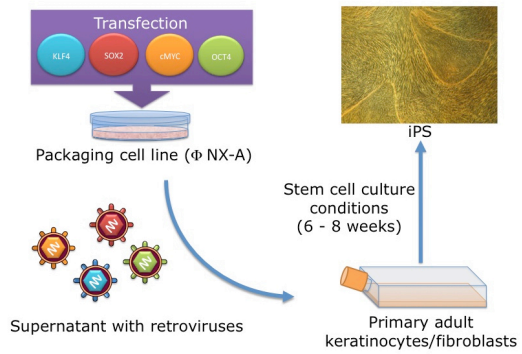

Figure 15: Schematic representation of the process of iPSC generation using retroviruses.

Importantly, it has been described that the efficiency of reprogramming could depend on the cell type employed (Aasen et al., 2008). In this study it was reported that when starting from keratinocytes, the reprogramming efficiency (1%) was significantly higher than that reported using fibroblasts (0.01%). Remarkably, the cell type of origin also determines the combination of transcription factors needed to generate iPSC; for example cord blood stem cells could be reprogrammed using only two factors (*OCT4* and *SOX2*) (Giorgetti et al., 2009, and reviewed in Stadtfeld et al., 2010). Human iPSC have very similar properties to hESC. These include similarities in their morphology, proliferation rate, gene expression profiles, and capacity to differentiate into cell types of the three embryonic germ layers *in vitro* and *in vivo* by the generation of teratomas, which are benign tumors formed after injection of hESC/iPSC into immunodeficient mice (reviewed in Stadtfeld et al., 2010)

"First-generation" iPSC were generated using retroviruses, which are efficiently silenced toward the end of reprogramming ,or lentiviruses (reviewed in Standtfelt et al., 2010). It is important to mention that the majority of reported iPSC lines have been generated using viral transduction with vectors encoding the reprogramming factors, resulting in multiple genomic integrations of the viral transgenes. While the potential for mutagenesis and tumorigenicity that result from these insertions may avoid the use of first-generation iPSC lines for cell therapy applications (Okita et al., 2007), initial proof-of-principle studies showed that they are useful for disease-modeling purposes (Raya et al., 2009; Ebert et al., 2009; Lee et al., 2009; Marchetto et al., 2010).

Importantly, strategies allowing the derivation of genetically unmodified human iPSC are emerging (reviewed in González et al., 2011), including the use of non-integrating RNA viruses or episomal vectors, the delivery of excisable lentiviral or transposon, the transduction of reprogramming proteins modified for cellular uptake , and the direct delivery of synthetic mRNAs modified to escape the endogenous antiviral cell response. It is important to mention that small molecules have been shown to increase the reprogramming efficiency, or even to replace the activity of one or more of the reprogramming factors. However, the generation of iPSC by using only small molecules has yet to be reported. Thus, the method used for deriving disease-specific iPSC lines heavily depends on their intended downstream use.

1.5.1- Modeling PD using iPSC

One of the biggest advantages of induced reprogramming is the readily generation of iPSC from patients with sporadic and familial forms of diseases. Therefore, this new cellular model allows the study of familial forms of PD and enables, for the first time, to model sporadic cases. iPSC have created powerful new opportunities for modeling human disease, screening new drugs, and also offer hope for personalized regenerative cell therapies. Multiple iPSC from patients of neurodegenerative, hematological, metabolic, cardiovascular, primary immunodeficiency and other diseases have been generated (reviewed in Robbinton and Daley, 2012). However, not in all the cases the disease was phenocopied in the iPSC-derived cells.

Modeling human disease using iPSC technology involves two steps. First, the generation of iPSC from representative patients; and second, the differentiation of patient-specific iPSC towards disease-relevant cell type(s). Hence, a critical issue for disease modeling with iPSC is the availability of reliable and reproducible protocols that could efficiently direct pluripotent stem cells towards the specific cell types affected in the disorder of interest.

iPSC technology, compared to precedent cellular models, offers the advantage to generate patient-specific iPS cells that carry the precise genetic variants, both known and unknown, that may contribute to the disease. Therefore, any cellular phenotypes observed could be correlated with clinical features such as progression and severity of the disease. Additionally, patient-specific iPSC may eventually serve as an autologous source for cell replacement therapies. The first derivation of patient-specific iPSC lines was achieved in 2008 and, since then, the number of diseases for which iPSC have been generated has grown exponentially (reviewed in Han et al., 2011). So far, the majority of iPSC lines reported in these studies have been generated using viral transduction of vectors encoding the reprogramming factors, as it is described to be the most efficient and rapid strategy.

In the case of neurological disorders, only a handful of iPSC-based models have generated convincing phenotypes, providing initial "proof of concept" for this approach: spinal muscular atrophy (Ebert et al., 2009), familial dysautonomia (Lee et al., 2009), and Rett syndrome (Marchetto et al., 2010). This could be due to several reasons. One of the most critical issues for modeling neurological diseases with iPSC is the availability of reliable and reproducible protocols that can efficiently direct pluripotent stem cells towards the specific neural cell types affected in the disorders of interest. Another important point is that neurodegenerative diseases do not become apparent until late in life. It has been postulated that neural cell types derived from pluripotent stem cells *in vitro* may represent early stages of nervous system development. Therefore, there is an urgent need to investigate if features typical from late stages of the neurodegenerative diseases could be reproduced *in vitro* using iPSC-derived cells. Finally, it is important to remark that many neurodegenerative diseases, such as PD, are thought to result from the combination of genetic factors with environmental stressors, so it is likely that diseased phenotypes could only emerge in iPSC-derived cells when they are subjected to environmental stressors.

To date, several studies have reported the generation of iPSC from patients suffering from sporadic and genetic forms PD (Table 4). The first reported generation of PD-specific iPSC was from a sporadic PD patient in 2008 (Park et al., 2008), although that study did not go beyond the generation of DA neurons from patient-specific iPSC. Over the following year, the Jaenisch's laboratory showed that iPSC derived from PD patients were

able to differentiate towards DA neurons, but no signs of neurodegeneration or disease-related phenotypes were observed in those cells (Soldner et al., 2009). Of note, the authors generated reprogramming-gene free iPSC lines from skin fibroblasts of 5 patients of idiopathic PD. Using *in vivo* experiments it was shown that DA neurons differentiated from PD-specific iPSC were able to survive and engraft in the rodent striatum for at least 12 weeks, with a small fraction of cells co-expressing TH and the G-protein-gated inwardly rectifying K+ channel subunit (GIRK2), a hallmark of A9 DA neurons (Hargus et al., 2010). Remarkably, injection of iPSC-derived DA neurons into the brains of 6-OHDA-lesioned rats resulted in improvement of the motor symptoms (Hargus et al., 2010). However, no PD-related neurodegeneration phenotypes were evident in the injected cells, raising the question as to whether relevant spontaneous phenotypes could be observed in the time frame of *in vitro* experiments or *in vivo* assays in short-lived organisms.

