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INSTITUTO DE CIÊNCIAS BÁSICAS DA SAÚDE
DEPARTAMENTO DE BIOQUÍMICA PROF. TUISKON DICK
PROGRAMA DE PÓS-GRADUAÇÃO EM CIÊNCIAS BIOLÓGICAS: BIOQUÍMICA

PARKINSON'S DISEASE: EXPERIMENTAL *IN VITRO* MODEL VALIDATION AND THE POTENTIAL ROLE OF COFILIN-1 IN THE PATHOPHYSIOLOGICAL MECHANISMS

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PORTO ALEGRE, MARCH, 2017

This thesis was carried out in Laboratory of Cellular Biochemistry, Biochemistry Department Prof. Tuiskon Dick, Institute of Health Sciences, Federal University of Rio Grande do Sul, Porto Alegre, RS, Brazil and in the Institute of Pharmaceutical Science, King's College London, London, United Kingdom. It was supported by Conselho Nacional de Desenvolvimento Científico e Tecnológico (CNPq- Projeto Neurodegenerativas #466989/2014-8), Michael J Fox Foundation (Rapid Innovations Awards- #1326-2014) and Coordenação de Aperfeiçoamento de Pessoal de Nível Superior (CAPES- Programa de Doutorado Sanduíche no Exterior- PDSE/CAPES #14581-13).

I dedicate this work to my beloved parents Graça and Leonel

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“The important thing is to never stop questioning”

Albert Einstein

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PART I

ABSTRACT

The dopaminergic neurodegeneration in the *substantia nigra pars compacta (SNpc)* is responsible for the marked motor impairment observed in Parkinson's disease (PD). However, the molecular mechanisms underlying this are not completely understood. Since by the time of diagnosis, 50-70% of the dopaminergic neurons of the nigrostriatal pathway have already been degenerated, it is difficult to investigate the early-stage events of disease pathogenesis.

Due to inaccessibility of the human brain to study initial pathogenic mechanisms of the disease, experimental models have been developed in an attempt to elucidate PD etiology and its progression. Nevertheless, PD models are a controversial issue in neuroscience research since it is challenging to mimic human neuronal complexity. Therefore, the lack of optimal models that recreate disease pathology is one of the causes of failure of clinical trials that have attempted to find new/better PD therapies. Taking this in consideration, the development of more suitable models is necessary to improve our knowledge regarding PD etiological mechanisms. Additionally, the understanding of the advantages and disadvantages of models already established would also be beneficial for PD research, which our group addressed by reviewing this subject. Considering this, we chose SH-SY5Y cells as a PD model for our studies. To investigate the initial stages of PD-induced neurodegeneration, our work focused in the role of cofilin-1, a protein involved in mitochondrial dysfunction caused by oxidant-induced-apoptosis, which are two pathogenic processes strongly related to PD. Hence, in the thesis, we aimed to validate the use of retinoic-acid-(RA)-differentiated SH-SY5Y cells as an *in vitro* model and use it to investigate the potential role of cofilin-1 in the initial molecular and cellular mechanisms of PD.

Although SH-SY5Y cells are widely used in PD research, their major drawback is their lack of important neuronal features, such as low levels of proliferation and stellate morphology. On the other hand, SH-SY5Y cells can acquire a neuronal phenotype when treated with differentiation agents such as RA. Since several protocols have been described, the consequence of which may be the discrepancies observed among studies regarding neuronal and dopaminergic features. In Chapter I, we aimed to validate a RA-differentiation protocol for SH-SY5Y cells previously established by our research group, focusing upon characterization of neuronal features and its subsequent response to 6-hydroxydopamine (6-OHDA), a toxin widely used to induce dopaminergic degeneration. RA-differentiated SH-SY5Y cells have low proliferative rates, a pronounced neuronal morphology and high expression of genes related to synapse vesicle cycle, dopamine synthesis/degradation, and dopamine transporter (DAT). After exploring phenotypic differences between these two models, we verified that RA-differentiated cells were more sensitive to 6-OHDA toxicity than undifferentiated cells, which could be related to an increase of DAT immunocontent. Many lines of evidence have showed that DAT is responsible for 6-OHDA uptake *in viv*o. Once inside the neuron, 6-OHDA underwent auto-oxidation causing a significant increase in oxidative stress. However, toxin uptake is not an essential step in undifferentiated SH-SY5Y cells, as auto-oxidation occurs extracellularly. We showed here, for the first time, that RA-differentiated SH-SY5Y cells can mimic, at least in part, an important mechanism of the 6-OHDA-induced cell death found in previous *in vivo* studies. Hence, the cellular

model established by our research group presents essential neuronal features, being a suitable model for PD research.

In Chapter II, RA-differentiated SH-SY5Y cells were used as cellular model to investigate disease molecular mechanisms, focusing upon cofilin-1. Our previous data have shown that oxidation of non-phosphorylate (activated) cofilin-1 leads to mitochondrial dysfunction and cell death induced by apoptosis in tumour cells. Here we found that cofilin-1 played a role in early stages of neuronal apoptosis induced by 6-OHDA in our cellular model since cofilin-1 mitochondrial translocation precedes organelle dysfunction. Overexpression of wild type *CFL1* resulted in increased sensitivity of SH-SY5Y cells to 6-OHDA-induced neuronal cell death. Furthermore, overexpression of non-oxidizable *CFL1* containing Cys-to-Ala mutations (positions 39, 80 and 139) increased neuronal resistance to this toxin, suggesting that oxidation is an important step in 6-OHDA toxicity. Follow-up experiments were performed in order to evaluate clinically whether cofilin-1 pathway proteins content is altered in PD *post mortem* human brain. Our findings showed a significant decrease in p-cofilin-1/cofilin-1 ratio in PD patients, which indicates an increase in the amount of activated cofilin-1 available for oxidation. Moreover, through principal component analysis, the immunodetection of cofilin-1 pathway proteins were able to discriminate controls and PD individuals during the early-stage of neuropathological changings. Hence, we demonstrated, for the first time, a possible role for cofilin-1 in PD pathogenesis and its potential use as biomarker.

Taken together, our data showed that RA-differentiated SH-SY5Y cells present terminally-differentiated dopaminergic neuron features, that are essential to mimic dopaminergic neurons. By using this cellular model and *post mortem* brain tissue, we also demonstrated a possible role for cofilin-1 in early steps of the neurodegeneration process found in PD, which it could impact drug and biomarker discovery researches.

Key words: SH-SY5Y; 6-hydroxydopamine; oxidative stress; mitochondrial dysfunction and neurodegeneration.

ABBREVIATIONS

PD - Parkinson's disease

SNpc - substantia nigra pars compacta

LB - Lewy bodies

DA - dopamine

ST - *striatum*

UPDRS - Unified Parkinson's disease rating scale

MDS - Movement Disorder Society

L-DOPA - levodopa

COMT- catechol-O-methyl transferase

MAO - monoamine oxidase

α-SYN - α-synuclein

Aβ - amyloid β

UPS - ubiquitin proteasome system

ROS - reactive oxygen species

CNS - central nervous system

VDAC - voltage-dependent-anion-channel

RBD - rapid eye movement disorder

MPTP - methyl-4-phenyl-1,2,3,6-tetrahydropyridine

6-OHDA - 6-hydroxydopamine

iPSC - induced pluripotent stem cells

TH - tyrosine hydroxylase

DAT - dopamine transporter

RA - retinoic acid

BDNF - Brain-derived neurotrophic factor

AD - Alzheimer’s disease

1. INTRODUCTION

1.1 Parkinson's Disease: an overview

Parkinson's disease (PD) is a chronic progressive neurogenerative disorder, with an incidence of 8-18 per 100,000 in the total population a year, and 160 per 100.000 aged 65 years or older (ASCHERIO; SCHWARZSCHILD, 2016; HIRTZ et al., 2007). The median onset of this disorder is 60 years and the mean duration is 15 years. As this is a chronic disease with long duration, prevalence is higher than incidence, which is about 0.3 % worldwide rising to 1% in population over 60 and to 3% of those over 80 (LEE; GILBERT, 2016; PRINGSHEIM et al., 2014; "WHO | Neurological Disorders: Public Health Challenges", 2012). In Brazil, since PD reports are not compulsory, there is only an estimation of the number of the cases of this disorder, which is 200,000 (BOVOLENTA et al., 2016; "Ministério da Saúde – Portal da Saúde - www.saude.gov.br - Principal", [s.d.]). The number of people affected worldwide by the disease is expected to double in the next 20 years as life expectancy increases (BARBOSA et al., 2006; DORSEY et al., 2007).

Epidemiological studies have revealed gender-differences in PD, being more common in men than women (1.5-2:1 ratio) (LEE; GILBERT, 2016). Other risk factors associated with this disease include traumatic brain injury, occupation-related toxin exposure (*e.g.* farmers exposed to pesticides), alcohol/drug abuse (*e.g.* metamphetamine), diabetes and high cholesterol levels. On the other hand, there are a low PD-onset-risk among smokers, coffee drinkers, those taking non-steroidal-anti-inflammatory drugs and physical activity (ASCHERIO; SCHWARZSCHILD, 2016; SMITH; DAHODWALA, 2014; WIRDEFELDT et al., 2011). The risk and the inverse risk factors are summarized

in Figure 1. Although several epidemiological studies that attempt to evaluate a PD patient profile, precise risk and protective factors and its causative mechanisms remain to be elucidated.

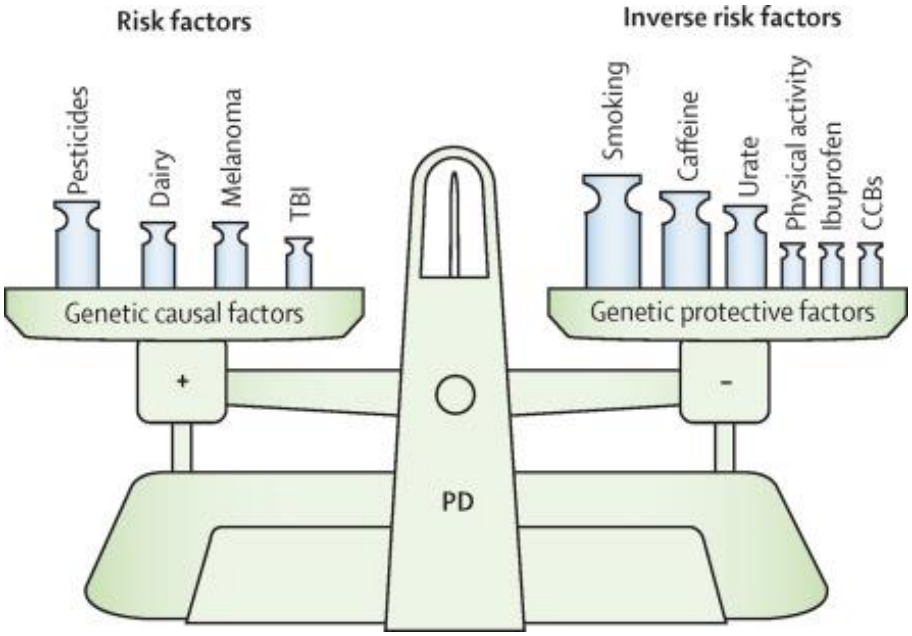

Figure 1: The balance of genetic and environmental factors that underlie PD occurrence. Larger weights have been used for those factors with stronger epidemiological evidence. TBI=traumatic brain injury. PD=Parkinson's disease. CCBs=calcium channel blockers. Image reproduced from (ASCHERIO; SCHWARZSCHILD, 2016).

PD was first described in 1817 by James Parkinson. In his monograph *An Essay on the Shaking Palsy,* he reported six cases where he observed "*an involuntary tremulous motion, with lessened muscular power; in parts not in action and even when supported; with a propensity to bend the trunk forward, and pass from a walking to a running pace"* (PARKINSON, 1817). Despite its brilliance, few physicians noticed Parkinson's work. Only fifty years later, the disease would gain wide-spread recognition. The French neurologist Jean-Martin Charcot explored Parkinson's data, where he described the clinical spectrum of

the disease. Then, he suggested to reject the earlier designation of the illness (Shaking Palsy) and he was the first one to use the term PD (GOETZ, 2011).

In 1919, more than 100 years after its discovery, Konstantin Tretiakoff examine fifty-four autopsied brains, and observed that PD subjects demonstrated damage to the *substantia nigra pars compacta* (*SNpc*) compared to non-disease controls. Moreover, he also verified that remaining neurons in this area contained small spherical structures surrounded by a clear halo. Tretiakoff named them as Lewy Bodies (LB), to acknowledge Frederick Lewy, the scientist who first described them (TRETIAKOFF, 1919).

In the early 1960's Oleh Hornykiewicz and Herbert Ehringer demonstrated that dopamine (DA) levels were decreased in the striatum (ST) of PD subjects. Later, researchers demonstrated that DA found in the ST arose from neurons projecting from the *SNpc*, compromises the nigrostriatal pathway (BERNHEIMER et al., 1973; UHL; HEDREEN; PRICE, 1985).

The dysfunction of the nigrostriatal pathway causes a marked motor impairment. The classical symptoms of PD are: tremor at rest, bradykinesia, which is the slowness of movement, rigidity, and postural instability (JANKOVIC; POEWE, 2012). As a consequence of the disease progression, motor features worsen over time and patients can develop another symptoms, for instance, freezing of gait and falls (GREFFARD et al., 2006). It has been estimated that motor symptoms develop after 50-70% of dopaminergic neuron degeneration (STOESSL, 2007).

According to United Kingdom PD Society Brain Bank – Clinical Diagnosis criteria, the identification of at least two of the classical motor symptoms is necessary for a possible diagnosis and three of them for a probable one. To

exclude motor cortical/corticospinal and cerebellar disorders, there are some exclusion features that need to be considerate, such as a history of repeated strokes, encephalitis, cerebral tumours, cerebellar signs, and neuroleptic treatment at onset of symptoms (HUGHES et al., 1992). In spite of the many efforts to improve clinical diagnosis, the confirmation of the disease only occurs after neuropathological assessment of patient brain at autopsy, where is verified the presence of moderate-to-severe neuronal loss in the *SNpc* (GELB; OLIVER; GILMAN, 1999). Due to the lack of available biomarkers, PD early diagnosis is still a challenge for neurologists.

To follow the course of disease progression, the unified Parkinson's disease rating scaled (UPDRS) was created. In 2007, the Movement Disorder Society (MDS) developed a new version of the scale termed MDS-UPDRS, which can be summarized in four parts: *i)* Non-motor signs in daily living; *ii)* motor experiences in daily living; *iii)* motor examination, and *iv)* motor complications. All items have 5 response scores varying from normal (0) to severe (4). Hence, higher scores describe an increase in disease severity (GOETZ et al., 2008).

After establishing diagnosis and severity, the management of PD begins. This decision will be based on the age of the patient, the presence of cognitive impairment, additional medical conditions and the wishes of the patient (DAVIE, 2008). Levodopa (*L*-DOPA), a DA precursor, is the gold-standard therapy for controlling PD symptoms. Also widely used are DA agonists and inhibitors of DA degradation (*e.g.* Catechol-o-methyl-transferase – COMT, monoamine oxidase - MAO inhibitors) (JANKOVIC; AGUILAR, 2008; JANKOVIC; POEWE, 2012). There are also surgical therapies (*e.g.* deep brain stimulation) for PD patients

who are experiencing decreased effects of medical DA therapy over time (KRACK et al., 2003; ZIBETTI et al., 2007) .

So far, only palliative treatments are available, and there is no cure. Although the existing therapies help during the early stages of the disease, the benefits gradually decreases and patients experience several side effects, such as on/off periods, dyskinesia and depression (FRANCO-IBORRA; VILA; PERIER, 2015). Hence, the development of new treatments for PD is fundamental.

1.2 Neuropathological alterations in PD brain

PD is associated with progressive loss of dopaminergic neurons in the *SNpc*, causing decreased DA levels in ST (GIBB, 1991), which is the main cause of the motor impairment in PD. These connections are named the nigrostriatal pathway, where the cells bodies of the neurons of the *SNpc* project their axons into the putamen and caudate nucleus, that are basal ganglia areas that composes the ST (Figure 2).

This pathway is involved in movement-influencing signals in the basal ganglia, which can be grouped into two: movement-generating direct pathway and movement-inhibiting indirect pathway. The first one promotes voluntary movements, and the second one inhibits unwanted movements. Hence, since a PD patient have decreased levels of DA in the ST, the direct pathway is inhibited and the indirect pathway hyperactivated. This explains the motor symptoms found in the disease (MILLER WC, 1988) (Figure 2).

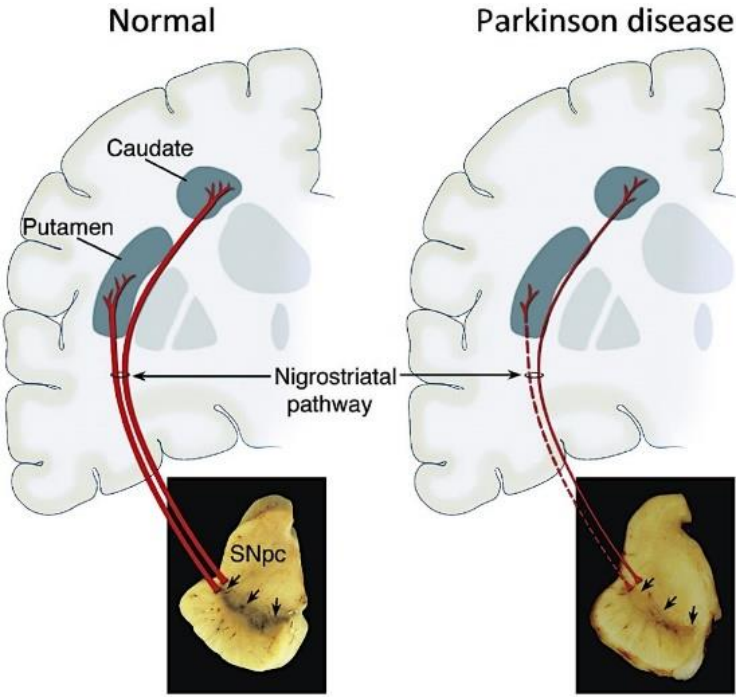

Figure 2: Schematic representation of the nigrostriatal pathway (in red). It is composed of dopaminergic neurons whose cell bodies are located in the *SNpc* (arrows). These neurons (thick solid red lines) project to the basal ganglia and synapse within the ST (*e.g.*., putamen and caudate nucleus). The photograph demonstrates the normal pigmentation of the *SNpc,* produced by neuromelanin within the dopaminergic neurons in normal subjects. Note the *SNpc* of a PD patient. The normal pigmentation fades as the dopaminergic degeneration progresses. Image reproduced from (DAUER; PRZEDBORSKI, 2003).

As mentioned above, definitive confirmation of the disease requires pathological examination at autopsy to evaluate the degree of dopaminergic degeneration in *SNpc* and the presence of LB in the remaining neurons present (BETHLEM; HARTOG; W A, 1960).

A classical LB is a globular eosinophilic inclusion of 8-30 µm in diameter in the cell body of degenerating neurons (Figure 3b, c, d). It has a condensed core surrounded by a halo of radiating fibrils. These structures are also found in the neurites named Lewy neurites (Figure 3a). There are also two other types of LB: the cortical and pale bodies. Cortical LBs do not present the obvious halo (SAKAMOTO et al., 2002) and pale LBs are rounded, pale and the eosinophilic granules lack the eosinophilic core of classic LBs (KANAZAWA et al., 2012). By

that time of their discovery, the major components of the structure were not known yet.

More than a century after their discovery, Spillantini and col. identified a strong staining in LB from idiopathic PD with antibodies for α-synuclein (α-SYN) (SPILLANTINI et al., 1997). This 140 amino acid protein is encoded by the *SNCA* gene (SPILLANTINI; DIVANE; GOEDERT, 1995) and is widely expressed in the brain tissue (JAKES; SPILLANTINI; GOEDERT, 1994). It comprises three domains: (*i*) N-terminal domain (aa 1-65), (*ii*) a non-β-amyloid (Aβ) domain (aa 66-95) and (*iii*) a C-terminal domain (aa96-140) (JAKES; SPILLANTINI; GOEDERT, 1994). It is hypothesized that its major form is either a monomer or an α-helix folded tetramer (BURRÉ et al., 2013). Lastly, this protein can be degraded by the ubiquitin-proteasome system (UPS) or autophagy-lysosomal pathway (SHI et al., 2010). Figure 3 demonstrates a typical α-SYN staining in LBs.

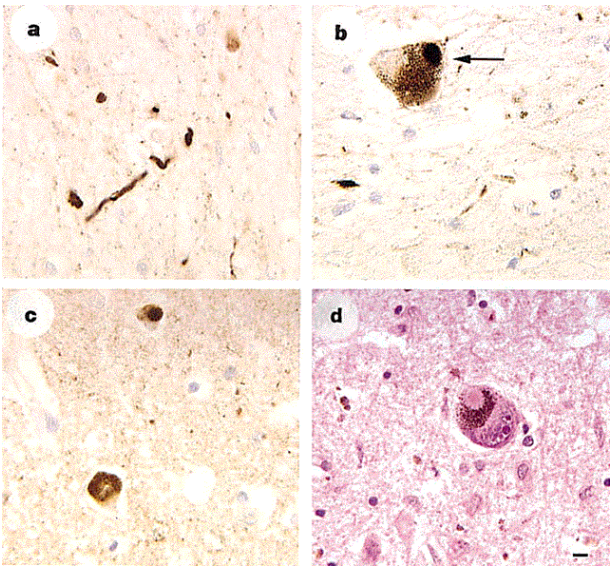

Figure 3: Representation of typical LB and Lewy neurites a) α-SYN-positive Lewy neurites in the *SNpc*. b) α-SYN-positive LB (arrow) in pigmented nerve cell of the *SNpc*. c) Two α-SYN-positive LB in the cingulate cortex. d) Hematoxylin and eosin-stained section of *SNpc* with a pigmented nerve cell containing a LB. Scale bar for a-d, 10 µm. Image reproduced from (SPILLANTINI et al., 1997).

α-SYN was first described within synaptic vesicles in the presynaptic nerve terminal (MAROTEAUX; CAMPANELLI; SCHELLER, 1988), hence it is normal physiological function is likely to be associated in neurotransmitter release (ALLEN REISH; STANDAERT, 2015). It was demonstrated that this protein modulates the stability of neuronal membrane, influences presynaptic signaling, and membrane trafficking through vesicular transport (IWAI et al., 1995; WANG; HAY, 2015). Its involvement in maintaining the SNARE complex by direct interaction with the C-terminus of synaptobrevin suggests that synaptic vesicles are likely the functional site for α-SYN where it appears to facilitate vesicle docking and fusion in the synaptic vesicle cycle (BURRÉ; SHARMA; SÜDHOF, 2014). Despite of the extensive studies, its physiological functions are poorly understood.