It has also been reported the derivation of iPSC from one patient with a triplication of the *SNCA* locus (Devine et al., 2011). The *SNCA* gene, encoding α-synuclein, was the first gene associated to familial PD (Polymeropoulos et al., 1997). Moreover, α-synuclein is the major component of LB (reviewed in Martin et al., 2011). DA neurons derived from these patient-specific iPSC produced double amount of α-synuclein transcript and protein. Again, however, no degeneration was observed in the iPSC-derived neurons. These results were independently confirmed by another group, which generated iPSC from one patient with a triplication in *SNCA* locus (Byers et al., 2012), and reported accumulation of α-synuclein, overexpression of oxidative stress markers, and increased sensitivity to peroxide-induced oxidative stress in iPSC-derived DA neurons. These combined results provide support for the usefulness of iPSC as valuable tools for studying synucleinopathies *in vitro*.

The development of methods to accelerate the emergence of diseased phenotypes *in vitro* will probably be essential for adult late-onset disorders. Cellular stressors described to favor parkinsonism, such as oxidative stress or specific neurotoxins, could be good candidates. Nguyen and colleagues derived iPSC from a patient carrying the G2019S mutation in the *LRRK2* gene (Nguyen et al., 2011). They showed that this iPSC line could be differentiated to DA neurons, and that mutant neurons were more sensitive to caspase-3 activation and cell death caused by exposure to stress agents, such as hydrogen peroxide, MG-132, and 6-hydroxydopamine, than control DA neurons; although the differences obtained were modest. Moreover, they also observed increased expression of key oxidative stress-response genes and α-synuclein protein in neurons from PD-patients compared to control iPSC or hESC. The fact that these results were based on cell lines generated from only one patient and one control cell line makes further validation warranted.

iPSC lines generated from mutations in genes associated with mitochondrial function, such as *PINK1* and *PARKIN* have also been reported. However, neurodegeneration phenotypes in iPSC-derived DA neurons have not been observed in any of these studies. The Krainc's laboratory generated iPSC from patients with three different mutations in the *PINK1* gene (Seibler et al., 2011). They showed that patient-specific iPSC lines were able to differentiate into DA neurons and that mutant neurons showed impaired recruitment of lentivirally expressed Parkin to mitochondria upon depolarization, increased mitochondrial copy number, and upregulation of PGC-1α, an important regulator of mitochondrial biogenesis. They also showed that ectopic expression of wild type PINK1 in mutant neurons corrected these alterations. Similarly, iPSC from two patients with mutations in the *PARKIN* gene have been generated (Jiang et al., 2012). In this case, iPSC derived-DA neurons showed increased spontaneous dopamine release, decreased dopamine uptake and increased ROS generation upon treatment with dopamine. Importantly, lentiviral transduction of Parkin

was able to rescue these phenotypes. Therefore, these two models represent valuable cellular models to study the mitochondrial role/involvement in PD pathogenesis.

Reference	Form of PD	Number Patients	Reprogramming strategy	Dopaminergic differentiation	PD-related phenotypes in dopaminergic neurons
Park et al	Sporadic	1	Retrovirus OSKM	Not performed	Not analyzed
Soldner et al	Sporadic	5	LV: Cre-excisable Dox-inducible: OSK and OSKM	5 % TH	No.
Nguyen et al	Genetic *LRRK2* (G2019S)	1	Retrovirus OSK	3-5% TH	Yes. Elevated α-synuclein expression and increased sensitivity to cellular stressors
Seibler et al	Genetic *PINK1* (C1366T, T509G)	3	Retrovirus OSKM	11-16% TH/TUJ1	Yes. Impairment of stress-induced mitochondrial translocation of Parkin
Davine et al	Genetic *SNCA* (triplication)	1	Retrovirus OSKM	28-37% TH/TUJ1	Yes. Doubling α-synuclein expression
Byers et al	Genetic *SNCA* (triplication	1	Retrovirus OSKM	6-11% TH+ (some A9 TH+GIRK2+)	Yes. α-synuclein accumulation, overexpression of oxidative stress markers and increased sensitivity to peroxide-induced stress
Jiang et al	Genetic *PARKIN* (deletions exon 3 and 5, deletion exon 3)	2	Lentivirus OSKMN	TH+ neurons	Yes. Reduction of dopamine uptake and increase spontaneous dopamine release. Increased ROS generation upon treatment with dopamine

OCT4(O); SOX2(S), KLF4(K); c-MYC(M), NANOG(N), Tyrosine hydroxylase (TH), b-III Tubulin (TUJ1) , G-protein-gated inwardly rectifying K+ channel subunit (Girk2) and reactive oxygen species (ROS)

Table 4: Published PD patient-specific iPSC lines

1.5.2-Direct lineage conversion approaches

A recent alternative to iPSC technology for disease modeling is the direct conversion approach. Direct lineage conversion is the conversion of one somatic cell type to another somatic cell type by using a combined expression of defined factors. To date, several studies have reported direct conversion of one cell type into another. Direct lineage conversion could provide new sources of human cells for modeling diseases, as well as for cell replacement therapies (reviewed in Vierbuchen and Wering, 2011).