Mutations in the *SNCA* gene (POLYMEROPOULOS et al., 1997) and (or) dysfunction of the UPS that degraded this protein (MCNAUGHT; JENNER, 2001) can lead to protein malfunction. Monomeric α-SYN is thermodynamically unstable and it readily unfolds and subsequently polymerizes in to high-molecular weight amyloid fibrils (BALDWIN et al., 2011). Furthermore, its aggregation is possibly mediated by the hydrophobic 12 amino acid sequence in the central part of the protein in non Aβ component (GIASSON et al., 2001).

It is hypothesized that overexpression of α-SYN and decrease of its degradation results in the formation of dimers, which it may growth by the addition of monomers generating oligomers. If the process continues, small amyloid fibrils are formed, as summarized in Figure 4. Accumulation of these fibrils are found in the LB (COLLA et al., 2012; PEELAERTS; BAEKELANDT, 2016). However, the initial mechanisms regarding α-SYN aggregation are still not elucidated.

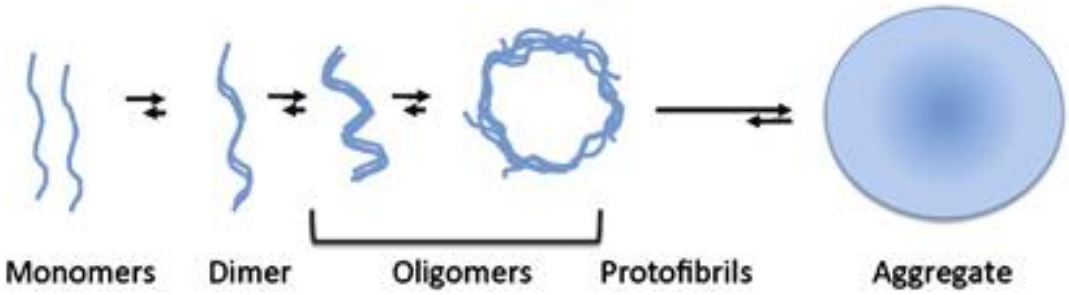

Figure 4: Schematic representation of the α-SYN aggregation process. Monomeric forms of α-SYN aggregate to form dimers and oligomers that grow into protofibrils and, finally, form mature fibrillar structures. Image reproduced from (MARQUES; OUTEIRO, 2012).

1.3 Pathogenesis

PD was not initially considered a genetic disease, as cases did not seem to have a discernible pattern or specific cause, as such it was considered for a long time a sporadic disease. However, in 1997, Polymeropulos and colleagues identified the first mutation related to PD in the *SNCA* gene (POLYMEROPOULOS et al., 1997). Since then, several genes have been associated with this disorder. These genetic discoveries have increased our understanding of cellular process that leads to dopaminergic degeneration. To date, 24 specific chromosomal *loci* in the human genome have been found to be associated with PD. All of them are termed *PARK* (to denote their putative link to PD), and numbered in chronological order of their identification (*PARK1*, *PARK2*, *PARK3*, etc.) (NALLS et al., 2014).

The PD-related genes with autosomal-dominant-inheritance are *SNCA (PARK 1 and 4)*, *LRRK2 (PARK 8)* and *VPS35 (PARK 17). PARK1* (*SNCA*) was first described in a missense mutation in an Italian family (Ala53Thr) (POLYMEROPOULOS et al., 1997). *LRRK2* is the most prevalent mutation found

in familial PD, leading to a gain of toxic function linked with an increase in kinase activity (ZIMPRICH et al., 2004). The last example of dominant inheritance is *VPS35*, where it may cause altered vesicle formation and trafficking (ZIMPRICH et al., 2011).

The recessive inheritance comprises 3 genes: *Parkin* (*PARK 2*), *DJ-1* (*PARK 7*) and *PINK1* (*PARK6*). *Parkin* and *PINK 1* mutations have been reported to be missense, nonsense or structural mutations (SHIMURA et al., 2000; VALENTE et al., 2004). Lastly, *DJ-1* was identified as a causative PD gene in two consanguineous families of Dutch and Italian origin (BONIFATI et al., 2003). These types of mutations are strongly associated with early-onset PD.

Although genetic PD cases have helped to elucidate some cellular mechanisms regarding dopaminergic degeneration, it represents just 10 % of the total number of PD cases. Several hypotheses regarding the pathogenesis of the disease (genetic and sporadic) suggest that the mechanisms underlying dopaminergic cell death and LB pathology might be the consequence of oxidative stress and mitochondrial dysfunction (FRANCO-IBORRA; VILA; PERIER, 2015).

The misbalance between reactive oxygen species (ROS) generation and antioxidants in the central nervous system (CNS) is widely known in neuroscience research. The susceptibility of neuronal cells can be explained by *i)* the high metabolic demand (brain is 2% of total body mass, however it consumes 20-30% of inspired oxygen); *ii)* high levels of polyunsaturated fatty acids and metals; and *iii)* low levels of antioxidants. These three conditions lead to a highly oxidative environment, which is prone to oxidative stress (KIM et al., 2015; SULTANA; PERLUIGI; ALLAN BUTTERFIELD, 2013).

In the dopaminergic system, the most affected system in PD, the high concentrations of both iron and DA itself in the *SNpc* contributes to oxidative stress. During DA metabolism, several ROS, such as H_2O_2, are generated and it can react with iron, forming $OH^{\cdot -}$, a potent oxidant via Fenton reaction (DEXTER et al., 1989b; ZECCA et al., 2001).

Such information leads to an investigation of oxidative stress markers in PD brains. Evidence from *post mortem* brain analyses showed an increase of oxidative damage markers. Lipid peroxidation products malondialdehyde (DEXTER et al., 1989a) and 4-hydroxynonenal (YORITAKA et al., 1996) were found to be elevated in the *SNpc*. Additionally, it has been shown that a product of nucleic acid oxidation, 8-hydroxyguanosine, is elevated in PD brain (ALAM et al., 1997). Lastly, the content of oxidized proteins (by carbonylation or nitrosylation) is significantly increased in PD (CHOI et al., 2005; MYTHRI et al., 2011; YORITAKA et al., 1996). With the respect to the antioxidant system, since levels of some peroxidases are low in control brains, glutathione play an important role in ROS detoxification within the CNS (KIM et al., 2015; MARKLUND, 1981). However, in PD brain, there is a decrease in glutathione content, which may contribute to the oxidative stress (SIAN et al., 1994a, 1994b).

Although substantial evidence points to the presence of oxidative stress in PD, it is not clear whether ROS is a primary event or a consequence of other cellular dysfunctions. The major source of the oxidative stress is the mitochondria mainly *via* the inhibition of respiration (HALLIWELL, 2006) (Figure 5 summarizes this hypothesis.). As neurons are heavily reliant on mitochondrial ATP production for the maintenance of ion gradients, axonal transport and synaptic neurotransmission, evidence suggests that dysregulation of mitochondrial

function plays a critical role in the pathogenesis of PD (CELARDO; MARTINS; GANDHI, 2014).

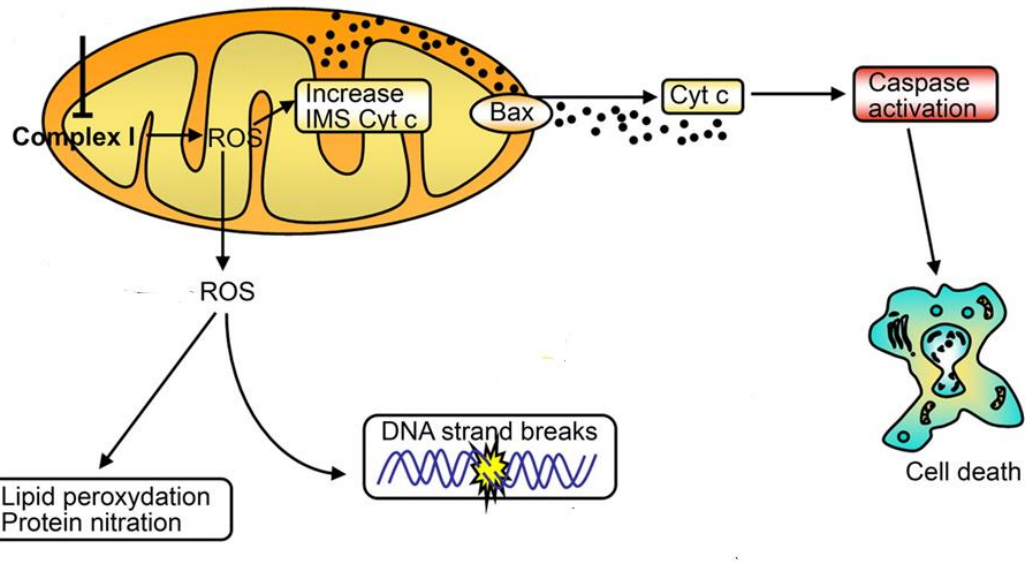

Figure 5: Mitochondrial dysfunction and oxidative stress as pathogenic mechanisms of PD. Proposed pathogenic scenario induced by complex I deficiency in which DA neuronal death results from a self-amplifying cascade of deleterious events that stat at the mitochondria with the alteration of oxidative stress phosphorylation and oxidative stress. Both processes lead to activation of the programmed cell death machinery. ROS – reactive species; Cyt c- cytochrome c. Image reproduced from (PERIER et al., 2007) with modifications.

Several lines of evidence sustain a link between PD and a dysfunctional respiratory chain, in particular a deficit in complex I activity (HATTORI et al., 1991; SCHAPIRA et al., 1990b). It was also demonstrated that catalytic subunits of complex I derived from PD frontal cortex mitochondria are oxidatively damaged, correlating with complex I dysfunction (KEENEY et al., 2006). Moreover, diminished activity in complex I has been reported in the *post mortem SNpc* other neuronal and non-neuronal regions, including cortex, skeletal muscles, fibroblasts and platelets of PD patients (PARKER; PARKS; SWERDLOW, 2008; SCHAPIRA et al., 1990a). Nevertheless, the relevance of complex I inhibition to disease pathogenesis remains uncertain.

Besides this, there are other important mitochondrial proteins misbalanced in PD, such as, the molecular chaperone prohibitin (FERRER et al., 2007), complexes II and III (MOISOI et al., 2009; PARKER; PARKS; SWERDLOW, 2008) and the outer mitochondrial membrane voltage-dependent anion channel (VDAC) (CHU et al., 2014). Lastly, it was also verified an impairment of oxidative phosphorylation in PD brains (NAVARRO et al., 2009; SCHAPIRA et al., 1989).

Although there are many lines of evidence showing the pivotal roles of oxidative stress and mitochondrial dysfunction, the upstream mechanisms still need to be elucidated.

1.4 Rebranding PD

Throughout history there have been patients who clearly displayed a wide range of symptoms completely unrelated to movement, such as cognitive impairment, sleep disorders and autonomic dysfunctions. This questioned the classic PD picture as DA-centered motor disease focused in one part of the brain (nigrostriatal pathway- basal ganglia).

Firstly, it was believed that non-motor symptoms were a late manifestation of the disease, however strong evidence has demonstrated that these features can precede the motor symptoms of PD by 20 years (GAENSLEN et al., 2011; PONT-SUNYER et al., 2015; SCHRAG et al., 2014). It is now accepted that PD has a premotor (or prodromal) stage, a period where dopaminergic degeneration has not begun but there are pathological changes (POSTUMA et al., 2012), which is summarized in Figure 6. Due to this, the MDS has been improving clinical

diagnosis by characterizing non-motor symptoms as well (POSTUMA et al., 2015).

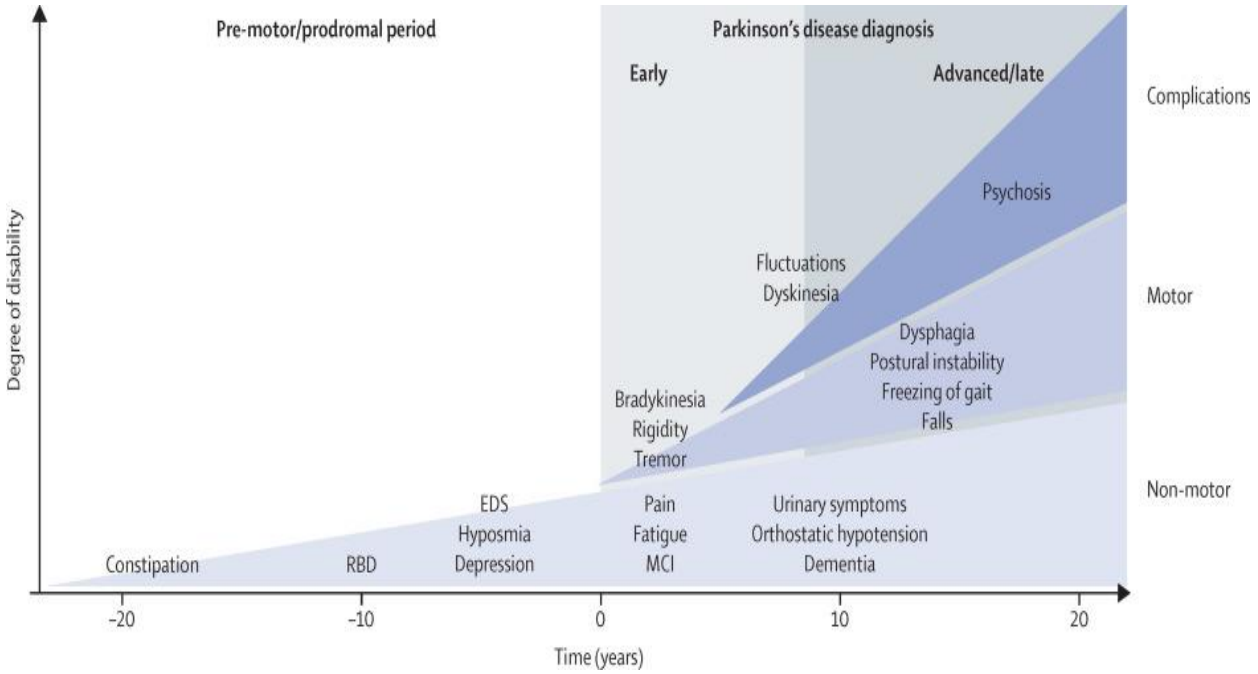

Figure 6: Time-line of PD symptoms onset. Clinical symptoms and time course of PD progression. Diagnosis of PD occurs with the onset of motor symptoms (time 0 years) but can be preceded by a premotor or prodromal phase of 20 years or more. This prodromal phase is characterised by specific non-motor symptoms. Additional non-motor features develop following diagnosis and with disease progression, causing clinically significant disability. Axial motor symptoms, such as postural instability with frequent falls and freezing of gait, tend to occur in advanced disease. Long-term complications of dopaminergic therapy, including fluctuations, dyskinesia, and psychosis, also contribute to disability. EDS=excessive daytime sleepiness. MCI=mild cognitive impairment. RBD=REM sleep behaviour disorder. Image reproduced from (KALIA; LANG, 2016).

The best characterized early non-motor features are idiopathic anosmia (olfactory dysfunction) (ROSS et al., 2008) and rapid eye movement sleep behavior disorders (RBD) (POSTUMA et al., 2009). Additionally, other non-motor clinical features has been described, such as depression, anxiety, cognitive impairment and autonomic dysfunctions (constipation and urinary difficulties) (KHOO et al., 2013; POSTUMA et al., 2012; SAVICA et al., 2009; SHIBA et al.,

2000). These symptoms usually do not respond to DA therapy, hence there is no appropriate management for them. This represents some of the greatest challenges to the quality of life for PD patients (CHAUDHURI; SCHAPIRA, 2009).

Thus the question arises -what kind of pathology can explain a long timeline prodromal stage before the onset of motor impairment?- In 2003, a German pathologist named Heiko Braak demonstrated that LB pathology was not confined to the midbrain. He demonstrated that LB and Lewy neurites are distributed in different brain areas in a manner which correlates with disease progression (BRAAK et al., 2003).

By using α-SYN immunohistochemistry, Braak undertook an anatomical autopsy investigation of 41 cases of PD, and 69 non-PD (but with incidental Lewy Bodies) and 58 age-related controls (with no PD diagnosis and no LB inclusions). Braak proposed six stages of pathology involving many areas of the brain: *stage I)* peripheral nervous system, olfactory system and medulla; *stage II)* pons (*locus ceruleus*, posterior raphe nuclei) and spinal cord grey matter; *stage III)* pons (pedunculopontine nucleus), midbrain, basal forebrain and limbic system; *stage IV)* thalamus, temporal and meso cortex; *stage V and VI)* multiple neocortical regions. Figure 7 represents this hypothesis and demonstrates that these stages have a rostrocaudal progression. With respect to the correlation with PD symptoms, stages 1 and 2 likely correspond with the onset of premotor symptoms, stage 3 corresponds to motor features, and stages 4–6 occur with the both motor and non-motor symptoms of advanced disease (BRAAK et al., 2003).

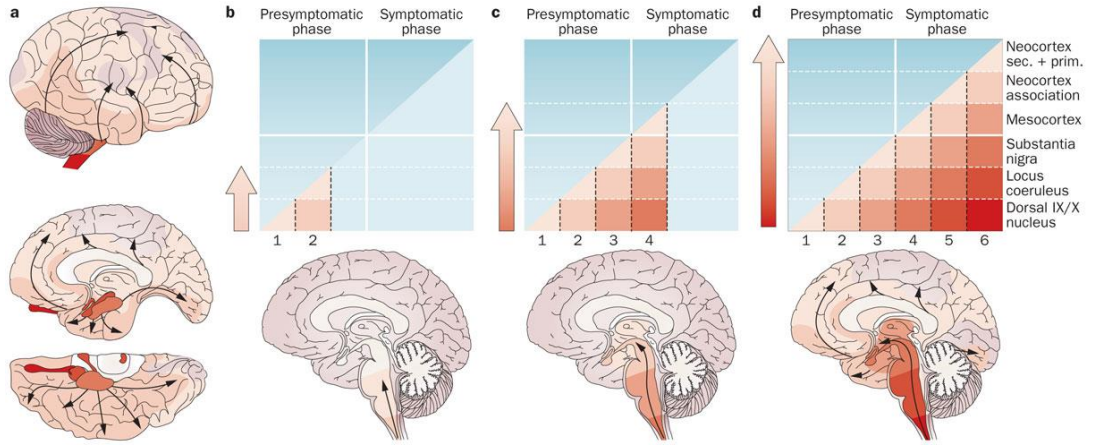

Figure 7: Braak staging and its correlation with symptoms onset. Cases with α-SYN inclusions fall into one of six groups according to the brain regions involved. Progression between groups involves additional brain areas and worsening of pathology in previously affected brain regions. a) Rostrocaudal progression of the pathological process (arrows). Variable red shading reflects the ascending disease process and increasing severity of pathology. b) Stage 1: lesions occur in the olfactory bulb, the anterior olfactory nucleus and/or the dorsal motor nuclei of the vagal and glossopharyngeal nerves in the brainstem. Stage 2: lesions are observed in the pontine tegmentum (*locus coeruleus*, magnocellular nucleus of the reticular formation, and lower raphe nuclei. c) Stages 3 and 4: lesions reach the pedunculopontine, nucleus, the cholinergic magnocellular nuclei of the basal forebrain, the pars compacta of the *SNpc* (stage 3), the hypothalamus, portions of the thalamus and, as the first cortical region, the anteromedial temporal mesocortex (stage 4). First clinical symptoms of the PD appear during stage 3 or early stage 4. d) Stages 5 and 6: lesions reach neocortical high-order association areas (stage 5), followed by first-order association areas and primary fields (stage 6). Image reproduced from (GOEDERT et al., 2012)

Recently, another study provided more evidence that PD is not just a simple motor disorder by showing that gut bacteria regulates motor dysfunction and pathophysiology of the synucleinopathies (SAMPSON et al., 2016). Here they tested whether bacteria derived from PD subjects could affect motor function using α-SYN overexpressing mouse. For that, they used fecal samples from control and PD individuals and transplanted in germ-free-animals. The authors demonstrated that PD-derived gut microbiota promoted motor deficits in mice and α-SYN aggregation, indicating that there is a specific disease dysbiosis. This data corroborates with Braak findings who showed LB pathology in vagus nerve, a long projection which connects the gut to the brain (BRAAK et al., 2003).

Hence, it has been shown that PD is not a single clinical disorder, but rather a heterogeneous group of diseases with various associated pathologies and a variable spectrum of clinical symptoms and signs ("Nature Outlook : Parkinson's disease", [s.d.]). The research progress in PD during the past 20 years not only indicates that simplistic branding of PD as a movement disorder was inappropriate but also that finding early markers of this disorder and better therapies are still a challenge.

1.5 The missing pieces of PD's puzzle: why have we failed to find a cure?

Although PD was first described 200 years ago, there are still no disease-modifying therapies. A great number of clinical trials have tested promising therapies, however none of them were successful and this is the most important unresolved issue in PD management (LANG et al., 2013; LÖHLE, 2010).

The difficulty in developing new/better therapies for this disease is attributed to lack of understanding of PD pathogenesis (ATHAUDA; FOLTYNIE, 2016; KIEBURTZ; OLANOW, 2015). In spite of the many hypotheses underlying dopaminergic degeneration and LB pathology, the precise mechanisms remain to be elucidated. Moreover, recent data demonstrated that the traditional view of PD as a simple motor disorder is out-of-date. As mentioned above in Section *1.3*, this disease cannot be considered as a simple disorder, since many brain areas are affected, which it makes it more difficult to understand PD etiology and find a suitable therapy.

Since PD affects only humans, studies into its pathogenesis are difficult to perform mainly because inaccessibility of the human brain for studies. Although

image analysis contributes to our understanding of PD, they are still very limited (MORGEN et al., 2011). To counter-act this, experimental models were developed in an attempt to elucidate the initial pathogenic mechanisms underlying PD and its progression.