Importantly, in 2010 the Wernig group described for the first time the conversion of mouse fibroblasts into cells that they called induced neurons (iN), with an efficiency of 19,5% (Vierbuchen et al., 2010). iN expressed multiple neuron-specific proteins, generated action potentials, and formed functional synapses. The authors used a combination of the transcription factors Ascl1, Brn2 and Myt11 to induce this conversion. In 2011, two independent groups provided the first proof-of principle of the conversion of human fibroblasts to dopaminergic neurons (iDA). Parmar's group achieved the conversion by the use of the three factors described by the Werning group, with the addition of two genes involved in DA neuron generation, *LMX1A* and *FOXA2* (Pfisterer et al., 2011). DA neurons obtained in this way expressed TH, Nurr1 and AADC, and generated action potentials. The efficiency of conversion in this case was reported to be around 10%. In contrast, the Broccoli's group described the conversion of human fibroblasts to DA neurons using three transcription factors: *ASCL1, NURR1* and *LMX1A* (Caiazzo et al., 2011). The DA neurons obtained by this group were able to release dopamine and showed spontaneous electrical activity. Moreover, DA neurons were able to engraft in neonatal mouse brain and retained the DA phenotype. Remarkably, they went one step further and

showed than adult fibroblasts from healthy donors, as well as from PD patients, were convertible to DA neurons with an efficiency of around 3%. In December of 2011, the Gearhart group described that iDA could also be generated from the conversion of astrocytes to DA neurons. In this case, they used a combination of three transcription factors: Ascl1, Lmx1b and Nurr1 (Addis et al., 2011).

iDA are postulated to present some advantages when compared to hESC- and iPSC-derived DA neurons. In the case of hESC, iDA would circumvent the ethical concerns related to the use of embryos needed for hESC derivation and potential issues of allogenic rejection. In addition to this, iDA would in principle reduce the risk of tumor formation after grafting into the brain, as these cells are generated in the absence of pluripotent intermediates. However, since the efficiency of iDA generation by direct conversion is low, and since both fibroblasts and iDA have limited proliferation ability, obtaining sufficient cell numbers is a key outstanding issue that currently limits the application of direct conversion strategies for disease modeling and cell therapy applications.

1.6. Generation of DA neurons from pluripotent stem cells.

Several protocols have been developed for the differentiation of pluripotent stem cells towards A9 DA neurons, the specific DA phenotype that is affected in PD (Schulz et al., 2004; Yan et al., 2005; Iacovitti et al., 2007; Cho et al., 2008; Chambers, et al., 2009; Friling et al., 2011). Importantly, most of these protocols are inspired in the microenvironment present in the developing brain during the generation of midbrain DA neurons.

1.6.1- Midbrain DA neuron development

Midbrain DA (mDA) neuron generation during embryo development could be divided in three different stages. The first stage is known as regional specification and it is characterized by the specification of neural stem/progenitor cell to mDA progenitors. Second, during early differentiation stage, mDA progenitors become mDA immature neurons. Finally, immature mDA neurons evolve into mature mDA neurons in the late maturation or late differentiation stage. Molecular markers allow the distinction of the sequential cell populations in the mDA lineage (Figure 16).

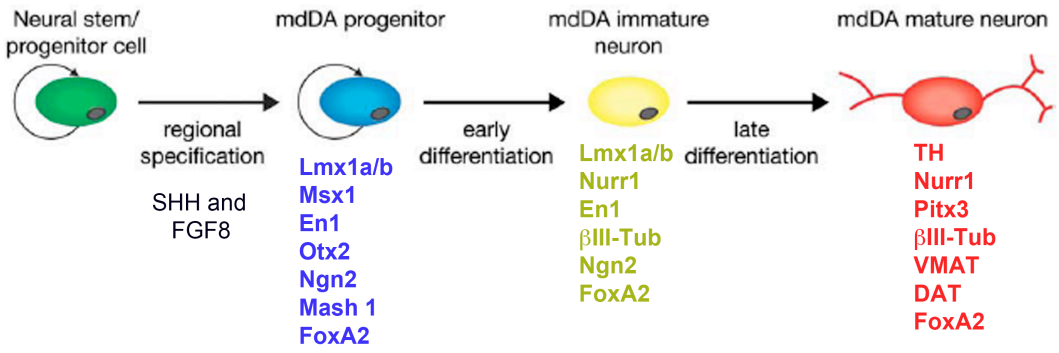

Figure 16. Stages of human midbrain dopaminergic neuron development and sequential timing of transcription factor expression during dopaminergic neuron development (Modified from Lin et al., 2009; Ang et al., 2006).

The specification of a permissive region for DA neuron generation is an essential event that occurs early in mDA neuronal development. The region where mDA neurons are born is characterized by the presence of two instructive signals: fibroblast growth factor 8 (FGF8) expressed at the midbrain-hindbrain border (isthmus) and sonic hedgehog (SHH) expressed in the ventricular zone (reviewed in Smidt and Burbach,2007). Wnt1 and Wnt5a are also expressed in this region and are essential for the formation of the midbrain (reviewed in Smidt and Burbach,2007). The presence of FGF8, SHH and Wnt1 within this area leads to the generation of mDA progenitors that express the following subset of transcription factors: LIM homeo box transcription factor one alpha and beta (LMX1a and LMX1b), MSX homoeobox 1 (MSX1), ortohdenticle homeobox 2 (OTX2), NK6 homeobox 1 (NKX6.1), Engrailed 1 (En1) and achaete-scute complex homolog 1 (MASH1). It has been described that SHH induces the expression of *LMX1a*, a crucial determinant of mDA neuron fate development (Andersson et al., 2006)(Figure 17). Andersson and colleagues showed that overexpression of *Lmx1a* in ventral midbrain of chick embryos promoted the generation of DA neurons over other neuronal cell types. This role was confirmed in mouse embryonic stem cells (mESC) overexpressing *Lmx1a*, where the authors showed higher levels of mDA neurons in overexpressing cells (Friling et al., 2009). It has also been described that Lmx1a starts a signaling cascade that leads to the activation of *Msx1a*, a transcription factor required for neuronal differentiation. Msx1a, in turn, activates the expression of *Ngn2*, a transcription factor that activates neurogenesis and inhibits the transcription factor *NKX6.1*, thus inhibiting alternate fates (Figure 17). Moreover, *LMX1A* also activates the expression of *NURR1* at early and late differentiation stages and of *PITX3* during the maturation stage, the latter controlling DA phenotype and survival (Chung et al., 2009). Additionally, SHH and FOXA2 reciprocally induce the expression of one another. FOXA2 is a transcription factor responsible for the activation of *NURR1*, an essential gene during DA neuron generation (Figure 17) (Chung et al., 2009). LMX1b belongs to the same family of LMX1a and has been shown to have some overlapping functions with LMX1a, although it is also expressed in other brain regions.

In addition, Wnt1 activates *OTX2*, a transcription factor expressed by mDA progenitors, which is required for regional and neuronal specification of mDA. It is responsible for the inhibition of *NKX2.2*, a transcription factor that induces serotonergic neural lineage and acts as a suppressor of DA lineage. Engrailed 1, a transcription factor expressed in progenitor, immature and mature DA neurons, has been described to play an important role in the generation and survival of mature DA neurons.

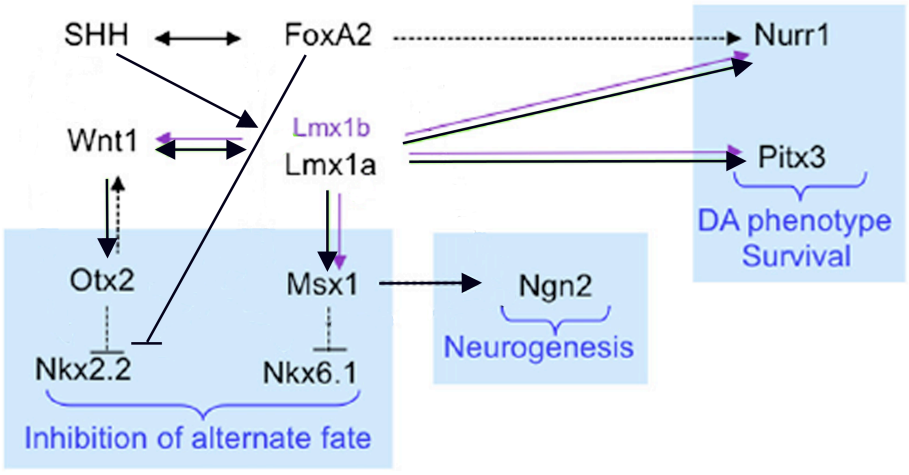

Figure 17: Signalling pathways involved in midbrain dopaminergic differentiation. Lmx1a and FoxA2 signaling pathways are crucial for (1) specification of DA fates and inhibition of alternate fates, (2) neurogenesis and (3) DA phenotype specification and survival. Dotted lines represent regulations that are not shown to be direct. Solid lines indicate regulations shown to be direct. Regulations of LMX1b are shown in purple. Modified from Chung et al., 2009.

During the early differentiation stage, immature neurons are generated that express the transcription factor *NURR1* (reviewed in Smidt and Burbach, 2007) and downregulate *SOX2* expression. These neurons start to express general neuronal markers such as ßIII-Tubulin, while continuing to express *LMa, EN1*, *NGN2* and *FOXA2*. Moreover, these neurons migrate to their specific ventral position and subsequently form VTA (A10 phenotype) and SNc (A9 phenotype) DA neuron populations.

Finally, the in the late differentiation stage, immature neurons became mature neurons and acquire a specific neurotransmitter phenotype. One of the most important genes involved in DA neuron maturation is NURR1, which regulates several proteins required for dopamine synthesis, release and regulation. Indeed, mDA neurons can be generated in the absence of NURR1, but they fail to be maintained and exhibit defects in neurotransmitter synthesis and release. NURR1 regulates the expression of *tyrosine hydroxylase* (*TH*), *vesicular monoamine transporter 2* (*VMAT2*), and *dopamine transporter* (*DAT*). Moreover, mature neurons also express *PITX3*, a transcription factor required for TH expression and for the survival of mature DA neurons (Maxwell et al., 2005).

It is important to note that mature A9 DA neurons are distinguishable from other subtypes of DA neurons because they express the *G-protein-gated inwardly rectifying K+ channel subunit* (*Girk2*).

1.6.2- Approaches pursued to differentiate pluripotent stem cells towards A9 DA neurons

The generation of pure and enriched cultures of DA neurons of A9 subtype is important to generate reliable PD cellular models, as well as to avoid adverse effects in cell replacement therapies. While many of the earlier differentiation protocols had a basic DA neuron phenotype (TH-positive cells) as target, more recent protocols tend to be designed for obtaining DA neurons of the specific A9 subtype (TH/GIRK2 double-positive neurons). It is also worth noting that the majority of the protocols described for the generation of DA neurons from pluripotent stem cells were implemented using hESC, and only recently attention has been drawn to designing protocols for both hESC and iPSC.

Published protocols for DA neuron differentiation from pluripotent stem cells differ in the culture conditions used (formation of embryoid bodies and/or co-culture), specific morphogens employed, or the use of genetic modifications in the pluripotent stem cells. Co-culture with stromal feeder cells is a system adopted by many groups. Specifically, PA6 and MS5 bone marrow-derived stromal cell lines, and primary astrocytes, have all been used to induce or support DA neuron differentiation. Zeng and colleagues cultured hES cells on PA6 stromal feeder cells for 3 weeks, resulting in 87% of the colonies showing TH-positive cells. DA neurons differentiated in this way were able to synthetize and release dopamine, and to survive in the brain of 6-OHDA-lesioned rats (Zeng et al. 2004). In contrast, Perrier and colleagues used the stromal cell line MS5 and a combination of morphogens (SHH, FGF8, BDNF, GDNF and dbcAMP) to induce DA differentiation; under these conditions, 30-50% of the differentiated cells were neurons, of which 64-79% were TH positive (Perrier et al., 2004).

The aggregation of hESC in embryoid bodies (EB) has been adopted for many laboratories (Schulz et al., 2004; Yan et al., 2005; Iacovitti et al., 2007; Cho et al., 2008). The

differentiation in EB tries to mimic the 3D environment in which DA neurons are generated during development. Interestingly, Schulz and colleagues described a relatively simple protocol that generated about 75% of TH-positive neurons out of the total neurons within the culture. Specifically, they cultured EBs for 1 month in suspension culture in serum-free media. In addition, Roy and colleagues developed a protocol combining EB formation with co-culture with immortalized human midbrain astrocytes. Basically, EBs were formed, and then fragmented and cultured to induce neural progenitors as neural rosettes. In a second step, neural rosettes were fragmented and co-cultured on the top of immortalized midbrain astrocytes, to obtain A9 DA neurons, which were GIRK2 positive. Cho and colleagues reported a highly-efficient protocol for the generation of TH-positive neurons based on the formation of neurosphere-like structures called spherical neural masses (SNM). The protocol involved the formation of EB, generation of neural rosettes and neural tube-like structures. These neural-like structures were fragmented and cultured in suspension giving rise to SNM, which could be passaged several times in suspension, frozen and thawed. These SNM when then allowed to differentiate in matrigel-coated dishes giving rise to DA neurons (Cho et al., 2008).