Animal models are a key part for preclinical research in drug discovery (JAGMAG et al., 2015b). As we are the only species that develop neurodegenerative disorders, it is necessary to induce the dopaminergic damage and/or LB pathology in animal models using genetic or toxin-based approached. Genetic models can be produced using molecular biology techniques such as gene knockout or overexpression of PD-related genes. Toxin models use neurotoxins which accumulates in the *SNpc* causing dopaminergic damage, *e.g.* 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine (MPTP) and 6-hydroxydopamine (6-OHDA) (BLESA; PRZEDBORSKI, 2014). Even though animal models are fundamental for PD research, it is the difficult to evaluate molecular mechanisms and translate these findings to humans.

To circumvent this issue, *in vitro* models are used (ALBERIO; LOPIANO; FASANO, 2012). Here we also use genetic-or-toxin-based in cellular models as well. To mimic dopaminergic neuron environment, it is used cell lines, primary cultures, organotypic cultures and inducible pluripotent cells (iPSC). Unfortunately, to date, there is no optimal *in vitro* model that recreates precisely the dopaminergic environment and PD pathology, which can create bias in PD research (FALKENBURGER; SCHULZ, 2006).

The lack of suitable models for PD is one of the reasons of the lack of understanding of PD pathogenesis. Hence, the development of accurate models is necessary to improve our knowledge regarding PD initial etiological

mechanisms. This thesis will address this shortfall by validating a retinoic acid (RA)-induced differentiated SH-SY5Y cell line as a dopaminergic model for studying PD pathogenesis. In order to demonstrate its suitability for such research, this thesis also describes studies which use this model to evaluate one such potential pathogenic mechanism, focusing in the role cofilin-1, a mediator of mitochondrial dysfunction in oxidant-induced-apoptosis.

1.5.1 In vitro experimental model of dopaminergic cells: the use of the neuroblastoma cell line SH-SY5Y in PD research

The human neuroblastoma SH-SY5Y cell line is widely used as PD *in vitro* model because it has human origin, it expresses low levels of several dopaminergic markers, including tyrosine hydroxylase (TH), dopamine-β-hydroxylase and the dopamine transporter (DAT) (XIE; HU; LI, 2010). However, there are some significant drawbacks of using this model for PD because they are considered as immature neuroblasts that proliferate over a long period of time in contrast to terminally-differentiated neurons that have low rates of proliferation. (LUCHTMAN; SONG, 2010).

In spite of its tumoral features, SH-SY5Y cells can acquire a neuronal-like phenotype when treated with differentiation agents, such as RA, brain-derived neurotrophic factor (BDNF) (AGHOLME et al., 2010; PÅHLMAN et al., 1984). Upon RA differentiation, SH-SY5Y cells increase the levels of DA (KORECKA et al., 2013; KUME et al., 2008) and changes their morphology by increasing neurite outgrowth (CHEUNG et al., 2009; FILOGRANA et al., 2015; LOPES et al., 2010).

However, the scientific literature present several differentiation protocols, which lead to variation among findings regarding neuronal and dopaminergic features (AGHOLME et al., 2010; CHEUNG et al., 2009; CONSTANTINESCU et

al., 2007; ENCINAS et al., 2000; FILOGRANA et al., 2015; KORECKA et al., 2013; LOPES et al., 2010). Hence, the validation of the best differentiation conditions is a necessary step to use SH-SY5Y as an *in vitro* model for PD research.

1.5.2 Cofilin-1

Cofilin-1 is a highly conserved ubiquitous protein (POPE et al., 2004) that mediates actin dynamics (HOTULAINEN et al., 2005; KIUCHI et al., 2007). Due to its pivotal importance in cytoskeleton physiology, this protein has a role in important biological functions as mitosis, cellular migration and synapses formation and function (ABE et al., 1996).

Since cofilin-1 is abundant in the brain and is important in synapses regulation (HOTULAINEN et al., 2005), it is expected that this protein has essential roles in normal neuronal physiology. However, stress conditions (*e.g* oxidative stress) can lead to an excessive expression of active cofilin-1 (non-phosphorylated form) and to the formation intermolecular bonds in this protein between Cys 39 and Cys 147. This can lead to cofilin-1-actin bundles, termed “rods”, which sequesters large amount of total cofilin-1, thus rendering it incapable of promoting actin filament disassembly and severing (Figure 8) (BERNSTEIN et al., 2012; NISHIDA et al., 1987). In neurodegenerative diseases such as PD, neuronal cytoplasmic rods accumulate within neurites, where they disrupt synaptic function and are a likely cause of synaptic loss without neuronal loss (BERNSTEIN et al., 2006; MINAMIDE et al., 2000).

Moreover, cofilin-1 is also involved with neuronal apoptosis. Evidences show that amyloid β (Aβ) results in cofilin-1 oxidation, which targets this protein

to the mitochondria in hippocampal cell lines leading to organelle dysfunction and cell death (Figure 8), suggests a role for cofilin-1 in another neurodegenerative disorder: Alzheimer's disease (AD) (WOO et al., 2015a, 2015b). Furthermore, our research group established a key role for cofilin-1 in mitochondrial dysfunction and oxidant-induced apoptosis in tumour cells (KLAMT et al., 2009), two biological processes considered as the main pathological mechanisms in PD. Hence, cofilin-1 may also mediate neurodegeneration in PD.

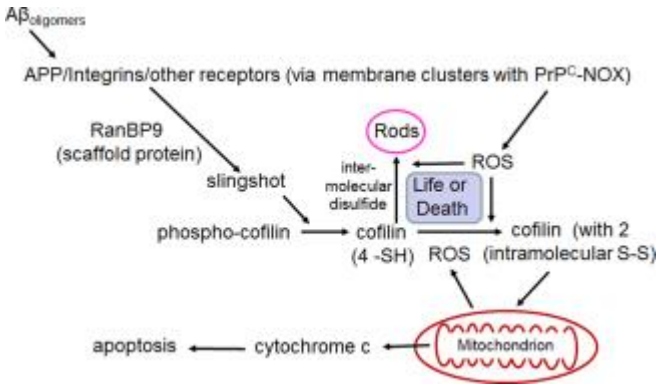

Figure 8: The role of cofilin-1 in AD. Schematic of pathway for the formation of Aβ oligomer-induced rods in neurons through the β1-integrin receptor. It is hypothesized that the formation of intermolecular disulfide bonds in cofilin-1 (leading to rod formation) in response to ROS production is competitive with cofilin-1's intramolecular oxidation, which targets it to mitochondria to trigger apoptosis. Rods are reversible if REDOX pathways can reduce the ROS levels and thus rods may form and disappear many times. It is hypothesized that eventually a large surge in ROS may trigger cofilin-1-induced apoptosis occurring later in AD progression. Image reproduced from (BAMBURG; BERNSTEIN, 2016)

2. JUSTIFICATION AND GENEREAL OBJECTIVE

To date, PD therapy is only palliative, and no disease-modifying therapies are available. This makes this disorder a socio-economic burden and a serious challenge for the public health system since the number of people affected worldwide by the disease is increasing as the life expectancy rises. The main reason of the failure of clinical trials for PD therapy is because its pathogenesis is not well elucidated, which, consequently, is attributed to the lack of reliable experimental models. Here we aimed to address this shortfall by validating RA-differentiated SH-SY5Y cells as PD experimental *in vitro* model and use it to investigate the molecular and cellular mechanisms of PD, focusing in the role of cofilin-1.

PART II

3. RESULTS

CHAPTER I

EXPERIMENTAL *IN VITRO* MODEL VALIDATION: THE USE OF RETINOIC-ACID-DIFFERENTIATED SH-SY5Y CELLS IN PARKINSON'S DISEASE RESEARCH

Article 1: *Mimicking Parkinson's disease in a plate dish: merits and pitfalls of the most used dopaminergic in vitro models.*

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Journal: NeuroMolecular Medicine

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JUSTIFICATION

The establishment of PD models is a controversial issue for neuroscience research because it is a challenge to mimic the progressive dopaminergic degeneration *in vivo* and *in vitro,* mainly because *a*nimal models rarely reproduce the full spectrum of the disease, such as symptoms, and *in vitro* models cannot mimic the central nervous system complexity.

The limitations of PD experimental models are the main cause of failure in PD studies, be they investigation of the basic pathological mechanisms or search for new therapeutic compounds. Although *in vivo* and *in vitro* models have achieved neuroprotection in drug screening research, clinical trials have shown little or none efficacy of these molecules. Since this is a major issue in neuroscience research, it is necessary not only to develop more suitable models, but also to acknowledge the features of the models that are already established.

Taking into account the great number of reviews regarding the use and the characteristics of animal models, no previous study has attempted to systematically review the literature to address the essential features necessary to establish PD *in vitro* models, which are widely used in drug screening research.

GENERAL OBJECTIVE

Highlight the importance of dopaminergic *in vitro* models and show the merits and limitations of the most used ones in order to help the researcher in choosing the most appropriated for PD studies.

SPECIFIC OBJECTIVES

- Description about how to establish a PD *in vitro* model;
- Description of dopaminergic neuronal features;
- Demonstrate the advantages and disadvantages of the most used PD *in vitro* models: primary culture, organotypic culture, cell lines and iPSC.

NeuroMolecular Medicine

Mimicking Parkinson's disease in a plate dish: merits and pitfalls of the most used dopaminergic cell in vitro models.

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Common.SubmissionDetails.Abstract:	Parkinson's disease (PD) is the second most-common neurodegenerative disorder, and has both unknown etiology and non-curative therapeutic options. Patients begin to present the classic motor symptoms of PD - tremor at rest, bradykinesia and rigidity - once 50-70% of the dopaminergic neurons of the nigrostriatal pathway have degenerated. As a consequence of this, it is difficult to investigate the early-stage events of disease pathogenesis. In vitro experimental models are used extensively in PD research because they present a controlled environment that enables the direct investigation of the early molecular mechanisms that are potentially involved with dopaminergic degeneration, as well as for the screening of potential therapeutic drugs. However, the establishment of PD in vitro models is a controversial issue for

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	neuroscience research not only because it is challenging to mimic, in isolated cell systems, neuronal physiological environment, but also the pathophysiological conditions experienced by human dopaminergic cells in situ during the progression of the disease. Since no previous study has attempted to systematically review the literature regarding the establishment of an optimal model, and the features presented by available models used in the PD field, this review aims to summarize the merits and limitations of the most used dopaminergic cell in vitro models in PD research, which may help the PD researcher to choose the most appropriate model for studies regarding the elucidation of the early-stage molecular events underlying PD initiation and progression.

Title Page

Title: Mimicking Parkinson's disease in a plate dish: merits and pitfalls of the most used dopaminergic *in vitro* models.

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Abstract

Parkinson's disease (PD) is the second most-common neurodegenerative disorder, and has both unknown etiology and non-curative therapeutic options. Patients begin to present the classic motor symptoms of PD – tremor at rest, bradykinesia and rigidity - once 50-70% of the dopaminergic neurons of the nigrostriatal pathway has degenerated. As a consequence of this, it is difficult to investigate the early-stage events of disease pathogenesis. *In vitro* experimental models are used extensively in PD research because they present a controlled environment that enables the direct investigation of the early molecular mechanisms that are potentially involved with dopaminergic degeneration, as well as for the screening of potential therapeutic drugs. However, the establishment of PD *in vitro* models is a controversial issue for neuroscience research not only because it is challenging to mimic, in isolated cell systems, neuronal physiological environment, but also the pathophysiological conditions experienced by human dopaminergic cells *in situ* during the progression of the disease. Since no previous study has attempted to systematically review the literature regarding the establishment of an optimal model, and the features presented by available models used in the PD field, this review aims to summarize the merits and pitfalls of the most used dopaminergic *in vitro* models in PD research, which may help the PD researcher to choose the most appropriate model for studies regarding the elucidation of the early-stage molecular events underlying PD initiation and progression.

Key Words: experimental models; cell lines; primary culture, organotypic culture; induced pluripotent stem cells; neurodegeneration.

Introduction

The pathological hallmark of Parkinson's disease (PD) is the degeneration of dopaminergic neurons of the *substantia nigra pars compacta* (SNpc) (Gibb, 1991) . The loss of this neuronal population results in functional imbalances in the nigrostriatal pathway, lowering the level of dopamine (DA) in the *striatum* (Gibb, 1992). This leads to a motor dysfunction and the development of the classical symptoms of PD such as bradykinesia, tremors and rigidity (Jankovic and Poewe, 2012). Along with the DA system, many lines of evidence have shown that PD involves the dysfunction of other brain areas and neurotransmitters (Braak et al., 2003). Thus, non-motor symptoms such as hallucinations, depression, sleep disorders, memory loss, anxiety and autonomic dysfunction have been identified as a part of the PD clinical phenotype (Sauerbier et al., 2015).

Even though the clinical symptoms of PD have been well characterized, early diagnosis of the disease is still a significant clinical challenge. Disease diagnosis can only be confirmed with *post-mortem* brain analysis, by the presence of intracytoplasmic and intra-axonal proteinaceous inclusions known as Lewy bodies in the remaining neurons of the SNpc (Price et al., 1978). These aggregated structures contain α-synuclein (Spillantini et al., 1997), a presynaptic neuronal protein whose function is not well understood (Burré et al., 2010).

Although the minority of PD cases are caused by mutations in specific genes (~10%) (Scott et al., 1997), the identification of genes associated with an increased risk of developing PD have contributed to the understanding of the molecular mechanisms that leads to dopaminergic degeneration (Polymeropoulos et al., 1997; Spatola and Wider, 2014). The genes related to the

genetic forms of PD are called *PARK* (Marder et al., 1996), and include both autosomal dominant (*e.g.*: *SNCA*- α-synuclein, *LRRK2*- leucine-rich repeat kinase 2,) as well as recessive (*e.g.*: *PARK2*- Parkin, *PINK1*- PTEN induced putative kinase 1, *DJ-1*- DJ-1 protein) inherited mutations (Ferreira and Massano, 2016). For sporadic forms of PD, which represent 90% of cases and for which no genetic mutations have been linked, several risk factors have been proposed, including age and environmental toxins (de Lau et al., 2005). Studies have shown that lifetime use of pesticides such as rotenone and paraquat are associated with a 2.5-fold increase in PD incidence (Tanner et al., 2011).

Although there are several studies showing risk factors for PD, the pathophysiological mechanisms of this disease are not well elucidated. *Post mortem* studies of SNpc derived from PD patients demonstrate significant (~30%) reduction in Complex I activity (the first enzyme of the mitochondrial respiratory chain responsible for the production of ATP in mitochondria) (Parker et al., 2008; Schapira et al., 1990). Another determinant of dopaminergic neuronal susceptibility in PD is oxidative stress. Neurons of the SNpc are exposed to chronic oxidative damage, mostly attributed to the high levels of iron and hydroxyl radical ($HO^{\bullet}$) produced by DA metabolism (Zhou et al., 2010). These data suggest that mitochondrial dysfunction and oxidative stress may play a role in disease pathogenesis.

Despite these evidences, the upstream molecular and cellular mechanisms underlying dopaminergic degeneration and Lewy body pathology have yet to be fully elucidated. The lack of understanding regarding the early-stages of PD is mostly related to the inaccessibility of the human brain for studies. A significant number of studies have used *post mortem* brain tissue to investigate

molecular mechanisms regarding the disease pathogenic process, but this approach can only address end-stage pathology and are not able to reveal the key stages of early disease etiology. Hence, to study its initial mechanisms and its progression, experimental PD models, both *in vivo* and *in vitro*, which reproduce both dopaminergic neuron and disease phenotype are essential (Olanow et al., 2008; Schüle et al., 2009).

Even though PD is considered a complex disorder, which many brain areas are involved, the dopaminergic degeneration is a common pathway in all PD cases, hence PD models aims to mimic this process. Animal models are suitable for investigating disease progression, because it is possible to assess motor behavior using standard behavioral tests such as rotor rod and amphetamine-induced rotational asymmetry (Lane and Dunnett, 2008; Vernon et al., 2011). However, to evaluate the molecular pathophysiological mechanisms, animal models are limited due to the difficulty in translating results to humans. On the other hand, *in vitro* models present a controlled environment that facilitate investigations of molecular and cellular pathophysiological mechanisms of dopaminergic degeneration in PD and the screening of potential therapeutics. Nonetheless, these models also present limitations, such as the difficulty in mimicking the central nervous sytem complexity *in vitro* (Freshney, 2001). Hence, not only the development of new models, but also enhance the understanding of the features of models that are already established are fundamental for PD research.

As there is a lack of understading of the initial pathogenic process in PD, many studies attempeted to investigate these mechanisms by using *in vitro* models. Since no previous study has attempted to review the literature regarding

the establishment of these experimental models, this review aims to highlight the importance of *in vitro* models in PD research and to address the essential features necessary to establish an *in vitro* model of PD. Moreover, this work discusses the merits and limitations of the most commonly-used dopaminergic *in vitro* models, in order to support the neuroscience community in choosing the most appropriate one.

How do we establish an in vitro model of PD?

To establish an *in vitro* model of PD, it is necessary to reproduce the pathological mechanisms of this disease (*e.g* dopaminergic degeneration) (Zheng et al., 2006). Hence, in this section, we will highlight important features of dopaminergic neurons that should be present in any PD *in vitro* model and also the most used models to reproduce the disease pathology.

Modeling the PD target cell: the attributes of a dopaminergic neuron

By definition, a "standard" neuron is an electrical and excitable cell, which processes and transmits information through chemical synapses. In this process, a pre-synaptic neuron releases neurotransmitter into the synaptic cleft that is adjacent to a post-synaptic neuron. Moreover, a neuron presents some distinguishing features compared to other cell types. Neurons possess dendritic processes radiating from the cell body, resulting in a stellate morphology. Another feature is that neurons do not undergo cell division and, as a result, are considered permanently post-mitotic (Kandel, 2013; Lodish et al., 2000).

Neuronal cells can be further classified according to the neurotransmitter they release. PD-target cells are mainly dopaminergic neurons in SNpc, since they synthesize and release the neurotransmitter DA. These neurons are large, have unmyelinated axons and give rise to hundreds of thousands of synapses

(each dopaminergic neuron from the SNpc innervates 6% of the dorsal striatum). Due to this, dopaminergic neurons have a high energy demand, which makes them more susceptible to impaired energy metabolism when compared to other types of neurons (Bolam and Pissadaki, 2012; Matsuda et al., 2009).

In SNpc, DA is synthesized from the aromatic amino acid tyrosine. In the first step of its synthesis, **tyrosine hydroxylase** (*TH*, E.C. 1.14.16.2*)* catalyzes the production of 3,4-dihydroxy-L-phenylalanine (*L*-DOPA) which is the rate-limiting step for catecholamine synthesis (Daubner et al., 2011). In the subsequent reaction, *L*-DOPA is converted to DA by ***L*-amino acid decarboxylase** (*DDC,* E.C. 4.1.1.28) (Lodish et al., 2000). Once DA is synthetized, it is stored in vesicles until release into the synaptic cleft by the onset of an action potential. The transport of DA from the cytosol to the membrane of presynaptic neuron occurs *via* the integral proteins **vesicular monoamine transporter (VMAT) 1** and **2** (*SLC18A1* and *SLC18A2*). Once DA is released into the synaptic cleft, it binds to DA receptors on the post-synaptic neuron (**D1, D2, D3 and D5** *DRD1, DRD2, DRD3, DRD5*) (Brichta et al., 2013; Nikolaus et al., 2007).

To prevent continuous stimulation of the postsynaptic neuron, DA must be removed from the synaptic cleft either *via* neurotransmitter reuptake by the presynaptic neuron from the synaptic or trough enzymatic degradation. The **dopamine transporter** (DAT, *SLC6A3*) is responsible for the majority of neurotransmitter reuptake back into the pre-synaptic neuron from the synaptic cleft (Ramamoorthy et al., 2011). Moreover, DA can be metabolized in the synaptic cleft by the enzymes **catechol *O*-methyl transferase** (COMT, EC 2.1.1.6) and **monoamine oxidase** (MAO, EC 1.4.3.4), which are produced by

neurons and glial cells (Lodish et al., 2000). Thus, it is fundamental that any *in vitro* dopaminergic cell model reproduces not only neuronal characteristics but also the metabolic machinery responsible for DA synthesis and the construction of the dopaminergic synapse (as illustrated in Fig. 1) (Cossette et al., 2005).

Modeling PD pathology

To mimic PD pathology, two approaches are used: genetic and toxin-based models.

Genetic Models

Although the majority of PD is sporadic, 10% of PD cases are caused by autosomal dominant or recessive mutations (Spatola and Wider, 2014). Therefore, modeling inheritable PD forms is a tool to evaluate the molecular pathways associated with alterations in the target gene (Jagmag et al., 2015).

This model is based on manipulation of PD- target genes (*PARK*), which can then be performed by either transgenic mutant or wild-type overexpression for autosomal dominant genes (*e.g.* α-synuclein) or knockout for autosomal recessive genes (*e.g. DJ-1*, *PINK-1*) in dopaminergic *in vitro* or *in vivo* models (Dawson et al., 2010). After genetic manipulation, it is evaluated some important endpoints, such as alterations in DA metabolism, alterations in synapse function and neurodegeneration.

Although genetic models helps the understanding of molecular mechanisms, transgenic *in vivo* rat and mouse models have consistently failed to reproduce the full pathological spectrum of PD, such as dopaminergic degeneration, suggesting that possession of a mutant gene is insufficient in itself

to result in the onset of PD, hence other confounding factors must also be involved (Bichler et al., 2013).

Neurotoxin Models

An extensive number of studies have shown that many toxins can mimic disease pathology by selectively damaging dopaminergic cells *in vitro* and *in vivo*, hence they have been used extensively to produce experimental models of PD (Corrigan et al., 2000; Richardson et al., 2009; Weisskopf et al., 2010). Here we will concentrate upon the two most used neurotoxins in PD research, 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine (MPTP) and 6-hydroxydopamine (6-OHDA), focusing on their features and their mechanisms of action.

MPTP

In 1979, an opioid substance abuser developed Parkinsonian symptoms due to the intravenously administration of meperidine analogues (Davis et al., 1979). Later, in 1982, Dr. William Langston diagnosed six patients with Parkinsonism caused by MPTP intoxication (Langston and Ballard, 1983). Hence, since them this toxin is used to induce dopaminergic damage.

This molecule is a highly lipophilic compound which readily crosses the blood-brain-barrier (BBB). Monoamine oxidase-B (MAOB) in glia cells converts MPTP into 1-methyl-4-phenylpyridinium ion (MPP^+) (Mizuno et al., 1987), which upon release from the glia is taken up specifically by the dopaminergic neuron *via* DAT due to its high affinity for the transporter, making it a high selective-dopaminergic-neurotoxin (Javitch and Snyder, 1984).