Several groups have tried to increase the efficiency of DA differentiation by genetically modifying hESC. The main candidates for this type of approaches have been genes involved in early specification/differentiation of mDA neurons and in cell survival. Martinat and colleagues transduced hESC with lentiviruses encoding Pitx3 and Nurr1. They observed that overexpression of these two genes increased the percentage of TH-positive cells, but the amount of neurons did not change. TH-positive cells resulted in robust behavioral improvement in PD rats (Martinat et al., 2006). Friling and colleagues showed that overexpression of Lmx1a in neural progenitor cells derived from mESC resulted in significant increases in the numbers of TH- and Girk2-positive neurons, compared to mock-transfected cells. When transplanted into the brain of neonatal rats intoxicated with 6-OHDA, TH-positive cells were able to engraft and survive. They also showed that constitutive overexpression of Lmx1a in hESC resulted in increased numbers of TH-positive neurons, although the specific subtype of DA neurons generated in this way was not addressed (Friling et al., 2009). Moreover, constitutive overexpression of Lmx1a in hESC is likely to impinge on their ability to self-renew as undifferentiated cells.

Another interesting way to enhance the efficiency of DA differentiation from pluripotent stem cells is the use of morphogens described to play a role during embryo development of mDA neurons, or modulating signaling pathways involved in neural induction and/or specification of mDA neurons. SHH and FGF8 have been described to be essential during the regional specification and generation of mDA progenitors, and therefore many published protocols use these factors (Perrier et al., 2004; Cho et al., 2008). It has also been described that antagonistic inhibition of the bone morphogenetic protein (BMP) signaling pathway enhances neural induction (Gerrard et al., 2005). Noggin, a peptide antagonist of BMP signaling, has thus been used in many protocols (Iacovitti et al., 2007; Sonntag et al., 2007). Importantly, Chambers and colleagues demonstrated that dual inhibition of BMP and TGFß signaling pathways through Noggin and SB43154 respectively, resulted in efficient neural induction (Chambers et al., 2009).

2- OBJECTIVES

1. Generation of an enriched population of nigrostriatal dopaminergic neurons from human embryonic stem cells and human induced pluripotent stem cells (iPSC).

2. Generation of nigrostriatal dopaminergic neurons, the specific subtype that degenerates in PD, from iPSC derived from PD patients carrying the G2019S *LRRK2* mutation, idiopathic PD patients, and healthy controls.

3. To develop a system for the long-term maintenance of nigrostriatal dopaminergic neurons in culture, aiming to mimic the aging process that occurs in the brain of PD patients.

4. To evaluate the appearance of PD-related neurodegeneration phenotypes in nigrostriatal dopaminergic neurons derived from PD patient-specific iPSC.

3-RESULTS

3.1- Paper 1: Efficient generation of A9 midbrain dopaminergic neurons by lentiviral delivery of LMX1A in human embryonic stem cells and induced pluripotent stem cells.

Sanchez-Danes A, Consiglio A, Richaud Y, Rodriguez-Piza I, Dehay B, Edel M, et al. Efficient generation of A9 midbrain dopaminergic neurons by lentiviral delivery of LMX1A in human embryonic stem cells and induced pluripotent stem cells. Hum Gene Ther. 2012 Jan;23(1):56-69.

3.2- Paper 2: Disease-specific phenotypes in dopamine neurons from human iPS-based models of genetic and sporadic Parkinson's disease

Sanchez-Danes A, Richaud-Patin Y, Carballo-Carbajal I, Jimenez-Delgado S, Caig C, Mora S, et al. Disease-specific phenotypes in dopamine neurons from human iPS-based models of genetic and sporadic Parkinson's disease. EMBO Mol Med. 2012 May;4(5):380-395.

Sanchez-Danes A, Richaud-Patin Y, Carballo-Carbajal I, Jimenez-Delgado S, Caig C, Mora S, et al. Disease-specific phenotypes in dopamine neurons from human iPS-based models of genetic and sporadic Parkinson's disease. Supplementary information. EMBO Mol Med. 2012 May;4(5):380-395.

4-DISCUSSION

In this thesis, we have investigated the usefulness of iPSC technology to generate novel experimental models for both genetic and sporadic forms of PD. We considered this goal of vital importance, as current animal and cellular models of PD do not faithfully recapitulate the pathology and typical progression of this disease. The first step to achieve this objective was the differentiation of pluripotent stem cells into DA neurons of the A9 subtype (Paper 1). The ultimate step was to generate a model for PD that recapitulates key pathological features of PD (Paper 2). Below, we will discuss in more detail these points.

4.1- Generation of DA neurons from human pluripotent stem cells

We considered essential to implement a protocol for the efficient generation of DA neurons of authentic A9 phenotype, as the majority of the protocols published until then had focused on the generation of DA neurons, defined as TH expressing neurons, but did not report the amount of A9 DA neurons achieved (reviewed in Hwang et al., 2010). Only one article described the generation GIRK2-positive DA neurons (Roy et al., 2006).

Our aim was to obtain an enriched population of A9 DA neurons. For this, we essentially tried to mimic the different events that occur during development for the generation of A9 DA neurons. Specifically, the aggregation of cells in EB resulted in higher DA yield, the addition of FGF2 during the neural progenitor stage enhanced the proliferation of progenitors (Maric et al., 2003), and the addition of SHH and FGF8 in the media permitted the regionalization and specification of mDA neurons (Roy et al,. 2006). Finally, the co-culture of DA progenitors with the stromal line PA6 enhanced the generation and maturation of DA neurons (Kawasaki et al., 2002). Under these culture conditions, 50% of hESC differentiated towards neurons, and around half of these neurons were DA. Importantly, this protocol was applicable both to hESC and iPSC. However, we considered necessary to improve the DA neuron yield obtained, as the values we obtained were much lower that the 86% reported by Cho and collegues (Cho et al., 2008).

For this purpose, we decided to take advantage of prior knowledge on LMX1A, a potent DA determinant during development (Andersson, et al., 2006). Indeed, it was described that transfection of the *LMX1A* gene under the control of the *Nestin* neural enhancer in mESC resulted in an increase in the numbers of TH-positive neurons generated, albeit only in the presence of SHH (Andersson, et al., 2006). Remarkably, Friling and colleagues nucleofected mESC with a vector expressing Lmx1a under the control of the *Nestin* neural enhancer and found an increase in the amount of mDA neurons obtained (Pitx3+, DAT+, TH+), up to 60-85% of the total number of neurons, ,compared to 20-30% achieved in mock-transfected cells. The authors also showed that hESC-derived neural progenitors transduced with a lentivirus expressing LMX1A from a ubiquitous promoter lead to an increase in the amount of mDA neurons, which reached 50% of all neurons in LMX1A-transduced cells, compared to 17% in mock-transduced cells. Building on this knowledge, we engineered hESC and iPSC with lentiviruses encoding LMX1A under the control of the *NESTIN* neural enhancer. Importantly, we have shown that overexpression of LMX1A in neural progenitors results in the efficient generation not only of DA neurons but also of A9-subtype DA neurons (Sánchez-Danes et al., 2012a). Specifically, our protocol achieved a high yield of DA neurons, as 80% of the neurons were TH positive, values similar to those obtained Cho and collegues (Cho et al., 2008). It is important to remark that we obtained 30% more DA neurons from hESC than Friling and collegues. These differences could be due to the fact that these authors transduced neural progenitors, rather than undifferentiated hESC as in our case, and also to the strong and ubiquitous promoter they used to drive LMX1A overexpression. Indeed, in a previous study it was reported that

strong expression of LMX1A in human midbrain progenitors resulted in a minor increase in TH-positive neurons (Roybon et al., 2008). Moreover, it has been described that *LMX1A* expression is restricted to neural and DA progenitors, and becomes downregulated as *TH* expression is activated in differentiating DA neurons in humans (Cai et al., 2009). Therefore, strong expression of *LMX1A* in a constitutive manner is likely to be detrimental for the generation of DA neurons, whereas a more physiological system such as the overexpression of *LMX1A* in neural progenitors would favor the generation of DA neurons.

Our results provide strong evidence that *LMX1A*, when expressed in neural progenitors, is a crucial transcription factor for the generation of A9 DA neurons from human pluripotent stem cells. Following our differentiation strategy, more than 60% of the neurons generated presented an A9 DA phenotype, the highest efficiency of A9 generation (GIRK2/TH double-positive neurons) from hESC described to date. Another important consideration is that DA neurons generated in in this way are able to survive, engraft and maintain a mature DA phenotype when transplanted into the brain of immunodeficient mice.

4.2- Applications of *in vitro* differentiated DA neurons

hESC and iPSC represent an unlimited source of DA neurons, since pluripotent stem cells can be expanded *in vitro* indefinitely and can be differentiated towards any cell type in the organism. hESC and iPSC-derived DA neurons have been shown to engraft and reduce motor asymmetry in parkinsonian rodents (Cho et al., 2008; Roy et al., 2006; Hargus et al. 2010; Cai et al.,2010). Transplantation experiments in rodents have highlighted the necessity of grafting differentiated DA cells devoid of undifferentiated pluripotent stem cells. Remarkably, iPSC offer important advantages over hESC, since they allow the generation of patient-specific DA neurons, thus overcoming, in principle, immune rejection issues. Nevertheless, the clinical translation of iPSC-based cell therapy applications appears as a distant goal that will require thorough pre-clinical testing of both efficacy and safety issues.

The overexpression of *LMX1A* in neural progenitor cells represents a potential strategy for the generation of DA neurons for cell replacement purposes. LMX1A has been described to play an essential role in midbrain dopaminergic neuron differentiation during development (Andersson et al., 2006). As it activates different pathways leading initially to neurogenesis, and later to the generation and maturation of DA neurons, LMX1A also inhibits the generation of other neuronal subtypes (Figure 17). Clinical translation of the pluripotent stem cells-derived DA neurons engineered with lentiviruses encoding *LMX1A* present one main limitation, which stems from the lentivirus-mediated genetic manipulation of these cells. Lentivirus vectors represent a potent tool to achieve robust expression of ectopic LMX1A in neural progenitor cells, but they could be tumorigenic has they integrate randomly into the cell genome. Therefore, a non-integrative strategy for the delivery of LMX1A, to neural progenitor cells at nearly "physiological" expression levels would need to be developed for its application to cell replacement therapies.

In the context of disease modeling, one of the most essential issues is to generate the specific cell type relevant for the disease. Hence, the protocol developed in this work represents a good starting point for the generation of a cell-based model for PD, as it establishes a new approach to generate an enriched population of A9 DA neurons. In

summary, the protocol described here could be directly applied to disease modeling of PD, but its application for cell replacement therapies would require further improvement.

4.3- Modeling PD using iPSC

The generation of iPSC from individuals suffering from genetic syndromes offers new opportunities for understanding the pathogenic mechanisms leading to the disease, as well as for screening novel therapeutic agents (reviewed in Unternaehrer and Daley, 2012). The obvious advantage of this approach is that patient-specific iPSC carry the precise genetic background that may lead to the disease. Additionally, any disease-related cell phenotype identified with this approach could be linked to the clinical manifestations and progression of the disease.

It is important to remark that not all the diseases are putative candidates for cell-based modeling through iPSC technology. Certain genetic alterations might affect the efficiency of the reprogramming process, thus making iPSC derivation more difficult or altering the nature of iPSC obtained. In some diseases, a correction of the genetic defect will be a prerequisite for the reprogramming, as it has been reported for Fanconi anemia (Raya et al, 2009). Reprogramming and derivation of iPSC requires cell division, therefore, iPSC from diseases affecting *in vitro* cell growth would be difficult to generate. As reprogramming requires epigenetic modifications, modeling epigenetic diseases could be problematic as well. Probably, the easiest diseases to model using iPSC would be highly penetrant and cell autonomous diseases. Although multigenic disorders could also be modeled, diseases heavily influenced by environmental factors would be more difficult to model. In the specific case of PD, iPSC from genetic and idiopathic PD patients have been reported (Hargus et al.,2010; Soldner et al., 2009; Nguyen et al., 2011; Seibler et al., 2011; Jiang et al., 2012), demonstrating that the reprogramming process is not altered by this disease.