Previous studies have reported that DAT is fundamental to MPP^+ uptake and neurotoxicity. Incubation of cells which express high levels of DAT (*e.g*

differentiated SH-SY5Y cells) with DAT antagonists significantly reduces the toxicity of MPP^+ towards these cells (Luchtman and Song, 2010). Furthermore, the kinetics of MPP^+ uptake by differentiated SH-SY5Y cells is almost completely dependent upon DAT expression (Presgraves et al., 2004). On the other hand, cells with low DAT expression (such as undifferentiated SH-SY5Y cells) are more resistant to this neurotoxin (Constantinescu et al., 2007; Presgraves et al., 2004), suggesting that such cells are unsuitable for use as a MPP^+-mediated PD toxin *in vitro* model.

Once inside the dopaminergic neuron, MPP^+ binds to the VMAT and is sequestered into DA vesicles, however a small but significant amount is taken up by mitochondria where it inhibits complex I activity (Nicklas et al., 1987). This process impairs the flow of electrons along the respiratory chain, leading to decreased levels of ATP production and increased ROS generation due the release of electrons from the Fe-S clusters of Complex I. These combined effects are most likely responsible for initiation of cell death-related signaling pathways such as p38 mitogen-activated kinase (Karunakaran et al., 2008), c-jun-N-terminal kinase (JNK) (Saporito et al., 2000) and Bax (Vila et al., 2001).

6-OHDA

6-OHDA is a hydroxylated metabolite of DA that shares structural similarities with this neurotransmitter, such as affinity to DAT (Laverty et al., 1965). This results in high selective uptake by catecholaminergic neurons *in vivo* (Luthman et al., 1989). Once inside the neuron, 6-OHDA accumulates in the cytosol, causing neuronal damage (Van Kampen et al., 2000). However, the *in vitro* uptake of this toxin and it neurotoxic mechanisms are not well elucidated.

The *in vitro* mechanism of 6-OHDA neurotoxicity is still controversial, but it is known that the auto-oxidation of 6-OHDA produces hydrogen peroxide (H_2O_2), hydroxyl radicals and quinones that are collectively related to its cytotoxicity (Soto-Otero et al., 2000). The auto-oxidation capacity of this toxin is one of the reasons of its molecular instability. Hence, in studies using 6-OHDA, it is commonly prepared with antioxidants such as ascorbic acid, which should be produced fresh each time (Lopes et al., 2010).

Using *in vitro* models with low levels of DAT (*e.g* undifferentiated SH-SY5Y cells) and high doses of 6-OHDA (*e.g* 100 - 150 µM), toxin's auto-oxidation occurs mainly in the extracellular medium, and the oxidants generated by this process leads to cell death. On the other hand, studies using cells with high levels of DAT expression (primary cells and/or differentiated SH-SY5Y cells) showed that DAT antagonists were able to prevent cell death caused by 6-OHDA (Cerruti et al., 1993) and that oxidative stress occurs mainly in the cytosol (Lopes et al., 2017). Hence, depending on the *in vitro* model used, it is possible to mimic more accurately DAT neuroprotection, which is a phenomenon found *in vivo*.

It has also been shown that 6-OHDA can accumulate in the mitochondria, where it inhibits the enzymes of the MRC (such as complex I) resulting in the reduction in ATP synthesis and consequently cell death (Glinka et al., 1997, 1996). In this context, neuronal death is due to cytochrome *c* release and caspase-3 activation (Saito et al., 2007). Moreover, P53, PUMA, Bax and p38 protein expressions increased significantly after 6-OHDA-induced cell death. In contrast, the level of Bcl-2 in the 6-OHDA-treated group were significantly decreased *in vitro* models (*e.g.* PC12 and SH-SY5Y) (Bernstein et al., 2011; Gomez-Lazaro et al., 2008; Pišlar et al., 2014). Although there are several

research papers regarding 6-OHDA cytotoxicity, its molecular mechanisms still need to be elucidate.

In vitro models

In this section, we will discuss the most used dopaminergic *in vitro* models in the scientific literature, describing methods, merits and limitations.

Cell lines

Cell lines are derived from population of cells from a multicellular organism. These cells undergo trough immortalization process, leading to the loss of some cell cycle checkpoint pathways and normal cellular senescence. (Maqsood et al., 2013; Shay et al., 1991) (Fig. 2a).

This model is suitable for evaluation of neuroprotective compounds for PD treatment, in particular in high-throughput screening approaches. Using cell lines, it is possible to test a large panel of drugs and select the most promising ones for further *in vivo* studies, which have been responsible for the identification of the vast majority of neuroprotective compounds that have progressed into pre-clinical trials (Bal-Price et al., 2010; Radio and Mundy, 2008). Besides this, molecular biology techniques (*e.g.* gene overexpression and knockout) are used to produce genetically-modified cell lines to evaluate the role of a gene of interest, a relatively easy procedure owing to the high transfection efficiency possible with immortalized cells (Stuchbury and Münch, 2010).

To mimic PD pathology in cell lines, it is used either genetic or toxin-based models. Firstly, as described above, PD-related genes can be overexpressed or knocked out with high efficiency. Secondly, both MPP^+ and 6-OHDA can be used to induce cell death in DA-producing cell lines.

The major advantages of this model are (*i*) their indefinite proliferation that allows high-throughput experimentation using a wide variety of experimental techniques and end points; (*ii*) high reproducibility when compared to primary and organotypic cultures because these cells represent homogenous population; (*iii*) expression of enzymes for DA metabolism and synapse formation (Fig. 2a) (Lopes et al., 2010; Schildknecht et al., 2009).

On the other hand, cell lines have many disadvantages. The major drawback of the model is its oncogenic features, because neurons does not undergo mitosis (Herrup and Yang, 2007; Luchtman and Song, 2010). Immortalized cells not only do not reproduce the morphology and physiology of a neuronal cell, but also they do not express significant levels of synaptic proteins compared to primary neurons and organotypic cultures. Also, constant proliferation produces a selection pressure by which mutations which promote proliferation and survival are favored, as such successive generations of cell lines demonstrate a significant loss of the dopaminergic phenotype compared to the parental cell line from which they were produced, hence many cell lines should not be used after a significant number of passages (Chang-Liu and Woloschak, 1997) (Fig. 2a).

There are many examples of cell lines used in PD research, such as MN9D, PC12, N2A, however in this review we will discuss about the two commercially-available dopaminergic cell lines with human origin: SH-SY5Y and LUHMES (Fig 2a).

SH-SY5Y

Neuroblastomas are malignant neuroendocrine tumors that release catecholamines, where the primary sites are located in the adrenal glands (Howman-Giles et al., 2007). The human neuroblastoma SH-SY5Y, first produced in 1978, is a subclone of the neuroblastoma SK-N-SH, which in turn was derived in 1973 from a bone marrow metastasis biopsy of a 4-year old girl with neuroblastoma. These cells, when cultured, resemble sympathetic neuroblasts, with epithelial morphology and sparse cytoplasm (Biedler et al., 1978). They express low levels of several dopaminergic markers, including TH, dopamine-β-hydroxylase and DAT, which enables them to synthesize and release DA, as a result of this, SH-SY5Y cells are widely used as PD model (Cheung et al., 2009; Filograna et al., 2015; Lopes et al., 2010).

Although SH-SY5Y cells have important dopaminergic characteristics (Gomez-Lazaro et al., 2008; Iglesias-González et al., 2012), they do not present neuronal features. This cell line is in the early stages of neuronal differentiation and is biochemically characterized by low levels of neuronal markers (Gilany et al., 2008), so their oncogenic features and continuous proliferation does not mimic neuronal physiology.

SH-SY5Y cells acquire a neuron-like phenotype when treated with differentiation agents such as retinoic acid (RA), and brain-derived-neurotrophic factor (BDNF) (Agholme et al., 2010; Encinas et al., 2000; Påhlman et al., 1984). The differentiation process leads to changes in morphology and biochemical alterations resulting in a phenotype which more closely replicates that of neurons. Furthermore, once SH-SY5Y cells are differentiated, there is a decrease in

proliferation rates, an increase in dopaminergic markers expression (*e.g.* DAT) and genes responsible for synapse machinery (Lopes et al., 2017). Cellular morphology also changes from epithelial to stellate due to the formation of cytoplasmic projections (neurites) (Filograna et al., 2015; Lopes et al., 2010; Schönhofen et al., 2015).

To replicate PD pathology in this cell model, we can use both genetic- and toxin-based approaches, for which 6-OHDA is the most commonly used. The majority of the studies are related to neuroprotection analysis in cells challenged with 6-OHDA, and also the characterization of differences between undifferentiated and differentiated cells (Lin and Tsai, 2016; Wei et al., 2015). However, there are studies which have evaluated the molecular mechanisms of this neurotoxin using undifferentiated SH-SY5Y cells (Izumi et al., 2005; Soto-Otero et al., 2000). As mentioned above, 6-OHDA enters dopaminergic neurons *via* DAT and causes a significant amount of oxidative stress. As undifferentiated cells express only low levels of DAT, they do not reproduce 6-OHDA-induced-cell death mechanisms which occur *in vivo* (Lopes et al., 2017). Hence, the use of RA-differentiated cells in PD mechanisms studies may avoid the bias caused by undifferentiated SH-SY5Y cells, especially those regarding the high proliferation rates and the low immunocontent of neuronal markers.

LUHMES

Lund human mesencephalic (LUHMES) cells are a subclone of tetracycline-controlled, *v-myc*-overexpressing human mesencephalic cell line MESC 2.10, which were derived from the ventral mesencephalic brain tissue obtained of an 8 week-old fetus (Lotharius et al., 2005). Unlike SH-SY5Y cells, LUHMES need to be differentiated to acquire a dopaminergic-like phenotype.

When this cell line is maintained in its proliferative state no measurable levels of DA and dopaminergic markers (*e.g.* TH and DAT) (Scholz et al., 2011; Zhang et al., 2014) are expressed, due to the expression of the *v-myc* oncogene under control of the *Tet* element promoter, which is silenced in the presence of tetracyclin. Addition of antibiotics and neurotrophins leads to cell line differentiation.

Differentiated LUHMES cells are phenotypically very similar to primary neurons. The expression of important dopaminergic markers, such as TH, DAT, VMAT and DRD2 increases upon differentiation, as does DA release. Moreover, it exhibits electrophysiological properties concurrent with primary dopaminergic neurons, plus they have a stellate morphology (Zhang et al., 2014).

To mimic PD pathology using LUHMES cells, neurotoxin-based models are often applied (Schildknecht et al., 2009; Stępkowski et al., 2015), with MPP^+ being the most used neurotoxin. It is also possible to produce genetic models of PD, since they are readily transfected. Genetic manipulation is performed using LUHMES in their proliferative state, and, after this, the differentiation process is performed. Post-mitotic neurons are able to maintain the overexpression of the gene of interest (Schildknecht et al., 2013).

LUHMES provide significant advantages over other cell lines such as SH-SY5Y because it is derived from normal neuronal cells and not from a tumor or site of metastasis (Scholz et al., 2011). Nevertheless, there are some disadvantages of using LUHMES as a PD model. Since these cells are similar to primary neurons, they have a long doubling-time period and grow slowly compared to other cell lines such as SH-SY5Y (Thomas et al., 2013). Also,

LUHMES culture medium needs significantly more supplementation than those for standard cell lines, requiring the addition of N2 supplement and fibroblast growth factor (FGF). Lastly, the plates need to be coated with poly-L-ornithine and human fibronectin because LUHMES do not attach to regular plastic culture plates and flasks (Lotharius et al., 2005). Therefore, LUHMES cell culture is more time-consuming and costly than standard cell line models.

Primary Cultures

Primary dopaminergic cultures are usually obtained from embryonic mice. The first step is to collect intrauterine horns from a pregnant dam at a specific embryonic stage, E14 for rats and E13 for mice. Embryos are dissected to access the ventral midbrain area (VMA). Then the fetus is decapitated and the brain is dissected. Once the VMA is removed, dopaminergic cells are isolated by using enzymatic digestion following differential centrifugations, and placed in a specific culture medium. Isolated neurons rapidly differentiate and form neurites and synapses (Fig. 2b) (Weinert et al., 2015). Compared to other *in vitro* models that are usually single-cell systems, primary dopaminergic cultures have a great amount of glia cells. These models are often used in studies investigating dopaminergic cell survival, neurite retraction and cellular regeneration (Schlachetzki et al., 2013; Tönges et al., 2012).

Unless the primary culture is obtained from PD transgenic animals, the PD pathology is induced *via* toxins such as 6-OHDA and MPP^+, since it is difficult to genetically manipulate primary neurons (Halterman et al., 2009). In this model, low doses of neurotoxins are used due to the high sensitivity of primary cultures compared to cell lines (Kanthasamy et al., 2006; Rodriguez-Pallares et al., 2007). Since this model has high contamination of glia cells, the most used neurotoxin

in primary cultures is MPP^+, since it can induce specific damage to dopaminergic cells rather than glia cells (Segura-Aguilar and Kostrzewa, 2015). The toxicity of 6-OHDA *in vitro* does not appear to be restricted to dopaminergic neurons, so it can cause bias in the research (Ding et al., 2004).

Primary cultures of neurons derived by murine VMA is by far one of the best for replicating the dopaminergic neuron phenotype because it reproduces much more closely the morphology and physiology of a neuronal cell. Primary cells exhibit a stellate morphology and also express the necessary proteins found in chemical synapses. In terms of proliferation rates, they are very similar to human neurons because they do not undergo mitosis and are considered terminally differentiated neurons. In respect of dopaminergic features, this cellular model demonstrates high TH and DAT expression (Fig. 2b) (Gaven et al., 2014).

Despite these advantages, primary cultures derived from murines are not a human genome-based model. Moreover, primary cultures present significant disadvantages regarding their culture technique. Firstly, the process is very time-consuming and not easy to perform. Since the area dissected is very small, the precision of the excision is fundamental in obtaining a highly-enriched dopaminergic neuron cultures. Hence, it is necessary to have experienced researchers who can perform this technique with accuracy in order to keep experimental error to a minimum (Falkenburger and Schulz, 2006). In addition, the VMA comprises a very heterogenous cell population, therefore there is a great deal of contamination with non-dopaminergic cells. The majority of cells in an isolated primary culture are glia cells plus other types of neuronal cells, hence only approximately 5% of the cell population are TH-positive (Weinert et al.,

2015). Thus, the difficulty in isolating dopaminergic cells will likely affect the reproducibility of experiments.

Since primary cultures are very time-consuming to perform, it is not suitable for screening libraries of compounds. Lastly, it is difficult to perform genetic manipulation in these cells, mostly due to primary neurons susceptibility to the vectors used in gene transfer (Halterman et al., 2009). Although the use of lentiviruses for gene delivery circumvents these problems, the low percentage of dopaminergic neurons in the primary cultures can create a significant bias in the interpretation of data (Fig. 2b) (Weinert et al., 2015).

Organotypic cultures

Organotypic cultures are defined as an *in vitro* model that maintains *in vivo* cellular interactions and three-dimensional structure (Studer, 2001). Newborn animals (*e.g.* 2 or 3-days-old rats) are used. The first step is the removal of the brain followed by dissection of the VMA. In contrast to the production of primary cultures, the tissue is not homogenized. Instead, the VMA is dissected transversely in 110 µm slices, placed in specific culture medium and maintained for 10-12 days (Fig. 2c) (Cavaliere et al., 2010; Stoppini et al., 1991). These models are often used to study connections between cortex, SNpc and *striatum,* neurogenesis and electrophysiological analysis (Plenz and Kitai, 1996).

The most common inducer of PD pathological mechanisms used in this model is toxins, where both MPP^+ and 6-OHDA are equally used (Kearns et al., 2006; Stahl et al., 2009). Frequently, the slices are incubated in a neurotoxin bath for up to 60 minutes (acute exposure) or for a few weeks (chronic exposure). These treatment cause degeneration of neurons in both SNpc and *striatum*, leading to decreased TH and NeuN expression (Daviaud et al., 2014; Larsen et

al., 2008). Furthermore, PD can be induced by mechanical damage of the nigrostriatal pathway. Such lesions can cause α-synuclein aggregation in organotypic slice models (Cavaliere et al., 2010).

The merits of organotypic models are the maintenance of the nigrostriatal pathway, and the preservation of brain region cytoarchitecture. Since *in vivo*-like situations are conserved in this model, it enables the study of drugs/compounds that do not cross the BBB in *in vivo* models (Humpel, 2015). Another advantage is its versatility for experiments that require long-term survival, such as chronic drug administration (Daviaud et al. 2013) and electrophysiology (Plenz and Kitai, 1996). Regarding dopaminergic neuronal features, neurons in tissue slices maintain high expression of TH and NeuN markers (Fig. 2c) (Daviaud et al., 2014).

Similar to primary neuron culture, organotypic models are very difficult to reproduce, necessitating a well-trained researcher to dissect the brain precisely. Moreover, this culture is derived from animals, and thus a non-human genome-based model (Stahl et al., 2009). Finally, there are ethical concerns regarding the use of animals in the preparation of such models (Fig. 2c).

iPSC – Induced pluripotent cells

Human pluripotent stem cells have a potentially limitless capacity for self-renewal and are able to differentiate to become any cell from the human body. Since they are derived from embryonic tissue and their isolation requires the destruction of the embryo in the pre-implantation stage, ethically there is a significant amount of controversy regarding their use. To overcome this, Takamashi et al. (2007) and Yu et al. (2007) described a method for preparing

iPSC cells that have almost the same pluripotency of embryonic stem cells (ES) but without the need to destroy embryos. Furthermore, their protocol allows the establishment of iPSC from any cell regardless of the donor's genetic background and health status (Takahashi et al., 2007; Yu et al., 2007).

iPSC can be obtained by introducing 4 genes (*Oct4, Sox2, Klf4, c-Myc*) into mature human cells, such as fibroblast isolated from skin biopsies. Once iPSC cells are generated, they can undergo an *in vitro* dopaminergic differentiation using, for instance, BDNF, cAMP and GDNF (Fig. 2d). The total culture time to produce mature neurons is about 4-10 weeks (Chambers et al., 2009; Cooper et al., 2010). After the differentiation process, neuronal cells derived from iPSC have significant expression levels of TH and DAT and high levels of DA. Moreover, these neurons acquire spontaneous synaptic activities (Hartfield et al., 2014).

The replication of PD pathology in neuronal models derived from iPSC cells is often reproduced using genetic models. Fibroblasts used for the generation of iPSC cells can be derived from PD or healthy patients. In the first case, PD patients carrying disease-associated mutations are used (*e.g* α-synuclein, *LRRK*). This allows the analysis of the pathological and genetic conditions that are observed *in situ.* Previous studies have shown that mitochondria of iPSC derived from PD patients have reduced basal oxygen consumption, altered mobility and are more vulnerable to the toxicity of pesticides (*e.g* paraquat) (Cooper et al., 2010; Ryan et al., 2013). In healthy patients, genome editing is necessary (*e.g.* overexpression or knockout of PD-related genes) (Imaizumi and Okano, 2014) or toxin treatment, but to a lesser extent (Hartfield et al., 2014), to mimic PD pathology.

Many lines of evidence demonstrate that this model is the gold-standard PD *in vitro* model, because not only does it replicate more accurately the dopaminergic neuron physiology, but also because of the possibility of using cells derived from PD patients (Jiang et al., 2012; Pu et al., 2012; Sánchez-Danés et al., 2012; Seibler et al., 2011). However, there are some disadvantages of using neuronal cell models derived from iPSC. The first one is the difficulty in performing the technique, which requires experienced researchers. Secondly, iPSC isolation and *in vitro* neuronal maturation is very time-consuming because the duration of culture lasts from 4 to 10 weeks. Thirdly, patients are required to provide samples (*e.g* fibroblasts), requiring written consent and ethics commission approval (Fig. 2d).

iPSC is also still a classical two-dimensional (2D) cell culture model. Therefore, the interpretation of cellular responses may be biased by the lack of cell-cell/cell-matrix interactions and cell morphology not present *in vivo*. However, 3D cell culture using iPSC-derived neural progenitor cells (NPCs) can overcome these drawbacks (Tieng et al., 2014), as they reproduce more accurately some *in vivo* developmental events (Lancaster and Knoblich 2014a). These structures, also called cerebral organoids, develop a variety of regional identities which are organized into domains capable of influencing one another and cerebral cortex regions that are similar to the developing human brain at early stages Lancaster et al. 2013;Lancaster and Knoblich 2014b). Although NPCs have yet to be used extensively in PD research, it is likely to be a useful model in the future for the investigation of early events in this disease (Tieng et al., 2014).

Conclusions

PD is still an incurable disease, with current treatments being palliative and only addressing the symptoms. Hence, to date, there is no disease-modifying therapies, which it can stop or slow PD progression. This is mainly attributed to the lack of understanding the pathogenesis underlying dopaminergic degeneration. A great amount of research data about PD pathophysiological mechanisms came from *in vitro* experimental models, which provide evidences and hypothesis about the initial steps of PD pathology. In spite of the importance of these *mo*dels, one of the causes of the lacking of understanding PD pathogenesis is associated with the difficulty in mimicking dopaminergic cells *in vitro*, which may cause bias in PD research. Here we discussed that the most commonly-used models present both significant advantages and disadvantages, and there is still no optimal *in vitro* model available. We also have highlighted the most important of features of the main *in vitro* models used in PD studies, which may help the researcher to choose the most appropriate model for elucidating the early-stage molecular events underlying PD initiation and progression (Fig. 2). This may contribute not only to the understanding of this disease, but also to find drug targets for a curative therapy.

Conflict of interests

The authors declare that they have no competing interests.

References

Agholme, L., Lindström, T., Kågedal, K., Marcusson, J., Hallbeck, M., 2010. An in vitro model for neuroscience: differentiation of SH-SY5Y cells into cells with morphological and biochemical characteristics of mature neurons. J. Alzheimers. Dis. 20, 1069–82. doi:10.3233/JAD-2010-091363

Bal-Price, A.K., Hogberg, H.T., Buzanska, L., Coecke, S., 2010. Relevance of in vitro neurotoxicity testing for regulatory requirements: challenges to be considered. Neurotoxicol. Teratol. 32, 36–41. doi:10.1016/j.ntt.2008.12.003

Bernstein, A.I., Garrison, S.P., Zambetti, G.P., O'Malley, K.L., 2011. 6-OHDA generated ROS induces DNA damage and p53- and PUMA-dependent cell death. Mol. Neurodegener. 6, 2. doi:10.1186/1750-1326-6-2

Bichler, Z., Lim, H.C., Zeng, L., Tan, E.K., 2013. Non-motor and motor features in LRRK2 transgenic mice. PLoS One 8, e70249. doi:10.1371/journal.pone.0070249

Biedler, J.L., Roffler-Tarlov, S., Schachner, M., Freedman, L.S., 1978. Multiple neurotransmitter synthesis by human neuroblastoma cell lines and clones. Cancer Res. 38, 3751–7.

Bolam, J.P., Pissadaki, E.K., 2012. Living on the edge with too many mouths to feed: why dopamine neurons die. Mov. Disord. 27, 1478–83. doi:10.1002/mds.25135

Braak, H., Tredici, K. Del, Rüb, U., de Vos, R.A.., Jansen Steur, E.N.., Braak, E., 2003. Staging of brain pathology related to sporadic Parkinson's disease. Neurobiol. Aging 24, 197–211. doi:10.1016/S0197-4580(02)00065-9

Brichta, L., Greengard, P., Flajolet, M., 2013. Advances in the pharmacological treatment of Parkinson's disease: targeting neurotransmitter systems. Trends Neurosci. 36, 543–554. doi:10.1016/j.tins.2013.06.003

Burré, J., Sharma, M., Tsetsenis, T., Buchman, V., Etherton, M.R., Südhof, T.C., 2010. Alpha-synuclein promotes SNARE-complex assembly in vivo and in vitro. Science 329, 1663–7. doi:10.1126/science.1195227

Cavaliere, F., Vicente, E.S., Matute, C., 2010. An organotypic culture model to study nigro-striatal degeneration. J. Neurosci. Methods 188, 205–12. doi:10.1016/j.jneumeth.2010.02.008

Cerruti, C., Walther, D.M., Kuhar, M.J., Uhl, G.R., 1993. Dopamine transporter mRNA expression is intense in rat midbrain neurons and modest outside midbrain. Brain Res. Mol. Brain Res. 18, 181–6.

Chambers, S.M., Fasano, C.A., Papapetrou, E.P., Tomishima, M., Sadelain, M., Studer, L., 2009. Highly efficient neural conversion of human ES and iPS cells by dual inhibition of SMAD signaling. Nat. Biotechnol. 27, 275–280. doi:10.1038/nbt.1529

Chang-Liu, C.M., Woloschak, G.E., 1997. Effect of passage number on cellular response to DNA-damaging agents: cell survival and gene expression. Cancer Lett. 113, 77–86.

Cheung, Y.-T., Lau, W.K.-W., Yu, M.-S., Lai, C.S.-W., Yeung, S.-C., So, K.-F., Chang, R.C.-C., 2009. Effects of all-trans-retinoic acid on human SH-SY5Y neuroblastoma as in vitro model in neurotoxicity research. Neurotoxicology 30, 127–35. doi:10.1016/j.neuro.2008.11.001

Constantinescu, R., Constantinescu, A.T., Reichmann, H., Janetzky, B., 2007. Neuronal differentiation and long-term culture of the human neuroblastoma line SH-SY5Y. J. Neural Transm. Suppl. 17–28.

Cooper, O., Hargus, G., Deleidi, M., Blak, A., Osborn, T., Marlow, E., Lee, K., Levy, A., Perez-Torres, E., Yow, A., Isacson, O., 2010. Differentiation of human ES and Parkinson's disease iPS cells into ventral midbrain

dopaminergic neurons requires a high activity form of SHH, FGF8a and specific regionalization by retinoic acid. Mol. Cell. Neurosci. 45, 258–66. doi:10.1016/j.mcn.2010.06.017

Corrigan, F.M., Wienburg, C.L., Shore, R.F., Daniel, S.E., Mann, D., 2000. Organochlorine insecticides in substantia nigra in Parkinson's disease. J. Toxicol. Environ. Health. A 59, 229–34.

Cossette, M., Lévesque, D., Parent, A., 2005. Neurochemical characterization of dopaminergic neurons in human striatum. Parkinsonism Relat. Disord. 11, 277–86. doi:10.1016/j.parkreldis.2005.02.008

Daubner, S.C., Le, T., Wang, S., 2011. Tyrosine hydroxylase and regulation of dopamine synthesis. Arch. Biochem. Biophys. 508, 1–12. doi:10.1016/j.abb.2010.12.017

Daviaud, N., Garbayo, E., Schiller, P.C., Perez-Pinzon, M., Montero-Menei, C.N., 2013. Organotypic cultures as tools for optimizing central nervous system cell therapies. Exp. Neurol. 248, 429–440. doi:10.1016/j.expneurol.2013.07.012

Daviaud, N., Garbayo, E., Lautram, N., Franconi, F., Lemaire, L., Perez-Pinzon, M., Montero-Menei, C.N., 2014. Modeling nigrostriatal degeneration in organotypic cultures, a new ex vivo model of Parkinson's disease. Neuroscience 256, 10–22. doi:10.1016/j.neuroscience.2013.10.021

Davis, G.C., Williams, A.C., Markey, S.P., Ebert, M.H., Caine, E.D., Reichert, C.M., Kopin, I.J., 1979. Chronic Parkinsonism secondary to intravenous injection of meperidine analogues. Psychiatry Res. 1, 249–54.

Dawson, T.M., Ko, H.S., Dawson, V.L., 2010. Genetic animal models of Parkinson's disease. Neuron 66, 646–61. doi:10.1016/j.neuron.2010.04.034

de Lau, L.M.L., Schipper, C.M.A., Hofman, A., Koudstaal, P.J., Breteler, M.M.B., 2005. Prognosis of Parkinson disease: risk of dementia and mortality: the Rotterdam Study. Arch. Neurol. 62, 1265–9. doi:10.1001/archneur.62.8.1265

Ding, Y.M., Jaumotte, J.D., Signore, A.P., Zigmond, M.J., 2004. Effects of 6-hydroxydopamine q. J. Neurochem. 89, 776–87. doi:10.1111/j.1471-4159.2004.02415.x

Encinas, M., Iglesias, M., Liu, Y., Wang, H., Muhaisen, A., Ceña, V., Gallego, C., Comella, J.X., 2000. Sequential treatment of SH-SY5Y cells with retinoic acid and brain-derived neurotrophic factor gives rise to fully differentiated, neurotrophic factor-dependent, human neuron-like cells. J. Neurochem. 75, 991–1003.

Falkenburger, B.H., Schulz, J.B., 2006. Limitations of cellular models in Parkinson's disease research. J. Neural Transm. Suppl. 261–8.

Ferreira, M., Massano, J., 2016. An updated review of Parkinson's disease genetics and clinicopathological correlations. Acta Neurol. Scand. doi:10.1111/ane.12616

Filograna, R., Civiero, L., Ferrari, V., Codolo, G., Greggio, E., Bubacco, L., Beltramini, M., Bisaglia, M., 2015. Analysis of the Catecholaminergic

Phenotype in Human SH-SY5Y and BE(2)-M17 Neuroblastoma Cell Lines upon Differentiation. PLoS One 10, e0136769. doi:10.1371/journal.pone.0136769

Freshney, I., 2001. Application of cell cultures to toxicology. Cell Biol. Toxicol. 17, 213–30.

Gaven, F., Marin, P., Claeysen, S., 2014. Primary culture of mouse dopaminergic neurons. J. Vis. Exp. e51751. doi:10.3791/51751

Gibb, W.R., 1992. Neuropathology of Parkinson's disease and related syndromes. Neurol. Clin. 10, 361–76.

Gibb, W.R., 1991. Neuropathology of the substantia nigra. Eur. Neurol. 31 Suppl 1, 48–59.

Gilany, K., Van Elzen, R., Mous, K., Coen, E., Van Dongen, W., Vandamme, S., Gevaert, K., Timmerman, E., Vandekerckhove, J., Dewilde, S., Van Ostade, X., Moens, L., 2008. The proteome of the human neuroblastoma cell line SH-SY5Y: an enlarged proteome. Biochim. Biophys. Acta 1784, 983–5. doi:10.1016/j.bbapap.2008.03.003

Glinka, Y., Gassen, M., Youdim, M.B., 1997. Mechanism of 6-hydroxydopamine neurotoxicity. J. Neural Transm. Suppl. 50, 55–66.

Glinka, Y., Tipton, K.F., Youdim, M.B., 1996. Nature of inhibition of mitochondrial respiratory complex I by 6-Hydroxydopamine. J. Neurochem. 66, 2004–10.

Gomez-Lazaro, M., Galindo, M.F., Concannon, C.G., Segura, M.F., Fernandez-Gomez, F.J., Llecha, N., Comella, J.X., Prehn, J.H.M., Jordan, J., 2008. 6-Hydroxydopamine activates the mitochondrial apoptosis pathway through p38 MAPK-mediated, p53-independent activation of Bax and PUMA. J. Neurochem. 104, 1599–612. doi:10.1111/j.1471-4159.2007.05115.x

Halterman, M.W., Giuliano, R., Dejesus, C., Schor, N.F., 2009. In-tube transfection improves the efficiency of gene transfer in primary neuronal cultures. J. Neurosci. Methods 177, 348–54. doi:10.1016/j.jneumeth.2008.10.023

Hartfield, E.M., Yamasaki-Mann, M., Ribeiro Fernandes, H.J., Vowles, J., James, W.S., Cowley, S.A., Wade-Martins, R., 2014. Physiological characterisation of human iPS-derived dopaminergic neurons. PLoS One 9, e87388. doi:10.1371/journal.pone.0087388

Herrup, K., Yang, Y., 2007. Cell cycle regulation in the postmitotic neuron: oxymoron or new biology? Nat. Rev. Neurosci. 8, 368–78. doi:10.1038/nrn2124

Howman-Giles, R., Shaw, P.J., Uren, R.F., Chung, D.K. V, 2007. Neuroblastoma and other neuroendocrine tumors. Semin. Nucl. Med. 37, 286–302. doi:10.1053/j.semnuclmed.2007.02.009

Humpel, C., 2015. Organotypic brain slice cultures: A review. Neuroscience 305, 86–98. doi:10.1016/j.neuroscience.2015.07.086

Iglesias-González, J., Sánchez-Iglesias, S., Méndez-Álvarez, E., Rose, S., Hikima, A., Jenner, P., Soto-Otero, R., 2012. Differential toxicity of 6-

hydroxydopamine in SH-SY5Y human neuroblastoma cells and rat brain mitochondria: protective role of catalase and superoxide dismutase. Neurochem. Res. 37, 2150–60. doi:10.1007/s11064-012-0838-6

Imaizumi, Y., Okano, H., 2014. Modeling human neurological disorders with induced pluripotent stem cells. J. Neurochem. 129, 388–99. doi:10.1111/jnc.12625

Izumi, Y., Sawada, H., Sakka, N., Yamamoto, N., Kume, T., Katsuki, H., Shimohama, S., Akaike, A., 2005. p-Quinone mediates 6-hydroxydopamine-induced dopaminergic neuronal death and ferrous iron accelerates the conversion of p-quinone into melanin extracellularly. J. Neurosci. Res. 79, 849–60. doi:10.1002/jnr.20382

Jagmag, S.A., Tripathi, N., Shukla, S.D., Maiti, S., Khurana, S., 2015. Evaluation of Models of Parkinson's Disease. Front. Neurosci. 9, 503. doi:10.3389/fnins.2015.00503

Jankovic, J., Poewe, W., 2012. Therapies in Parkinson's disease. Curr. Opin. Neurol. 25, 433–47. doi:10.1097/WCO.0b013e3283542fc2

Javitch, J.A., Snyder, S.H., 1984. Uptake of MPP(+) by dopamine neurons explains selectivity of parkinsonism-inducing neurotoxin, MPTP. Eur. J. Pharmacol. 106, 455–6.

Jiang, H., Ren, Y., Yuen, E.Y., Zhong, P., Ghaedi, M., Hu, Z., Azabdaftari, G., Nakaso, K., Yan, Z., Feng, J., 2012. Parkin controls dopamine utilization in human midbrain dopaminergic neurons derived from induced pluripotent stem cells. Nat. Commun. 3, 668. doi:10.1038/ncomms1669

Kandel, E., 2013. Principles of Neural Science, Fifth Edition.

Kanthasamy, A.G., Anantharam, V., Zhang, D., Latchoumycandane, C., Jin, H., Kaul, S., Kanthasamy, A., 2006. A novel peptide inhibitor targeted to caspase-3 cleavage site of a proapoptotic kinase protein kinase C delta (PKCdelta) protects against dopaminergic neuronal degeneration in Parkinson's disease models. Free Radic. Biol. Med. 41, 1578–89. doi:10.1016/j.freeradbiomed.2006.08.016

Karunakaran, S., Saeed, U., Mishra, M., Valli, R.K., Joshi, S.D., Meka, D.P., Seth, P., Ravindranath, V., 2008. Selective activation of p38 mitogen-activated protein kinase in dopaminergic neurons of substantia nigra leads to nuclear translocation of p53 in 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine-treated mice. J. Neurosci. 28, 12500–9. doi:10.1523/JNEUROSCI.4511-08.2008

Kearns, S.M., Scheffler, B., Goetz, A.K., Lin, D.D., Baker, H.D., Roper, S.N., Mandel, R.J., Steindler, D.A., 2006. A method for a more complete in vitro Parkinson's model: slice culture bioassay for modeling maintenance and repair of the nigrostriatal circuit. J. Neurosci. Methods 157, 1–9. doi:10.3201/eid1204.050756

Lancaster, M.A., Knoblich, J.A., 2014a. Organogenesis in a dish: Modeling development and disease using organoid technologies. Science (80-.). 345, 1247125–1247125. doi:10.1126/science.1247125

Lancaster, M.A., Knoblich, J.A., 2014b. Generation of cerebral organoids from

human pluripotent stem cells. Nat. Protoc. 9, 2329–2340. doi:10.1038/nprot.2014.158

Lancaster, M., Renner, M., Martin, C.-A., Wenzel, D., Bicknell, L.S., Hurles, M.E., Homfray, T., Penninger, J.M., Jackson, A.P., Knoblich, J.A., 2013. Cerebral organoids model human brain development and microcephaly. Nature 501, 373–9. doi:10.1038/nature12517

Lane, E., Dunnett, S., 2008. Animal models of Parkinson's disease and L-dopa induced dyskinesia: how close are we to the clinic? Psychopharmacology (Berl). 199, 303–12. doi:10.1007/s00213-007-0931-8

Langston, J.W., Ballard, P.A., 1983. Parkinson's disease in a chemist working with 1-methyl-4-phenyl-1,2,5,6-tetrahydropyridine. N. Engl. J. Med. 309, 310.

Larsen, T.R., Söderling, A.-S., Caidahl, K., Roepstorff, P., Gramsbergen, J.B., 2008. Nitration of soluble proteins in organotypic culture models of Parkinson's disease. Neurochem. Int. 52, 487–494. doi:10.1016/j.neuint.2007.08.008

Laverty, R., Sharman, D.F., Vogt, M., 1965. Action of 2, 4, 5-trihydroxyphenylethylamine on the storage and release of noradrenaline. Br. J. Pharmacol. Chemother. 24, 549–560. doi:10.1111/j.1476-5381.1965.tb01745.x

Lin, C.-Y., Tsai, C.-W., 2016. Carnosic Acid Attenuates 6-Hydroxydopamine-Induced Neurotoxicity in SH-SY5Y Cells by Inducing Autophagy Through an Enhanced Interaction of Parkin and Beclin1. Mol. Neurobiol. doi:10.1007/s12035-016-9873-7

Lodish, H., Berk, A., Zipursky, S.L., Matsudaira, P., Baltimore, D., Darnell, J., 2000. Neurotransmitters, Synapses, and Impulse Transmission.

Lopes, F.M., da Motta, L.L., De Bastiani, M.A., Pfaffenseller, B., Aguiar, B.W., de Souza, L.F., Zanatta, G., Vargas, D.M., Schönhofen, P., Londero, G.F., de Medeiros, L.M., Freire, V.N., Dafre, A.L., Castro, M.A.A., Parsons, R.B., Klamt, F., 2017. RA Differentiation Enhances Dopaminergic Features, Changes Redox Parameters, and Increases Dopamine Transporter Dependency in 6-Hydroxydopamine-Induced Neurotoxicity in SH-SY5Y Cells. Neurotox. Res. doi:10.1007/s12640-016-9699-0

Lopes, F.M., Schröder, R., da Frota, M.L.C., Zanotto-Filho, A., Müller, C.B., Pires, A.S., Meurer, R.T., Colpo, G.D., Gelain, D.P., Kapczinski, F., Moreira, J.C.F., Fernandes, M. da C., Klamt, F., 2010. Comparison between proliferative and neuron-like SH-SY5Y cells as an in vitro model for Parkinson disease studies. Brain Res. 1337, 85–94. doi:10.1016/j.brainres.2010.03.102

Lotharius, J., Falsig, J., van Beek, J., Payne, S., Dringen, R., Brundin, P., Leist, M., 2005. Progressive degeneration of human mesencephalic neuron-derived cells triggered by dopamine-dependent oxidative stress is dependent on the mixed-lineage kinase pathway. J. Neurosci. 25, 6329–42. doi:10.1523/JNEUROSCI.1746-05.2005

Luchtman, D.W., Song, C., 2010. Why SH-SY5Y cells should be differentiated. Neurotoxicology 31, 164-5-6. doi:10.1016/j.neuro.2009.10.015

Luthman, J., Fredriksson, A., Sundström, E., Jonsson, G., Archer, T., 1989. Selective lesion of central dopamine or noradrenaline neuron systems in the neonatal rat: motor behavior and monoamine alterations at adult stage. Behav. Brain Res. 33, 267–77.

Maqsood, M.I., Matin, M.M., Bahrami, A.R., Ghasroldasht, M.M., 2013. Immortality of cell lines: challenges and advantages of establishment. Cell Biol. Int. 37, 1038–45. doi:10.1002/cbin.10137

Marder, K., Tang, M.X., Mejia, H., Alfaro, B., Côté, L., Louis, E., Groves, J., Mayeux, R., 1996. Risk of Parkinson's disease among first-degree relatives: A community-based study. Neurology 47, 155–60.

Matsuda, W., Furuta, T., Nakamura, K.C., Hioki, H., Fujiyama, F., Arai, R., Kaneko, T., 2009. Single nigrostriatal dopaminergic neurons form widely spread and highly dense axonal arborizations in the neostriatum. J. Neurosci. 29, 444–53. doi:10.1523/JNEUROSCI.4029-08.2009

Mizuno, Y., Sone, N., Saitoh, T., 1987. Effects of 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine and 1-methyl-4-phenylpyridinium ion on activities of the enzymes in the electron transport system in mouse brain. J. Neurochem. 48, 1787–93.

Nicklas, W.J., Youngster, S.K., Kindt, M. V, Heikkila, R.E., 1987. MPTP, MPP+ and mitochondrial function. Life Sci. 40, 721–9.

Nikolaus, S., Antke, C., Kley, K., Poeppel, T.D., Hautzel, H., Schmidt, D., Müller, H.-W., 2007. Investigating the dopaminergic synapse in vivo. I. Molecular imaging studies in humans. Rev. Neurosci. 18, 439–72.

Olanow, C.W., Kieburtz, K., Schapira, A.H. V, 2008. Why have we failed to achieve neuroprotection in Parkinson's disease? Ann. Neurol. 64 Suppl 2, S101-10. doi:10.1002/ana.21461

Påhlman, S., Ruusala, A.I., Abrahamsson, L., Mattsson, M.E., Esscher, T., 1984. Retinoic acid-induced differentiation of cultured human neuroblastoma cells: a comparison with phorbolester-induced differentiation. Cell Differ. 14, 135–44.

Parker, W.D., Parks, J.K., Swerdlow, R.H., 2008. Complex I deficiency in Parkinson's disease frontal cortex. Brain Res. 1189, 215–8. doi:10.1016/j.brainres.2007.10.061

Pišlar, A.H., Zidar, N., Kikelj, D., Kos, J., 2014. Cathepsin X promotes 6-hydroxydopamine-induced apoptosis of PC12 and SH-SY5Y cells. Neuropharmacology 82, 121–31. doi:10.1016/j.neuropharm.2013.07.040

Plenz, D., Kitai, S.T., 1996. Organotypic cortex-striatum-mesencephalon cultures: the nigrostriatal pathway. Neurosci. Lett. 209, 177–80.

Polymeropoulos, M.H., Lavedan, C., Leroy, E., Ide, S.E., Dehejia, A., Dutra, A., Pike, B., Root, H., Rubenstein, J., Boyer, R., Stenroos, E.S., Chandrasekharappa, S., Athanassiadou, A., Papapetropoulos, T., Johnson, W.G., Lazzarini, A.M., Duvoisin, R.C., Di Iorio, G., Golbe, L.I., Nussbaum, R.L., 1997. Mutation in the alpha-synuclein gene identified in families with Parkinson's disease. Science 276, 2045–7.

Presgraves, S.P., Ahmed, T., Borwege, S., Joyce, J.N., 2004. Terminally differentiated SH-SY5Y cells provide a model system for studying neuroprotective effects of dopamine agonists. Neurotox. Res. 5, 579–98.

Price, K.S., Farley, I.J., Hornykiewicz, O., 1978. Neurochemistry of Parkinson's disease: relation between striatal and limbic dopamine. Adv. Biochem. Psychopharmacol. 19, 293–300.