Several iPSC lines have been generated from patients affected by different pathologies. However, only in some cases it has been possible to reproduce the diseased phenotype *in vitro*. It has been postulated that developmental phenotypes are easier to detect that degenerative phenotypes (Han et al., 2011). In the case of neurological diseases, diseased phenotypes have been reproduced for pediatric or early-onset diseases such as familial dysautonomia (Lee et al., 2009) or Rett syndrome (Marchetto et al., 2010). In contrast, neurodegenerative disease with late onset, such as PD, have not been reported to reproduce the diseased phenotype *in vitro* (Hargus et al., 2010; Soldner et al., 2009; Nguyen et al., 2011; Seibler et al., 2011; Jiang et al., 2012).

To our knowledge, for the first time we have been able to recapitulate the pathological features of PD, characterized by α-synuclein accumulation and DA neuron loss, in DA neurons derived from PD patients, but not in neurons from healthy controls (Sánchez-Danés et al., 2012b). We have described that the appearance of diseased phenotypes critically depends on the time-span of the cultured neurons, as we did not observed any differences in DA neuron morphology in short-term cultures. We postulate that DA neurons cultured for up to 75 days suffer from culture-related stress conditions mimicking *in vivo* aging of patients, and consequently develop PD-related phenotypes *in vitro*.

Although, DA neurons derived from G2019S LRRK2 carriers and idiopathic PD patients show a similar diseased phenotype when cultured for up to 75 days, the mechanism by which they degenerate appears to be, at least in part, different in either case. DA neurons from LRRK2-PD showed strong accumulation of α-synuclein at early time-points, thus

indicating that the pathological mechanisms have started to be active but the morphological phenotype was not present until longer time points. This observation, make us hypothesize that the altered phenotype could result from cumulative alterations of cellular processes that would start early on. These results are in line of those reported from Nyguren and collegues, who found accumulation of α-synuclein in DA neurons from a carrier of the G2019S LRRK2 mutation in neurons cultured for 60 days. However these authors did not observe loss of DA neurons, probably due to the fact that their cultures were not aged enough (Nyguren et al., 2011). Our results also show that DA neurons from idiopathic PD were similar to those of control individuals in short-term cultures, but that ID-PD DA neurons showed diseased phenotype in long-time cultures. In this case, it seems that aging-associated alterations in the cellular functions are, at least partially, responsible for this DA neuron degeneration. Consistent with our results, Soldner and collegues did not find any differences between DA neurons derived from ID-PD and from control iPSC, and hypothesized that this could be due to the short time-span of cultured neurons in their study (32-42 days) (Soldner et al., 2009).

Another important factor that could influence the appearance of diseased phenotypes in iPSC-derived DA neurons is the efficient generation of A9 DA neurons, as this DA neuron subtype has been described to be more sensitive to nitrosative, oxidative and dopamine catabolism stresses, and more prone to degeneration. We believe that our combination of LMX1A-mediated cell engineering and culture conditions allowed us to obtain an enrichment of A9 DA neurons in our cultures, compared to previous published attempts. Specifically, percentages of 10% of TH-positive neurons were reported for ID-PD iPSC (Soldner et al., 2009), and of less than 1% of A9 DA neurons in the case of LRRK2-PD iPSC (Nguyen et al., 2011).

In order to validate our model, we also investigated whether patient-specific iPSC-derived DA neurons recapitulated pathogenic mechanisms leading to PD. We decided to focus on autophagy, as many neurodegenerative diseases have been associated with defects in the autophagy compartment (Wong and Cuervo, 2010), and neurite retraction has been linked to activation of autophagy (Plowey et al., 2006). Since neurite retraction was one of the main characteristics of the diseased phenotype observed in long-term cultures, we studied the possible involvement of autophagy at that time-point. Importantly, we demonstrated that DA neurons from PD patients presented defects in autophagy clearance and found a correlation between neurite retraction and autophagy impairment in disease-specific DA neurons. These results highlight the usefulness of our iPSC-based model of PD for elucidating the pathogenic mechanisms underlying this disease. Importantly, our cell-based model of PD is not only applicable to genetic PD but also to idiopathic PD, the latter being the most common cause of PD. Thus, our results indicate that idiopathic PD is encoded in the patients' genome, at least in the cases analyzed in our study, and that multifactorial diseases of unknown origin could be modeled using iPSC.

4.4- Future goals

The generation of useful models of genetic and idiopathic PD opens the door to the elucidation of the mechanisms leading to PD. Our results are specifically relevant for idiopathic PD since, to the best of our knowledge, our study represents the first successful attempt at modeling idiopathic PD through iPSC technology. It is important to remark that sporadic PD likely represents a heterogeneous category of disease-causing mechanisms, though to result from complex interactions between gene susceptibility and environmental factors (Corti et al., 2011), and that specific interactions could be different among patients

but in all cases eventually lead to PD. Indeed, it has been postulated that sporadic PD results from the alteration of different cellular mechanisms converging in all the cases to the same result of DA neuron degeneration and loss. Therefore, being able to analyze the specific contribution of mechanisms proposed to be involved in PD pathogenesis, such as protein misfolding and aggregation, oxidative and nitrosative stress, mitochondrial dysfunction, or autophagy, in DA neurons from sporadic PD patients will surely help understanding the relative contribution of these mechanisms to PD pathogenesis. Additionally, the effect of different environmental stressors in DA neuron survival and degeneration could help elucidating the interactions between genetic and environmental factors. In the case of LRRK2-associated PD, studies comparing DA neurons from healthy individuals and carriers of the G2019S mutation would help investigating the biological function of LRRK2 and, specifically, the role of its protein kinase activity. It would be also important to identify the different protein complexes associated to LRRK2 in DA neurons and, as in the case of ID-PD, to analyze the specific contribution of the different mechanisms described to be altered in PD.

Importantly, modeling diseases using iPSC consists in two main steps: reprogramming and differentiation towards the relevant cell type. The differentiation step could be seen as mimicking the different stages of DA neuron development. So we can take advantage of this knowledge and investigate whether DA neuron differentiation or maturation is compromised in any way in cells derived from patient specific iPSC.

The protocol for differentiation and culture of DA neurons used in our work appears to be a good model for A9 DA neuron aging, which could also be applied to other PD-associated mutations. iPSC from carriers of mutations in *PINK1* and *PARKIN* have been generated and differentiated towards DA neurons but no obvious alterations has been observed in these cells (Seibler, et al 2011; Jiang et al., 2012). These patient-specific iPSC lines provide good systems to study mitochondrial dysfunction and dopamine utilization, respectively, but we believe that they would benefit from the possibility of generating aged A9 DA neurons.

The discovery of the mechanisms involved in the different sporadic and genetic cases of PD would be of vital importance to develop specific therapeutic strategies to correct the malfunction of the cellular processes individually or collectively. Therefore, it is of crucial importance to find safe and reliable drugs specific for each dysfunction. We consider that the PD model generated here, which recapitulates the key pathological features of PD, represents an invaluable platform for screening drugs that may prevent, ameliorate, or rescue neurodegeneration in PD.

The generation of DA neurons derived from PD patient-specific iPSC is of great importance, as it could allow establishing a link between the phenotype observed *in vitro* and the clinical manifestations and progression of the disease *in vivo* in the patients, both for genetic and idiopathic forms of PD. Discovering relevant associations between specific clinical features of PD patients and alterations in cellular functions such as protein misfolding and aggregation, oxidative and nitrosative stresses, mitochondrial dysfunction, or autophagy, would provide a rational basis for personalized treatments for PD patients.

5- CONCLUSIONS

1. We have implemented a protocol for the generation of an enriched population of nigrostriatal dopaminergic neurons from human embryonic stem cells and induced pluripotent stem cells (iPSC), based on the transduction of lentiviruses encoding LMX1A and specific culture conditions.

2. We have successfully generated nigrostriatal dopaminergic neurons from iPSC representing patients of idiopathic PD, of genetic PD associated to the LRRK2 G2019S mutation, and healthy controls.

3. The co-culture of iPSC-derived neural progenitor cells with cortical murine astrocytes allowed us to maintain dopaminergic neurons in culture for 2.5 months and to study aged neurons.

4. We have identified the presence of α-synuclein accumulation in dopaminergic neurons derived from carriers of the LRRK2 G2019S mutation, and neurodegeneration phenotypes in dopaminergic neurons from genetic and idiopathic PD.

5. We have identified that dopaminergic neurons from PD patients present altered autophagy, thus validating the model as a potential tool to elucidate PD pathological mechanisms.

6-LIST OF ABBREVIATIONS

5HT	Serotonin
6-OHDA	6-hydroxydopamine
AADC	L-amino acid decarboxylase
ALDH1A1	Aldehyde dehydrogenase 1
APG	Autophagosomes
APGL	Autophagolysomes
cAMP	Cyclic adenosine monophosphate
CMA	Chaperone-mediated autophagy
CRT	Cell replacement therapy
Ctrl	Healthy individuals
DA	Dopaminergic / Dopamine
DAT	Dopamine transporter
DBH	Dopamine-b-hydroxylase
DBS	deep brain stimulation
DOPAC	3,4-Dihydroxyphenylacetic acid
EB	Embryoid bodies
eIF4E	Eukaryotic initiation factor 4E
EN1	Engrailed 2
ETC	Electron transfer chain
FGF8	Fibroblast growth factor 8
GDC1	GTP cyclohydrolase 1
GDNF	Glial-derived neurotrophic factor
GIRK2	G-protein-gated inwardly rectifying K+ channel subunit
GPe	Globus pars externa
GPi	Pallidus pars interna
GTP	Guanosine triphosphate
GWAS	Genome-wide association studies
hESC	Human embryonic stem cells
HSV tk	Herpes virus thymidine kinase
ID-PD	Idiopathic Parkinson's disease
iDA	Induced dopaminergic neurons
iN	Induced neurons
iPSC	Induced pluripotent stem cell
KRS	Kufor-Rakeb syndrome
L-DOPA	L-dyhidroxypenilalanina
LB	Lewy Bodies
LC3	Light chain type 3 protein
LMX1a	LIM homeo box transcription factor one alpha
LMX1b	LIM homeo box transcription factor one beta
LRRK2	Leucine rich repeat kinase-2
LRRK2-PD	familial Parkinson's disease associated to the mutation g1029S in the LRRK2 gene
LVs	Lentiviral vectors
MAO B	Monoamine oxidase B
MASH1	Achaete-scute complex homolog 1
mDA	Midbrain dopaminergic
mESC	Mouse embryonic stem cells
MFB	Medial forebrain bundle
MOI	Multiplicity of infection

MPP+	Metabolite 1-methyl-4-phenylpyridinium
MPTP	1-Methyl-4-phenyl-1,2,3,6-tetrahydropyridine
MSX1	MSX homoeobox 1
mtDNA	Mitochondrial DNA
NesE	*NESTIN* enhancer
NKX6.1	NK6 homeobox 1
NPCs	Neural precursors cells
NTN	Neurturin
NURR1	Nuclear receptor-related 1
OMM	Outer mitochondrial membrane
OTX2	Ortohdenticle homeobox 2
PARIS	Parkin-interacting substrate
PD	Parkinson's disease
PGK	Phospho glycerate kinase
PINK1	PTEN induced putative kinase 1
RNS	Reactive nitrogen species
ROS	Reactive oxygen species
SCID	Severe combined immunodeficient
SHH	Sonic hedgehog
SN	Substantia nigra
SNCA	α-synuclein
SNpc	Substantia nigra pars compacta
SPr	Substantia nigra pars reticulate
STN	Subthalamic nucleus
T.U	Transducing units
TH	Tyrosine hydroxylase
TPA	Tetradecanoyl-phorbol-13-acetate
TUJ1	Class III-β-tubulin
UPS	Ubiquitin and proteosomal system
Vim	Ventral medial nucleus of the thalamus
VMAT2	Vesicular monoamine transporter 2
vmDAn	Ventral midbrain dopaminergic neurons

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8-ANNEXES

Articles resulted from the participation of the PhD candidate in projects related to the thesis research

8.1-Paper 3: Rem2 GTPase controls proliferation and apoptosis of neurons during embryo development.

Edel MJ, Boué S, Menchon C, Sánchez-Danés A, Izpisua Belmonte JC. Rem2 GTPase controls proliferation and apoptosis of neurons during embryo development. Cell Cycle. 2010 Sep 1;9(17):3414-22.

8.2-Paper 4: Increased dosage of tumor suppressors limits the tumorigenicity of iPS cells without affecting their pluripotency.

Menendez S, Camus S, Herreria A, Paramonov I, Morera LB, Collado M, Pekarik V, Maceda I, Edel M, Consiglio A, Sanchez A, Li H, Serrano M, Belmonte JC. Increased dosage of tumor suppressors limits the tumorigenicity of iPS cells without affecting their pluripotency.Aging Cell. 2012;11(1):41-50.

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