Pu, J., Jiang, H., Zhang, B., Feng, J., 2012. Redefining Parkinson's disease research using induced pluripotent stem cells. Curr. Neurol. Neurosci. Rep. 12, 392–8. doi:10.1007/s11910-012-0288-1

Radio, N.M., Mundy, W.R., 2008. Developmental neurotoxicity testing in vitro: models for assessing chemical effects on neurite outgrowth. Neurotoxicology 29, 361–76. doi:10.1016/j.neuro.2008.02.011

Ramamoorthy, S., Shippenberg, T.S., Jayanthi, L.D., 2011. Regulation of monoamine transporters: Role of transporter phosphorylation. Pharmacol. Ther. 129, 220–38. doi:10.1016/j.pharmthera.2010.09.009

Richardson, J.R., Shalat, S.L., Buckley, B., Winnik, B., O'Suilleabhain, P., Diaz-Arrastia, R., Reisch, J., German, D.C., 2009. Elevated serum pesticide levels and risk of Parkinson disease. Arch. Neurol. 66, 870–5. doi:10.1001/archneurol.2009.89

Rodriguez-Pallares, J., Parga, J.A., Muñoz, A., Rey, P., Guerra, M.J., Labandeira-Garcia, J.L., 2007. Mechanism of 6-hydroxydopamine neurotoxicity: the role of NADPH oxidase and microglial activation in 6-hydroxydopamine-induced degeneration of dopaminergic neurons. J. Neurochem. 103, 145–56. doi:10.1111/j.1471-4159.2007.04699.x

Ryan, S.D., Dolatabadi, N., Chan, S.F., Zhang, X., Akhtar, M.W., Parker, J., Soldner, F., Sunico, C.R., Nagar, S., Talantova, M., Lee, B., Lopez, K., Nutter, A., Shan, B., Molokanova, E., Zhang, Y., Han, X., Nakamura, T., Masliah, E., Yates, J.R., Nakanishi, N., Andreyev, A.Y., Okamoto, S., Jaenisch, R., Ambasudhan, R., Lipton, S.A., 2013. Isogenic human iPSC Parkinson's model shows nitrosative stress-induced dysfunction in MEF2-PGC1α transcription. Cell 155, 1351–64. doi:10.1016/j.cell.2013.11.009

Saito, Y., Nishio, K., Ogawa, Y., Kinumi, T., Yoshida, Y., Masuo, Y., Niki, E., 2007. Molecular mechanisms of 6-hydroxydopamine-induced cytotoxicity in PC12 cells: involvement of hydrogen peroxide-dependent and -independent action. Free Radic. Biol. Med. 42, 675–85. doi:10.1016/j.freeradbiomed.2006.12.004

Sánchez-Danés, A., Richaud-Patin, Y., Carballo-Carbajal, I., Jiménez-Delgado, S., Caig, C., Mora, S., Di Guglielmo, C., Ezquerra, M., Patel, B., Giralt, A., Canals, J.M., Memo, M., Alberch, J., López-Barneo, J., Vila, M., Cuervo, A.M., Tolosa, E., Consiglio, A., Raya, A., 2012. Disease-specific phenotypes in dopamine neurons from human iPS-based models of genetic and sporadic Parkinson's disease. EMBO Mol. Med. 4, 380–95. doi:10.1002/emmm.201200215

Saporito, M.S., Thomas, B.A., Scott, R.W., 2000. MPTP activates c-Jun NH(2)-terminal kinase (JNK) and its upstream regulatory kinase MKK4 in

nigrostriatal neurons in vivo. J. Neurochem. 75, 1200–8.

Sauerbier, A., Jenner, P., Todorova, A., Chaudhuri, K.R., 2015. Non motor subtypes and Parkinson's disease. Parkinsonism Relat. Disord. doi:10.1016/j.parkreldis.2015.09.027

Schapira, A.H., Mann, V.M., Cooper, J.M., Dexter, D., Daniel, S.E., Jenner, P., Clark, J.B., Marsden, C.D., 1990. Anatomic and disease specificity of NADH CoQ1 reductase (complex I) deficiency in Parkinson's disease. J. Neurochem. 55, 2142–5.

Schildknecht, S., Karreman, C., Pöltl, D., Efrémova, L., Kullmann, C., Gutbier, S., Krug, A., Scholz, D., Gerding, H.R., Leist, M., 2013. Generation of genetically-modified human differentiated cells for toxicological tests and the study of neurodegenerative diseases. ALTEX 30, 427–44.

Schildknecht, S., Pöltl, D., Nagel, D.M., Matt, F., Scholz, D., Lotharius, J., Schmieg, N., Salvo-Vargas, A., Leist, M., 2009. Requirement of a dopaminergic neuronal phenotype for toxicity of low concentrations of 1-methyl-4-phenylpyridinium to human cells. Toxicol. Appl. Pharmacol. 241, 23–35. doi:10.1016/j.taap.2009.07.027

Schlachetzki, J.C.M., Saliba, S.W., Oliveira, A.C.P. de, 2013. Studying neurodegenerative diseases in culture models. Rev. Bras. Psiquiatr. (São Paulo, Brazil 1999) 35 Suppl 2, S92-100. doi:10.1590/1516-4446-2013-1159

Scholz, D., Pöltl, D., Genewsky, A., Weng, M., Waldmann, T., Schildknecht, S., Leist, M., 2011. Rapid, complete and large-scale generation of post-mitotic neurons from the human LUHMES cell line. J. Neurochem. 119, 957–71. doi:10.1111/j.1471-4159.2011.07255.x

Schönhofen, P., de Medeiros, L.M., Bristot, I.J., Lopes, F.M., De Bastiani, M.A., Kapczinski, F., Crippa, J.A.S., Castro, M.A.A., Parsons, R.B., Klamt, F., 2015. Cannabidiol Exposure During Neuronal Differentiation Sensitizes Cells Against Redox-Active Neurotoxins. Mol. Neurobiol. 52, 26–37. doi:10.1007/s12035-014-8843-1

Schüle, B., Pera, R.A.R., Langston, J.W., 2009. Can cellular models revolutionize drug discovery in Parkinson's disease? Biochim. Biophys. Acta 1792, 1043–51. doi:10.1016/j.bbadis.2009.08.014

Scott, W.K., Staijich, J.M., Yamaoka, L.H., Speer, M.C., Vance, J.M., Roses, A.D., Pericak-Vance, M.A., 1997. Genetic complexity and Parkinson's disease. Deane Laboratory Parkinson Disease Research Group. Science 277, 387–8; author reply 389.

Segura-Aguilar, J., Kostrzewa, R.M., 2015. Neurotoxin Mechanisms and Processes Relevant to Parkinson's Disease: An Update. Neurotox. Res. 27, 328–354. doi:10.1007/s12640-015-9519-y

Seibler, P., Graziotto, J., Jeong, H., Simunovic, F., Klein, C., Krainc, D., 2011. Mitochondrial Parkin recruitment is impaired in neurons derived from mutant PINK1 induced pluripotent stem cells. J. Neurosci. 31, 5970–6. doi:10.1523/JNEUROSCI.4441-10.2011

Shay, J.W., Wright, W.E., Werbin, H., 1991. Defining the molecular mechanisms of human cell immortalization. Biochim. Biophys. Acta 1072, 1–7.

Soto-Otero, R., Méndez-Alvarez, E., Hermida-Ameijeiras, A., Muñoz-Patiño, A.M., Labandeira-Garcia, J.L., 2000. Autoxidation and neurotoxicity of 6-hydroxydopamine in the presence of some antioxidants: potential implication in relation to the pathogenesis of Parkinson's disease. J. Neurochem. 74, 1605–12.

Spatola, M., Wider, C., 2014. Genetics of Parkinson's disease: the yield. Parkinsonism Relat. Disord. 20 Suppl 1, S35-8. doi:10.1016/S1353-8020(13)70011-7

Spillantini, M.G., Schmidt, M.L., Lee, V.M.-Y., Trojanowski, J.Q., Jakes, R., Goedert, M., 1997. [alpha]-Synuclein in Lewy bodies 388, 839–840.

Stahl, K., Skare, Ø., Torp, R., 2009. Organotypic cultures as a model of Parkinson s disease. A twist to an old model. ScientificWorldJournal. 9, 811–21. doi:10.1100/tsw.2009.68

Stępkowski, T.M., Wasyk, I., Grzelak, A., Kruszewski, M., 2015. 6-OHDA-Induced Changes in Parkinson's Disease-Related Gene Expression are not Affected by the Overexpression of PGAM5 in In Vitro Differentiated Embryonic Mesencephalic Cells. Cell. Mol. Neurobiol. 35, 1137–47. doi:10.1007/s10571-015-0207-5

Stoppini, L., Buchs, P.A., Muller, D., 1991. A simple method for organotypic cultures of nervous tissue. J. Neurosci. Methods 37, 173–82.

Stuchbury, G., Münch, G., 2010. Optimizing the generation of stable neuronal cell lines via pre-transfection restriction enzyme digestion of plasmid DNA. Cytotechnology 62, 189–94. doi:10.1007/s10616-010-9273-1

Studer, L., 2001. Culture of substantia nigra neurons. Curr. Protoc. Neurosci. Chapter 3, Unit 3.3. doi:10.1002/0471142301.ns0303s00

Takahashi, K., Okita, K., Nakagawa, M., Yamanaka, S., 2007. Induction of pluripotent stem cells from fibroblast cultures. Nat. Protoc. 2, 3081–9. doi:10.1038/nprot.2007.418

Tanner, C.M., Kamel, F., Ross, G.W., Hoppin, J.A., Goldman, S.M., Korell, M., Marras, C., Bhudhikanok, G.S., Kasten, M., Chade, A.R., Comyns, K., Richards, M.B., Meng, C., Priestley, B., Fernandez, H.H., Cambi, F., Umbach, D.M., Blair, A., Sandler, D.P., Langston, J.W., 2011. Rotenone, paraquat, and Parkinson's disease. Environ. Health Perspect. 119, 866–72. doi:10.1289/ehp.1002839

Thomas, M.G., Saldanha, M., Mistry, R.J., Dexter, D.T., Ramsden, D.B., Parsons, R.B., 2013. Nicotinamide N-methyltransferase expression in SH-SY5Y neuroblastoma and N27 mesencephalic neurones induces changes in cell morphology via ephrin-B2 and Akt signalling. Cell Death Dis. 4, e669. doi:10.1038/cddis.2013.200

Tieng, V., Stoppini, L., Villy, S., Fathi, M., Dubois-Dauphin, M., Krause, K.-H., 2014. Engineering of midbrain organoids containing long-lived dopaminergic neurons. Stem Cells Dev. 23, 1535–47. doi:10.1089/scd.2013.0442

Tönges, L., Frank, T., Tatenhorst, L., Saal, K.A., Koch, J.C., Szego, É.M., Bähr, M., Weishaupt, J.H., Lingor, P., 2012. Inhibition of rho kinase enhances survival of dopaminergic neurons and attenuates axonal loss in a mouse model of Parkinson's disease. Brain 135, 3355–70. doi:10.1093/brain/aws254

Van Kampen, J.M., McGeer, E.G., Stoessl, A.J., 2000. Dopamine transporter function assessed by antisense knockdown in the rat: protection from dopamine neurotoxicity. Synapse 37, 171–8. doi:10.1002/1098-2396(20000901)37:3<171::AID-SYN1>3.0.CO;2-R

Vernon, A.C., Crum, W.R., Johansson, S.M., Modo, M., 2011. Evolution of extra-nigral damage predicts behavioural deficits in a rat proteasome inhibitor model of Parkinson's disease. PLoS One 6, e17269. doi:10.1371/journal.pone.0017269

Vila, M., Jackson-Lewis, V., Vukosavic, S., Djaldetti, R., Liberatore, G., Offen, D., Korsmeyer, S.J., Przedborski, S., 2001. Bax ablation prevents dopaminergic neurodegeneration in the 1-methyl- 4-phenyl-1,2,3,6-tetrahydropyridine mouse model of Parkinson's disease. Proc. Natl. Acad. Sci. U. S. A. 98, 2837–42. doi:10.1073/pnas.051633998

Wei, L., Ding, L., Mo, M.-S., Lei, M., Zhang, L., Chen, K., Xu, P., 2015. Wnt3a protects SH-SY5Y cells against 6-hydroxydopamine toxicity by restoration of mitochondria function. Transl. Neurodegener. 4, 11. doi:10.1186/s40035-015-0033-1

Weinert, M., Selvakumar, T., Tierney, T.S., Alavian, K.N., 2015. Isolation, culture and long-term maintenance of primary mesencephalic dopaminergic neurons from embryonic rodent brains. J. Vis. Exp. e52475. doi:10.3791/52475

Weisskopf, M.G., Knekt, P., O'Reilly, E.J., Lyytinen, J., Reunanen, A., Laden, F., Altshul, L., Ascherio, A., 2010. Persistent organochlorine pesticides in serum and risk of Parkinson disease. Neurology 74, 1055–61. doi:10.1212/WNL.0b013e3181d76a93

Yu, J., Vodyanik, M.A., Smuga-Otto, K., Antosiewicz-Bourget, J., Frane, J.L., Tian, S., Nie, J., Jonsdottir, G.A., Ruotti, V., Stewart, R., Slukvin, I.I., Thomson, J.A., 2007. Induced pluripotent stem cell lines derived from human somatic cells. Science 318, 1917–20. doi:10.1126/science.1151526

Zhang, X.-M., Yin, M., Zhang, M.-H., 2014. Cell-based assays for Parkinson's disease using differentiated human LUHMES cells. Acta Pharmacol. Sin. 35, 945–56. doi:10.1038/aps.2014.36

Zheng, K., Liu, T.-Q., Dai, M.-S., Ge, D., Li, X.-Q., Ma, X.-H., Cui, Z.-F., 2006. Comparison of different culture modes for long-term expansion of neural stem cells. Cytotechnology 52, 209–18. doi:10.1007/s10616-006-9037-0

Zhou, Z.D., Lan, Y.H., Tan, E.K., Lim, T.M., 2010. Iron species-mediated dopamine oxidation, proteasome inhibition, and dopaminergic cell demise: implications for iron-related dopaminergic neuron degeneration. Free Radic. Biol. Med. 49, 1856–71. doi:10.1016/j.freeradbiomed.2010.09.010

Figure Captions:

Figure 1: The dopaminergic neuron and the nigrostriatal pathway. Dopaminergic neuron within the *substantia nigra pars compacta* of the midbrain stimulate the caudate and putamen of the striatum, forming the nigrostriatal pathway. In the dopaminergic synapse, dopamine synthesis (*via* TH and DDC) and dopamine vesicle cycling (*via* DAT) occur in the pre-synpatic terminus. In the post-synaptic terminus, dopamine released into the synaptic cleft bind to and stimulates dopamine receptors (D1, D2, D3 and D5). The degradation of dopamine by MAO and COMT enzymes occurs both within the synaptic cleft and pre-synaptic terminal.

Figure 2: *In vitro* models of Parkinson's disease, shown in order of lowest to the highest difficulty in production and maintenance. (a) Cell line models are derived from biopsies obtained from multicellular organisms, in which a population of cells is selected by clonal expansion and are immortal *via* transformation with *e.g.* large T antigen. Hence, cell lines proliferate indefinitely. They can be used as undifferentiated or differentiated cells. (b) In primary cultures, intrauterine horns from a pregnant dam (E14 for rats and E13 for mouse) are used. The embryos are dissected to obtain the VMA. Dopaminergic cells are then isolated using mechanical and enzymatic digestion and placed in an optimized culture medium. (c) In organotypic cultures, the *in vivo* cellular interactions and three-dimensional structure of the tissue is maintained. Brains from newborn animals are used, from which the ventral midbrain area (VMA) is dissected out. This is then cut transversely into slices and placed into optimized culture medium. (d) iPSC are obtained by transforming mature human cells

(usually fibroblasts) with 4 genes - *Oct4, Sox2, Klf4 and c-Myc*. Once generated, these cells can undergo differentiation to acquire a dopaminergic phenotype.

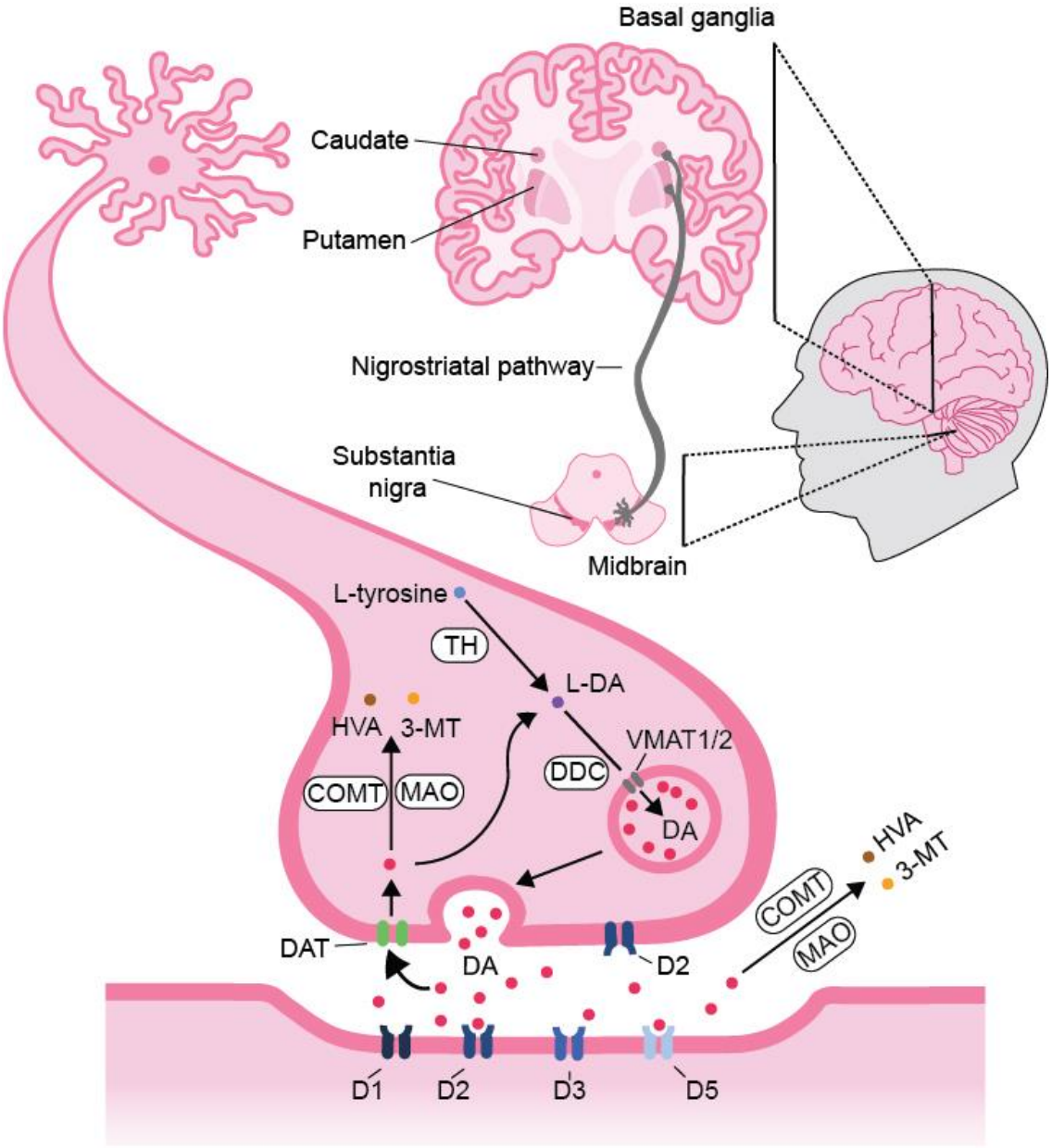
Basal ganglia
Caudate
Putamen
Nigrostriatal pathway
Substantia nigra
Midbrain
L-tyrosine
TH
L-DA
HVA
3-MT
VMAT1/2
DDC
COMT
MAO
DA
DAT
DA
D2
COMT
MAO
HVA
3-MT
D1
D2
D3
D5

Figure 1

a. Cell lines
Level of difficulty
PROS
- Large-scale
- Reproducibility
- Genetic manipulation
- Human-based genome
CONS
- Oncogenic
- Requires differentiation
- Single-cell system
+ PD related genes
b. Primary Cultures
Level of difficulty
PROS
- Neuronal physiology
- Neuronal morphology
CONS
- Time-consuming
- Non-human-based gene
- Glia contamination
- Not suitable for genetic manipulation
c. Organotypic Cultures
Level of difficulty
PROS
- Mimic in vivo connections
CONS
- Time-consuming
- Non-human-based gene
- Glia contamination
- Not suitable for genetic manipulation
d. Induced Pluripotent Cells
Level of difficulty
PROS
- Neuronal physiology
- Cells can be derived from PD patients
-Neuronal morphology
CONS
- Time-consuming
- Consent/ethical approval
Control
PD
Oct4
Sox2
Klf4
c-Myc

Figure 2

Article 2: *RA-Differentiation Enhances Dopaminergic Features, Changes Redox Parameters and Increases Dopamine Transporter Dependency in 6-Hydroxydopamine-Induced-Neurotoxicity in SH-SY5Y cells*

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JUSTIFICATION:

The human neuroblastoma cell line SH-SY5Y is the most used *in vitro* PD model because not only it expresses the catecholamine synthesis machinery, but also it is easy to cultivate when compared with other *in vitro* models (*e.g.* iPSCs). However, there are some significant drawbacks of using this model for PD since they are considered as immature neuroblasts that proliferate over a long period of time in contrast to terminally-differentiated neurons that have low rates of proliferation.

In spite of its tumoral features, SH-SY5Y cells can acquire a neuronal-like phenotype when treated with differentiation agents, such as RA. However, the scientific literature present several protocols, which lead to variation among findings regarding neuronal and dopaminergic features. Hence, an understanding of the neuronal features of SH-SY5Y cell line in differentiated and undifferentiated states would be beneficial for PD research.

GENERAL OBJECTIVE

Characterization of relevant neuronal features and 6-OHDA response in undifferentiated and RA-differentiated SH-SYSY cells.

SPECIFIC OBJECTIVES

- Neuronal characterization by neurite outgrowth evaluation, proliferation rates and gene expression of cell cycle and synapse vesicle cycle networks in undifferentiated and RA-differentiated SH-SY5Y cells;
- Dopaminergic characterization through gene expression of dopaminergic synapse network, DA immunoreactivity and DAT immunocontent in undifferentiated and RA-differentiated SH-SY5Y cells;
- Investigation of antioxidant gene expression and activities in undifferentiated and RA-differentiated SH-SY5Y cells;
- Evaluation of 6-OHDA-induced cell death in undifferentiated and RA-differentiated SH-SY5Y cells;
- Verification of the role of thiol-reducing agents in 6-OHDA-induced cytotoxicity in undifferentiated and RA-differentiated SH-SY5Y cells;
- Evaluation of the role of DAT in 6-OHDA-induced cell death in undifferentiated and RA-differentiated SH-SY5Y cells.

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ORIGINAL ARTICLE

RA Differentiation Enhances Dopaminergic Features, Changes Redox Parameters, and Increases Dopamine Transporter Dependency in 6-Hydroxydopamine-Induced Neurotoxicity in SH-SY5Y Cells

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Abstract Research on Parkinson's disease (PD) and drug development is hampered by the lack of suitable human in vitro models that simply and accurately recreate the disease conditions. To counteract this, many attempts to differentiate cell lines, such as the human SH-SY5Y neuroblastoma, into dopaminergic neurons have been undertaken since they are easier to cultivate when compared with other cellular models. Here, we characterized neuronal features discriminating undifferentiated and retinoic acid (RA)-differentiated SH-SYSY cells and described significant differences between these cell models in 6-hydroxydopamine (6-OHDA) cytotoxicity. In contrast to undifferentiated cells, RA-differentiated SH-SY5Y cells demonstrated low proliferative rate and a pronounced neuronal morphology with high expression of genes related to synapse vesicle cycle, dopamine synthesis/degradation, and of dopamine transporter (DAT). Significant differences between undifferentiated and RA-differentiated SH-SY5Y cells in the overall capacity of antioxidant defenses were found; although RA-differentiated SH-SY5Y cells presented a higher basal antioxidant capacity with high resistance against H_2O_2 insult, they were twofold more sensitive to 6-OHDA. DAT inhibition by 3α-bis-4-fluorophenyl- methoxytropane and dithiothreitol (a cell-permeable thiol-reducing agent) protected RA-differentiated, but not undifferentiated, SH-SY5Y cells from oxidative damage and cell death caused by 6-OHDA. Here, we demonstrate that undifferentiated and RA-differentiated SH-SY5Y cells are two unique phenotypes and also have dissimilar mechanisms in 6-OHDA cytotoxicity. Hence, our data support the use of RA-differentiated SH-SY5Y cells as an in vitro model of PD. This study may impact our understanding of the pathological mechanisms of PD and the development of new therapies and drugs for the management of the disease.

Keywords SH-SY5Y cells · Retinoic acid · Parkinson's disease · Experimental model · 6-hydroxydopamine · Dopamine transporter

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Introduction

Dopaminergic degeneration found in Parkinson's disease (PD) (Gibb 1991) is mainly associated with oxidative stress (Fariello 1988) and mitochondrial dysfunction (Schapira et al. 1990). However, the functional changes operating during the initial stage of PD remain unknown (Mullin and Schapira 2015). The lack of understanding the molecular mechanisms of PD has many causes (Olanow et al. 2008; Olanow 2009), which one of them is attributed to the difficulty to reproduce the complex physiological features of a human dopaminergic neuron in vitro (Schüle et al. 2009; Bal-Price et al. 2010). Hence, there are limited reliable neuronal in vitro cell models to study PD pathophysiological mechanisms (Radio and Mundy 2008; Haggarty and Perlis 2014).

In this context, the human neuroblastoma cell line SH-SY5Y is the most used in vitro model of dopaminergic neurons (Xie et al. 2010) because it does not only express the catecholamine synthesis machinery but is also easy to cultivate when compared with another in vitro models (e.g., primary culture and inducible pluripotent stem cells (iPSC) (Biedler et al. 1978; Kovalevich and Langford 2013). Even though these cells are widely used in PD research, they are epithelial cells and do not present neuronal properties such as a terminal post-mitotic state and the expression of synaptic proteins (Radio and Mundy 2008). Interestingly, the in vitro differentiation induced by retinoic acid (RA) of this cell line into a neuron-like phenotype was established more than 30 years ago (Påhlman et al. 1984).

However, there is no consensus which differentiation protocol is more suitable for this cell line. The scientific literature shows a divergence not only in serum concentration (1–10% fetal bovine serum (FBS)), which neurotrophin to be used (e.g., RA, BDNF, and TPA), but also in differentiation length (4–12 days). Hence, depending on the protocol used, there are several discrepancies among findings regarding neuronal and dopaminergic markers (e.g., tyrosine hydroxylase (TH) and dopamine transporter (DAT)) (Presgraves et al. 2004; Cheung et al. 2009; Agholme et al. 2010; Lopes et al. 2010; Korecka et al. 2013). This brings discussion whether SH-SY5Y cells must be differentiated (Luchtman and Song 2010; Xie et al. 2010).

Furthermore, different protocols also may cause changing in cell susceptibility to neurotoxins, such as 6-hydroxydopamine (6-OHDA) (Cheung et al. 2009; Lopes et al. 2010; Forster et al. 2016). In vivo, it is widely accepted that this toxin enters into the dopaminergic neuron via DAT and causes a massive oxidative stress (Ljungdahl et al. 1971). However, 6-OHDA mechanism of action is still controversial in in vitro studies. Although DAT inhibitors provide a partial protection against 6-OHDA toxicity towards primary dopaminergic neurons (Cerruti et al. 1993; Abad et al. 1995), many lines of evidence showed no protection in undifferentiated SH-SY5Y cells from cell death induced by this toxin (Storch et al. 2000; Izumi et al. 2005; Hanrott et al. 2006). Regarding RA-differentiated SH-SY5Y cells, no study showed the effect of DAT inhibition in 6-OHDA-induced cell death.

Even though with the emergence of new, more physiologically relevant models such as iPSC as in vitro models for PD (Hartfield et al. 2014), it is clear that the majority of studies have been undertaken using cell lines such as SH-SY5Y due to considerations such as availability of iPSC and the necessary expertise in their differentiation into dopaminergic neurons (Filograna et al. 2015; Forster et al. 2016; Lin and Tsai 2016). Hence, an understanding of the potential differences in SH-SY5Y cell line RA-differentiated and undifferentiated states and their response to 6-OHDA are imperative as this remains the most commonly used in vitro model (Kovalevich and Langford 2013).

In the present work, we aimed to validate a differentiation protocol previously described by our research group (Lopes et al. 2010) comparing undifferentiated and RA-differentiated SH-SY5Y cells regarding gene expression of important cellular networks related to dopaminergic neuronal machinery, morphology, redox metabolism, and 6-OHDA cytotoxicity. To further investigate 6-OHDA operating mechanisms in both models, DAT inhibition and pre-treatment with thiol-reducing agents were performed. Here, we demonstrate critical differences between models, such as DAT dependency of 6-OHDA-induced cell death in RA-differentiated SH-SY5Y cells.

Materials and Methods

Cell Culture

Human neuroblastoma cell line SH-SY5Y (ATCC, Manassas, VA, USA) was maintained in a 1:1 mixture of Ham's F12 and Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% heat-inactivated FBS (Cripion®, São Paulo, SP, Brazil), 2 mM of glutamine, 100 U/mL of penicillin/streptomycin, and antimycotic (cat. no. 10378016, Thermo Fisher Scientific®, Waltham, MA, USA) in a humidified atmosphere of 5% of CO_2 at 37 °C.

In our cellular differentiation protocol (as described in Fig. 1), only attached cells were maintained and floating cells were discarded; 3×10^4 cells/cm^2 were seeded in 10% FBS medium. After 24 h (day 1), medium was replaced with medium in which the FBS concentration was reduced to 1% and supplemented with 10 μM of RA (all-trans-retinoic acid, Enzo®—East Farmingdale, NY, USA) and incubated for 7 days. At day 4, the medium was replaced, and at day 7, cells were harvested and used for experiments.

It is important to note that successful differentiation depends upon (at least) three factors: (i) the confluence of the cells in day 1 must be around 75% (higher confluence inhibits

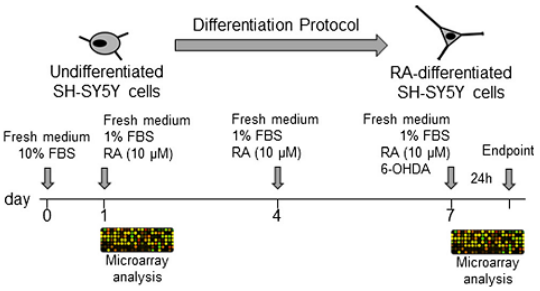


Fig. 1 Protocol design of the RA-induced differentiation. At day 0, exponentially growing SH-SY5Y cells were cultured in cell medium containing 10% FBS. After 24 h (day 1), the medium was removed and fresh medium containing 1% FBS and 10 μM RA (differentiation medium) was added. Three days later (day 4), the differentiation medium was replaced with fresh differentiation medium. At day 7, SH-SY5Y cells were used in experiments

neurite outgrowth, and lower confluence leads SH-SY5Y cells to detach); (ii) the cell medium should only be used for a maximum of 2 weeks to avoid glutamine decomposition; and (iii) RA powder is diluted in absolute ethanol to prepare the stock solution. The concentration of this solution was determined using E^{M} (351 nm) = 45,000 at the day of the medium replacement (i.e., days 1 and 4) to control any changes in the concentration that may occur during storage (Lopes et al. 2010; Sharow et al. 2012).

RNA Isolation and Microarray Assay

Cells were harvested and the RNA was isolated using TRIzol Reagent (Thermo Fisher Scientific®, Waltham, MA, USA) following by purification (Qiagen RNeasy Mini Kit no. 74104 and no. 79254—Free RNase DNase Set Qiagen,Hilden, Germany). Microarray analysis was performed using the chip GeneChip® PrimeView™ Human Gene Expression Array (Affymetrix™). The samples were collected at day 0 (undifferentiated cells) and day 7 (RA-differentiated cells) (Fig. 1), and raw data was deposited on GEO repository (GEOID: GSE71817).

Gene Set Enrichment Analysis and Expression Values

Four gene networks were analyzed in both undifferentiated and RA-differentiated SH-SY5Y cells: cell cycle, synapse vesicle cycle, dopaminergic synapse, and antioxidant (extracted from KEGG platform; KEGG Pathway Database 2016). Gene set enrichment analysis was used to identify genes that contribute to global changes in expression levels in a given microarray dataset comparison. Gene set enrichment analysis (GSEA) considers experiments with genome-wide expression profiles from two classes of samples (e.g., RA-differentiated cells vs. undifferentiated). Genes were ranked based on the correlation between their expression and the class distinction. Given a prior-defined network (e.g., synaptic vesicle cycle), the GSEA determines if the members of these sets of genes are randomly distributed or primarily found at the top or bottom of the ranking (Subramanian et al. 2005).

To access the logarithm of gene expression, raw CEL files were analyzed using the R/Bioconductor pipeline. The data was normalized by robust multi-array average (RMA) using the AFFY package, log (base 2) transformed, and batch corrected with ComBat using the SVA package.

Cell Cycle and Cellular Growth

DNA composition was measured using propidium iodide (PI; cat. no. P3566, Thermo Fisher Scientific®, Waltham, MA, USA), flow cytometry (BD Accuri™ C6 Flow Cytometer, USA). The results were express as percentage of cells in each cell cycle phase (G0/G1, S, and G2/M). Cellular proliferation was measured by cell counting using a Neubauer Chamber. Undifferentiated cells reach the confluency at day 4, forming a monolayer. After this, cells continued to proliferate, as shown in Fig. 2a, but as floating cells.

Neurite Density

Neurite density was evaluated by immunofluorescence. Cells were washed with PBS, fixed with methanol/acetone solution (1:1) for 20 min in room temperature, and permeabilized with PBS/Tween 0.2%. The blocking was performed with 1% BSA solution for 1 h in room temperature. Then, cells were incubated with anti-βIII tubulin antibody (dilution, 1:250, Alexa 488 conjugated, cat. no. ab204605, Abcam®, Cambridge, UK) for 2 h in room temperature and with Nuclear dye DAPI (dilution, 0.25 μg/μL, cat. no. D1306, Thermo Fisher Scientific®, Waltham, Massachusetts, USA) for 5 min. Randomly selected images were captured using an Olympus IX70 inverted microscope and analyzed with NIS-element software. Neurite density was assessed using the AutoQuant Neurite software (implemented in *R*) and expressed as arbitrary units (A.U.) (Schönhofen et al. 2015).

Dopamine Immunoreactivity

Dopamine reactivity was evaluated using an anti-dopamine antibody (dilution, 1:250, cat. no. ab6427, Abcam®, Cambridge, UK) followed by incubation with Alexa 488-conjugated antibody (dilution, 1:500, cat. no. A11008, Thermo Fisher Scientific®, Waltham, MA, USA). Randomly selected images were captured using an EVOS FLoid® Cell Imaging (Korecka et al. 2013).

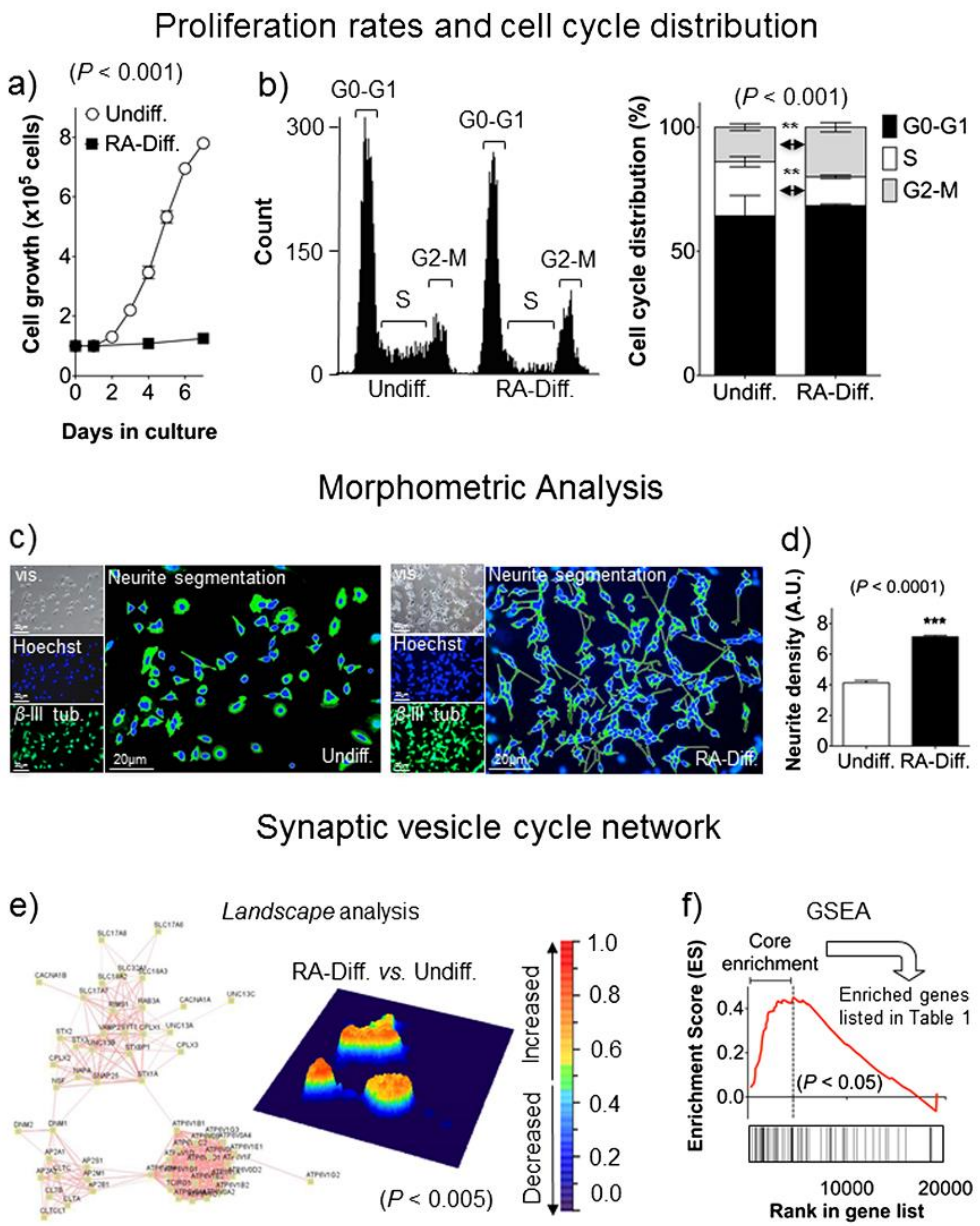


Fig. 2 Neuronal characterization of undifferentiated and RA-differentiated SH-SY5Y cells. **a** Cellular growth in undifferentiated and RA-differentiated cells. **b** Cell cycle analysis. Representative image of the cell cycle analysis in undifferentiated cells and RA-differentiated cells, in which results were expressed as percentage of cells in each cell cycle phase (G0/G1, S, and G2/M). Neurite density was evaluated by immunofluorescence. **c** Representative images of immunocytochemical detection of tubulin in undifferentiated and RA-differentiated SH-SY5Y cells. **d** Quantification of the neurite density per cell body using AutoQuant Neurite software. Expression of synaptic vesicle cycle network in undifferentiated and RA-differentiated SH-SY5Y cells. **e** STRING representation of synaptic vesicle cycle network gene interactions and landscape analysis, generated with ViaComplex® V1.0. Color gradient (*z*-axis), demonstrating elevated expression of this network in 7-day-RA-differentiated, compared with undifferentiated, SH-SY5Y cells. *P* value refers to bootstrap analysis comparing cell lines. **f** Enrichment analysis used to identify the genes that contributed individually to the global changes in expression levels observed in RA-differentiated cells in the syntactic vesicle cycle network. Data are presented as mean ± SD of four independent experiments ($n = 4$), each carried out in triplicates. $^*p < 0.05$ (Student's *t* test). Transcripts obtained as described in "Materials and Methods." Nominal *p* value of enrichment analysis obtained from GSEA ($p < 0.05$)

Cytotoxicity Parameters

Undifferentiated and RA-differentiated SH-SY5Y cells were treated for 24 h with 6-OHDA and H_2O_2. Cell viability were analyzed using 3-(4,5-dimethylthiazol- 2-yl)-2,5-diphenyltetrazolium bromide (MTT; cat. no. M5655, Sigma®) reduction assay as previously described (Lopes et al. 2010).

Oxidative Stress Parameters

We evaluated the redox status in both undifferentiated and RA-differentiated SH-SY5Y cells by measuring reduced thiol and reduced glutathione (GSH) levels as well as the following antioxidant enzyme activities: glutathione peroxidase (GPx), catalase (CAT), superoxide dismutase (SOD), thioredoxin reductase (TrxR), glutathione reductase (GR), and glutathione-*S*-transferase (GST) as described previously (Lopes et al. 2012). H_2O_2 generation was measured using AmplexRed® (cat. no. a12222, Thermo Fisher Scientific®, Waltham, MA, USA).

Reducing Thiol Agents Treatment

The role of reducing agents in 6-OHDA cytotoxicity was assessed via pre-treatment with dithiothreitol (DTT; cat. no. D0632, Sigma®) or *tris*(2-carboxyethyl)phosphine (TCEP; cat. no. C4706, Sigma®) in both cell models for 1 h in 37 °C. Cells were then incubated with the median toxic dose (TD_{50}) of 6-OHDA. The cytotoxicity was analyzed using MTT reduction.

DAT Immunocontent

To evaluate changes in DAT immunocontent during the RA-differentiation process, western blot analysis was performed using anti-DAT antibody (dilution, 1:1000; cat. no. 9299, Santa Cruz® Biotechnology, Dallas, TX, USA) and rabbit anti-glyceraldehyde-3-phosphate dehydrogenase (GAPDH) antibody (dilution, 1:5000; cat. no. ab9485, Abcam®, Cambridge, UK) as loading control.

Molecular Docking

The calculations performed in this study have taken full advantage of the X-ray crystal structure of the *Drosophila melanogaster* DAT (PDB ID 4M48) at 3.0 Å of resolution (Penmatsa et al. 2013).

Molecular docking was performed using Autodock4 and the protocol-adopted validated through the redocking of nortriptyline in the DAT binding site, as describe elsewhere (Halperin et al. 2002; Mohammad et al. 2008), which was employed to obtain the molecular structures of dopamine, 6-OHDA, *p*-quinone and 3α-bis-4-fluorophenylmethoxytropane (DATi; cat. no. 0918, Tocris®, Avonmonth, Bristol, UK) for docking input. Upon completion, a thousand poses were obtained (50 poses per output) and clustered within a RMSD tolerance of 1.0 Å using Autodock Tools. The best results obtained were based upon visual inspection and the calculated binding energy. Binding energy (E_{OPT}) was recalculated, using Forcite code, through the equation "E_{OPT} = EDAT + L − (EDAT + EL)" where EDAT + L is the total energy of the system formed by ligand bond in DAT; EDAT is the total energy of the DAT alone, while EL is the total energy of the ligand molecule alone.

DAT Pharmacological Inhibition

To investigate the DAT dependency of 6-OHDA-induced cell death in both models, cells were pre-incubated for 30 min with 20 μM of DATi (cat. no. 0918, Tocris®, Avonmonth, Bristol, UK). Following this, cells were exposed to TD_{50} 6-OHDA for 24 h (Lopes et al. 2010). Cell viability was assessed using MTT assay. H_2O_2 generation was measured using AmplexRed®.

Statistical Analysis

Data were expressed as means ± SD of at least three independent experiments carried out in triplicate, and P values were considered significant for $P < 0.05$. Differences within the experimental groups were determined by Student's t test or one-way analysis of variance (ANOVA). Comparison among means was carried out using Newman-Keuls multiple comparisons test as post hoc (GraphPad® Software 5.0).

Results

RA-Differentiation Protocol Induces Neuronal Features in SH-SY5Y Cells

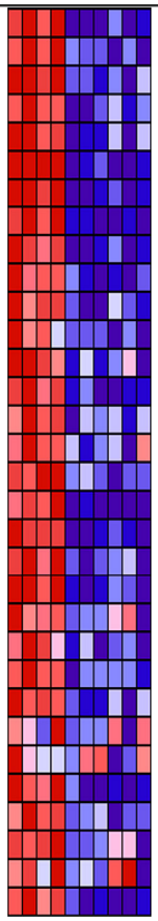
PD-target cells are neurons derived from *substantia nigra pars compacta*, which are specialized cells that process and transmit information through electrical and chemical synapses, with stellate morphology and do not undergo to cell divisions (Kandel 2013). To evaluate these relevant features to mimic more accurately the neuronal cell physiology, we explored the effect of RA-differentiation protocol on cell growth, morphology, and the expression of gene sets associated with cell cycle and synapse vesicle cycle (protocol description in Fig. 1).

Here, we showed a significant decrease in the proliferation rates of RA-differentiated SH-SY5Y cells (Fig. 2a) ($n = 3$; $P < 0.001$) mainly associated with a decrease in S phase in combination with an arrest in G2-M (Fig. 2b) ($n = 3$; $P < 0.001$). Further, we investigated gene expression of the

cell cycle network (KEGG pathways entry no. hsa04110) using microarray analysis in undifferentiated and RA-differentiated cells. Although no statically significant differences were observed between the two phenotypes, there are genes associated with G2-M arrest, such as *GDD45G* and *SMAD3* (Herrup and Yang 2007), upregulated in RA-differentiated SH-SY5Y cells as shown in Electronic Supplementary Material Fig. 1.

Upon the decrease in proliferation rate and cell cycle arrest, a significant change in morphology with increased neurite density was verified in RA-differentiated cells (Fig. 2c, d) ($n = 3$; $P < 0.0001$), suggesting a change from epithelial (as defined by ATCC for SH-SY5Y cells) (ATCC 2016) to a stellate neuronal morphology. After morphological characterization, we analyzed which cellular model possessed the appropriate molecular machinery to support the synaptic transmission, using the synaptic vesicle cycle gene list (extracted from KEGG pathways entry no. hsa04728). We found a significant enrichment of this gene set in RA-differentiated, compared with undifferentiated, SH-SY5Y cells (Fig. 2e, f) ($n = 4$; $P < 0.05$). Enriched genes are listed in Table 1.

Table 1 Core enrichment from the synaptic vesicle network in 7-day RA-differentiated SH-SY5Y cells compared with undifferentiated cells

Heat map	Gene symbol	Gene name
	*SLC18A1*	Solute carrier family 18 (vesicular), member 1
	ATP6V1G2	ATPase, H+ transporting, V1 subunit G2
	NSF	*N*-ethylmaleimide-sensitive factor
	ATP6V0D1	ATPase, H+ transporting, V0 subunit d1
	ATP6V0E2	ATPase, H+ transporting V0 subunit e2
	SNAP25	Synaptosomal-associated protein, 25 kDa
	ATP6V0E1	ATPase, H+ transporting, V0 subunit e1
	STXBP1	Syntaxin binding protein 1
	DNM1	Dynamin 1
	ATP6V1C1	ATPase, H+ transporting, V1 subunit C1
	DNM3	Dynamin 3
	CPLX3	Complexin 3
	CPLX1	Complexin 1
	AP2A2	Adaptor-related protein complex 2, alpha 2 sub.
	ATP6V0C	ATPase, H+ transporting, V0 subunit c
	RIMS1	Regulating synaptic membrane exocytosis 1
	STX3	Syntaxin 3
	ATP6V1H	ATPase, H+ transporting, V1 subunit H
	ATP6V1D	ATPase, H+ transporting, V1 subunit D
	AP2B1	Adaptor-related protein complex 2, beta 1 sub.
	ATP6V1B2	ATPase, H+ transporting, V1 subunit B2
	CACNA1B	Calcium channel, L type, alpha 1B subunit
	SLC18A3	Solute carrier family 18 (vesicular), member 3
	AP2M1	Adaptor-related protein complex 2, mu 1 subunit
	CLTC	Clathrin, heavy chain (Hc)
	SLC17A8	Solute carrier family 17, member 8
	ATP6V1G3	ATPase, H+ transporting, V1 subunit G3
	ATP6V1A	ATPase, H+ transporting, V1 subunit A
	CLTA	Clathrin, light chain (Lca)
	STX2	Syntaxin 2
	UNC13A	Unc-13 homolog A (*C. elegans*)
	ATP6V1E1	ATPase, H+ transporting, V1 subunit E1

Data generated with gene set enrichment analysis (GSEA) comparing 7-day RA-differentiated cells ($n = 4$) vs. undifferentiated SH-SY5Y cells ($n = 6$) transcripts obtained as described in "Materials and Methods"

RA-Differentiation Potentiates Dopaminergic Features in SH-SY5Y Cells

After studying the differences in general neuronal properties obtained from RA-differentiation protocol, we investigated dopaminergic features of both phenotypes of SH-SY5Y cells. At first, we evaluated global differences in gene expression of the dopaminergic synapse network, where we found no significant differences between the two models (Fig. 3a). However, there are genes upregulated in RA-differentiated cells listed in the Electronic Supplementary Material Table 1.

Using differential gene expression analysis, we verified the expression levels of the most common dopaminergic markers (Korecka et al. 2013), associated with catecholamine synthesis (dopa decarboxylase (*DDC*), GTP cyclohydrolase (*GCH1*), and *TH*), degradation (monoamine oxidase A (*MAOA*) and B (*MAOB*), catechol-*O*-methyltransferase (*COMT*)), and synaptic function (vesicular monoamine transporter 1 (*SLC18A1*) and 2 (*SLC18A2*), dopamine transporter (*SLC6A3*), and dopamine receptor D2 (*DRD2*)). Both models present the same level of expression in all genes studied except for *DRD2*, *GHC*, and *SLC18A1,* which have higher expression in the RA-differentiated cells (Fig. 3b).

Lastly, dopamine immunocontent was investigated using an immunocytochemical approach in both SH-SY5Y phenotypes. In Fig. 3c, we confirmed that both models have immunochemical detection of this neurotransmitter. Hence, in spite of both models of SH-SY5Y cells present dopamine content, neuronal dopaminergic features are potentiated after RA differentiation (e.g., *DRD2* and *SLC18A1*).

RA-Differentiation Induces Changes in Oxidative Status and 6-OHDA-Mediated Neurotoxicity in SH-SY5Y Cells

Due to the pivotal role played by reactive species in PD (Fariello 1988), the endogenous machinery responsible for the basal redox status should be characterized when establishing any relevant in vitro cell model of PD. To do so, we firstly evaluated the gene expression levels of the human antioxidant network (according to KEGG pathways). There were no differences in gene expression in antioxidant network. However, some antioxidant genes were upregulated in RA-differentiated cells (e.g., *GPX3*, *TMX4*, and *GRLX*) (Fig. 4a).

To better characterize these redox differences, we evaluated the activity of several enzymes involved in first- line antioxidant defenses and the level of non-enzymatic antioxidant defenses in both SH-SY5Y phenotypes. Our in vitro validation revealed that RA-differentiated cells have higher antioxidant enzyme activities and lower levels of H_2O_2 production (Table 2).

After investigating the basal redox metabolism in undifferentiated and RA-differentiated SH-SY5Y cells, we aimed to examine their susceptibility to oxidative stress induced by H_2O_2 and 6-OHDA. RA-differentiated SH-SY5Y cells were more resistant to H_2O_2, yet they were twofold more susceptible to 6-OHDA cytotoxicity (Table 2). It is well known that 6-OHDA toxicity acts via the induction of oxidative stress; however, the higher endogenous antioxidant capacity observed was not able to protect RA-differentiated cells from the cell death, suggesting a dissimilar mechanism of 6-OHDA detoxification in this cellular model.

The Role of Thiols in 6-OHDA-Induced-Cell Death in Undifferentiated and RA-Differentiated SH-SY5Y Cells

Previous data have shown that 6-OHDA uptake is not an essential process and the auto-oxidation occurs extracellularly in undifferentiated SH-SY5Y cells (Storch et al. 2000; Hanrott et al. 2006; Iglesias-González et al. 2012), suggesting that this toxin has different mechanisms from animal and primary culture models. Hence, in order to understand our previous results regarding the susceptibility of 6-OHDA in RA-differentiated cells, we evaluate the role of cell-permeable and cell-impermeable reducing agents in 6-OHDA-induced cell death.

We first pre-incubated undifferentiated and RA-differentiated cells with two thiol-reducing agents TCEP (a cell-impermeable compound) and DTT (a cell-permeable molecule), before challenging cells with 6-OHDA (Fig. 4b, d) (Hsu et al. 2005). Interestingly, no differences were found between both cellular models when TCEP were used to protect cells against 6-OHDA-oxidant insult (Fig. 4c). On the other hand, DTT was able to prevent 60% of 6-OHDA-dependent cytotoxicity in RA-differentiated cells (Fig. 4e) ($n = 3$; $P < 0.0005$), in contrast to only 24% in undifferentiated cells, indicating that, in RA-differentiated cells, an intracellular oxidation step of the neurotoxin is associated with the cell death caused by 6-OHDA (Fig. 4b, F (3, 8) = 126.5, $n = 3$; $P < 0.0001$).

The Role of DAT in 6-OHDA-Induced-Cell Death in Undifferentiated and RA-Differentiated SH-SY5Y Cells

To investigate more accurately the role of intracellular auto-oxidation, we evaluated the role of DAT in the toxicity induced by 6-OHDA in both cellular models because the activity of this transporter is fundamental for toxin uptake. Figure 5a shows an increase in DAT immunocontent in RA-differentiated cells ($n = 3$; $P < 0.01$).

We then verified whether the inhibition of this transporter interfered in the cell death caused by 6-OHDA. First, we examined how DATi and 6-OHDA interact with DAT by using molecular docking followed by classical refinement of geometries (Fig. 5b) and compared the binding energy (E_{OPT}) of those compounds with the corresponding values obtained for

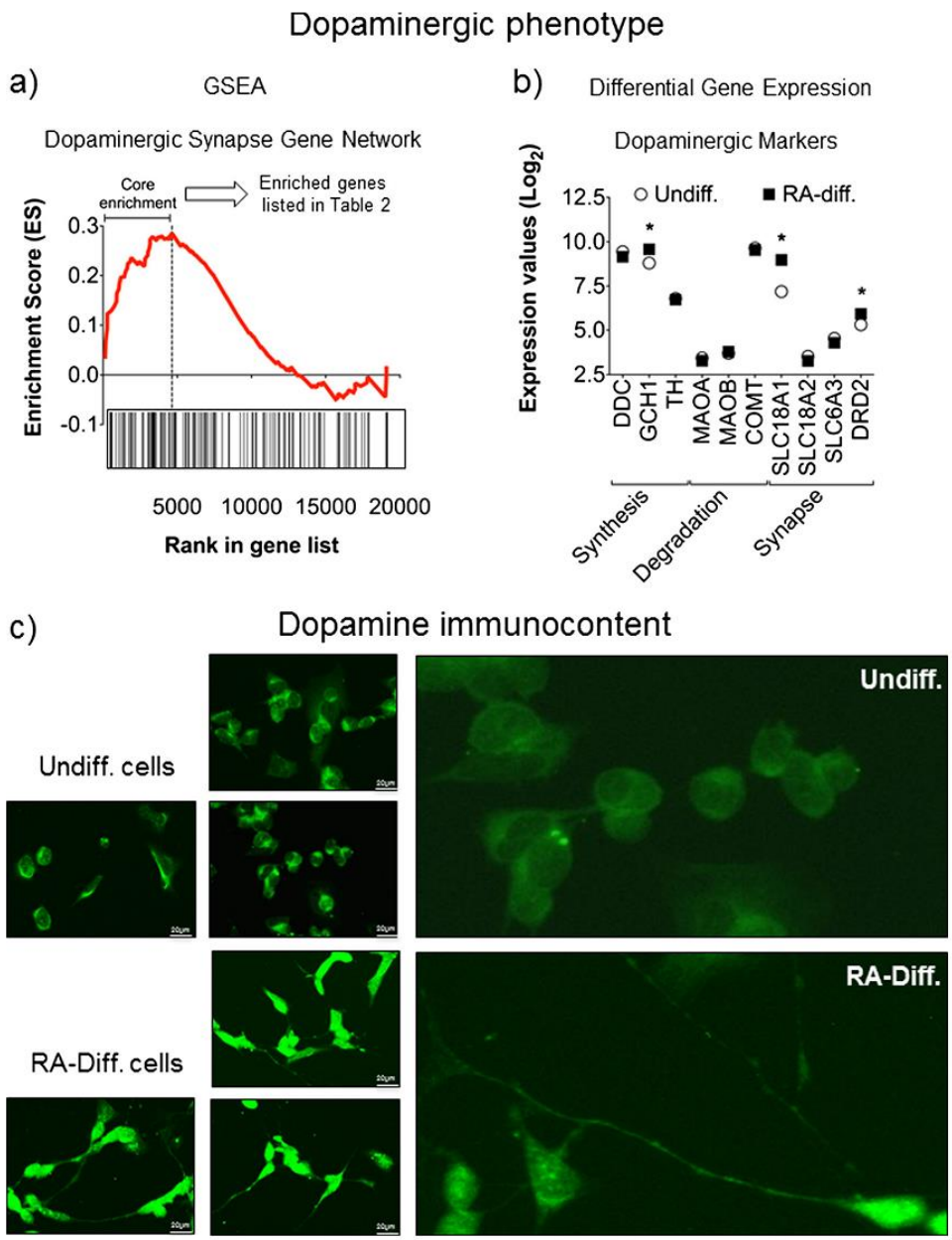


Fig. 3 Dopaminergic characterization of undifferentiated and RA-differentiated SH-SY5Y cells. **a** Enrichment analysis used to identify the genes that contributed individually to the global changes in expression levels observed in RA-differentiated cells in the dopaminergic synapse network using GSEA. **b** Differential expression levels of pre-synaptic dopaminergic markers in undifferentiated and RA-differentiated cells. **c** Immunocytochemical detection of dopamine. Representative fluorescence microscopy images of undifferentiated and RA-differentiated SH-SY5Y cells. Data are presented as mean ± SD of four independent experiments ($n = 4$), each carried out in triplicates ($n = 4$). *$P < 0.05$ (Student's t test)

dopamine and *p*-quinone (Electronic Supplementary Material Fig. 2 and Table 2 for the raw docking data). Our data suggests that DATi inhibits DAT by preventing substrate binding and stabilizing the outward-open conformation. Furthermore, we

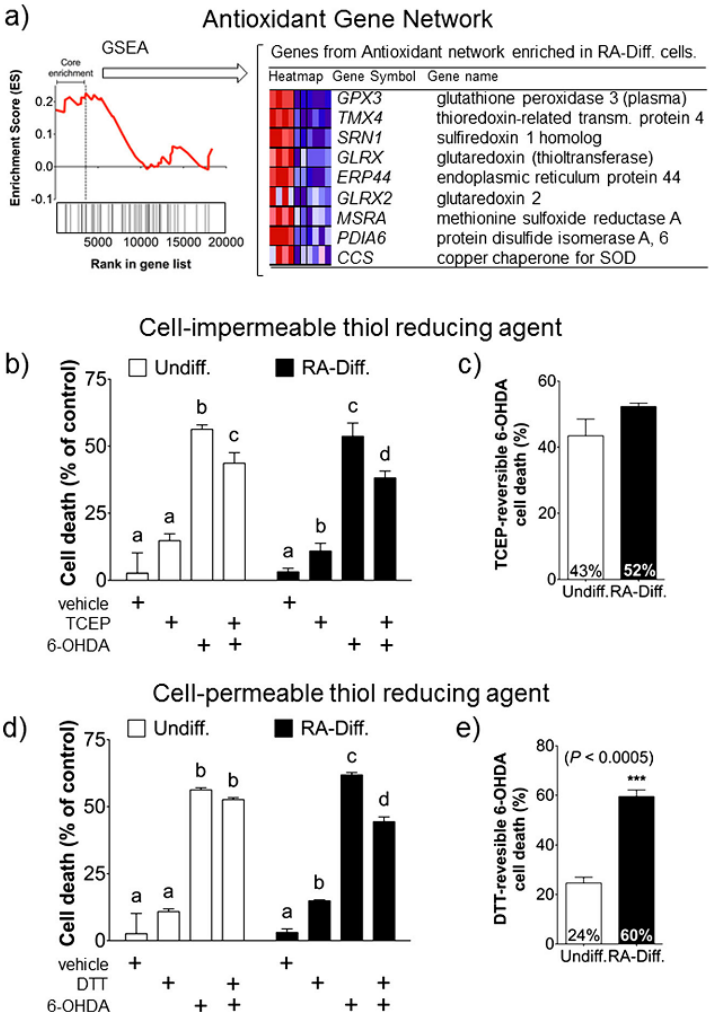


Fig. 4 Redox characterization of undifferentiated and RA-differentiated SH-SY5Y cells. **a** Enrichment analysis used to identify the genes that contributed individually to the global changes in expression levels observed in RA-differentiated cells in the antioxidant network using GSEA. The role of cell-impermeable (**b**) and cell-permeable (**d**) thiol-reducing agents pre-treatment in 6-OHDA-induced cell death in undifferentiated and RA-differentiated SH-SY5Y cells. The results were expressed as a percentage of the control ± SD. Significant differences are expressed by *letters*, where *equal letters* represent no significant differences and *different letters* represent significant differences ($P < 0.05$) (one-way analysis of variance). **c**, **e** Analysis of the inhibition of 6-OHDA-induced cell death for each thiol-reducing agent in both cellular models. Data are presented as mean ± SD of four independent experiments ($n = 4$), each carried out in triplicates. *$P < 0.05$ (Student's t test)

found that dopamine, DATi, *p*-quinone, and 6-OHDA, all compete sterically for the same binding site via the spatial blockage of Asp46 residue (Asp79 in DAT from *Homo sapiens*). This steric blockage of the same binding site demonstrates a competitive inhibition mechanism of action for DATi (Fig. 5b). Due to the lower ligation energy of DATi for DAT in comparison with *p*-quinone and 6-OHDA, but higher for dopamine, our docking data showed that DATi blocks completely the interaction of dopamine with DAT, but only partially in *p*-quinone and 6-OHDA (Electronic Supplementary Material Table 2). Thus, it suggests that DATi inhibits DAT by preventing substrate binding and stabilizing the outward-open conformation.

Based on these findings, we pharmacologically inhibited DAT in both cellular models via incubation with DATi prior to challenging cells with 6-OHDA. Our data showed that DAT inhibition resulted in a significant decrease in cell death (41%) (Fig. 5f) and H_2O_2 production (48%) (Fig. 5d) by 6-OHDA treatment only in RA-differentiated cells with no effect observed in undifferentiated cells, suggesting a specific role played by DAT in the cell death caused by this neurotoxin in the neuronal phenotype (Fig. 5c, F (3, 12) = 9.571, n = 3; $P < 0.01$) (Fig. 5e, F (3, 8) = 201.4, n = 3; $P < 0.0001$).

Discussion

The difficulty in mimicking neuronal features in vitro has always been an issue in neuroscience studies, thus the development of more suitable models is necessary since they are

Table 2 In vitro evaluation of redox parameters in undifferentiated and 7-day RA-differentiated human SH-SY5Y neuroblastoma cells

	Undifferentiated	RA-differentiated	Fold change	*P*
Antioxidant enzymes defenses				
CAT (U/mg)	0.43 ± 0.07	**1.43 ± 0.16**	3.33	0.046
GPx (U/mg)	2.83 ± 0.50	3.85 ± 0.99	1.36	0.2336
SOD (U/mg)	10.18 ± 4.42	19.52 ± 3.09	1.92	0.0803
GR (nmol/mg)	16.47 ± 1.86	**25.46 ± 1.94**	1.55	0.0291
TrxR (nmol/mg)	**23.51 ± 1.59**	11.08 ± 0.54	0.47	0.0003
GST (U/mg)	9.96 ± 2.57	**25.31 ± 1.62**	2.54	0.0031
Non-enzymatic defenses				
Thiol levels (nmol/mg)	17.57 ± 3.95	**39.16 ± 3.70**	2.23	0.0026
GSH levels (nmol/mg)	**16.39 ± 1.00**	6.96 ± 0.98	0.42	0.0008
Reactive species				
H_2O_2 production (RFU/min/cell)	**13.05 ± 0.47**	9.57 ± 0.60	0.73	0.0024
TD_{50} (μM)				
H_2O_2	573.37 ± 31.52	**740.00 ± 30.55**	1.29	0.0024
6-OHDA	**35.00 ± 2.03**	15.00 ± 0.866	0.43	0.0001

Data represent mean ± SD of at least four independent experiments ($n = 4$). *P* values indicate statistic differences between experimental groups (Student's *t* test). Fold changing indicates the ratio of values found in RA-differentiated to undifferentiated cells

Bold entries indicate which phenotype has higher absolute values when there is statistical significance in oxidative stress parameters

Abbreviations: *CAT* catalase, *GPx* glutathione peroxidase, *SOD* superoxide dismutase, *GR* glutathione reductase, *TrxR* thioredoxin reductase, *GST* glutathione-*S*-transferase, *GSH* glutathione, TD_{50} median toxic dose, *6-OHDA* 6-hydroxydopamine

fundamental to study molecular mechanism of neurodegenerative disease, such as PD. In this regard, the most in vitro experimental model used for PD is the human neuroblastoma SH-SY5Y cell because they express dopaminergic markers and are easy to cultivate when compared with other models (Xie et al. 2010; Kovalevich and Langford 2013). We previously established a catecholaminergic differentiation protocol for this cell line (Lopes et al. 2010). Here, we focused in explore neuronal features in both cellular models.

There are many lines of evidence showing the effect of RA differentiation in SH-SY5Y regarding the evaluation of proliferation rates (Ross 1996; Pezzini et al. 2016; Kunzler et al. 2016). Previous studies have demonstrated that RA-induced differentiation can cause cell cycle arrest either in G1/G0 phase or in G2/M phase and a decrease in proliferation rates, which leads to terminal differentiation of neuroblastoma cells (Qiao et al. 2012; Hämmerle et al. 2013).

We verified decreased cellular growth in RA-differentiated cells was associated with a decrease in S phase in combination with G2-M arrest (Fig. 2b). This data corroborates with our findings regarding gene expression of the cell cycle network. Genes upregulated in RA-differentiated cells are associated with cell cycle arrest, for instance, cyclin-dependent protein kinases (CDK) inhibitors (e.g., p18, p19, p21, and p27) and to G2-M arrest, such as *GDD45G* and *SMAD3* (Herrup and Yang 2007) (summarized in the Electronic Supplementary Material Fig. 1). Moreover, the cell cycle arrest in G2-M is commonly found in neurodegenerative diseases such as PD, where some populations of neurons complete DNA synthesis and are able to pass through the S phase but are arrested at the G2/M (Frade and Ovejero-Benito 2015).

Another important neuronal parameter is cellular morphology. Neurons present neurites, which refers to axons and dendrites extended by neuronal cell lines, thus their quantification is an important morphological parameter of neuronal differentiation (Radio and Mundy 2008; Bal-Price et al. 2010). Here, we showed an increase in neurite density in RA-differentiated cells, representing a significant advantage of this cellular model, since these structures form synapses and can be used as an endpoint in neurotoxicological evaluations (Lopes et al. 2012).

Besides low proliferation rates and stellate morphology, dopaminergic neuronal cells process their information through chemical synapses. The biological event related to neurotransmitter release is the synaptic vesicle cycle (Kandel 2013). This pathway consists of exocytosis followed by endocytosis and recycle (Rizo and Xu 2015). At first, vesicles are loaded with neurotransmitters, which require the presence of an active transporter along with a proton pump to provide the required pH and electrochemical gradients. Fundamental to this is the role of H^+-ATPase transporters and solute carriers such as *SLC18A1*, *SLC18A3*, and *SLC17A8* (Beyenbach and Wieczorek 2006). Once the vesicles are loaded, they are tethered near to the

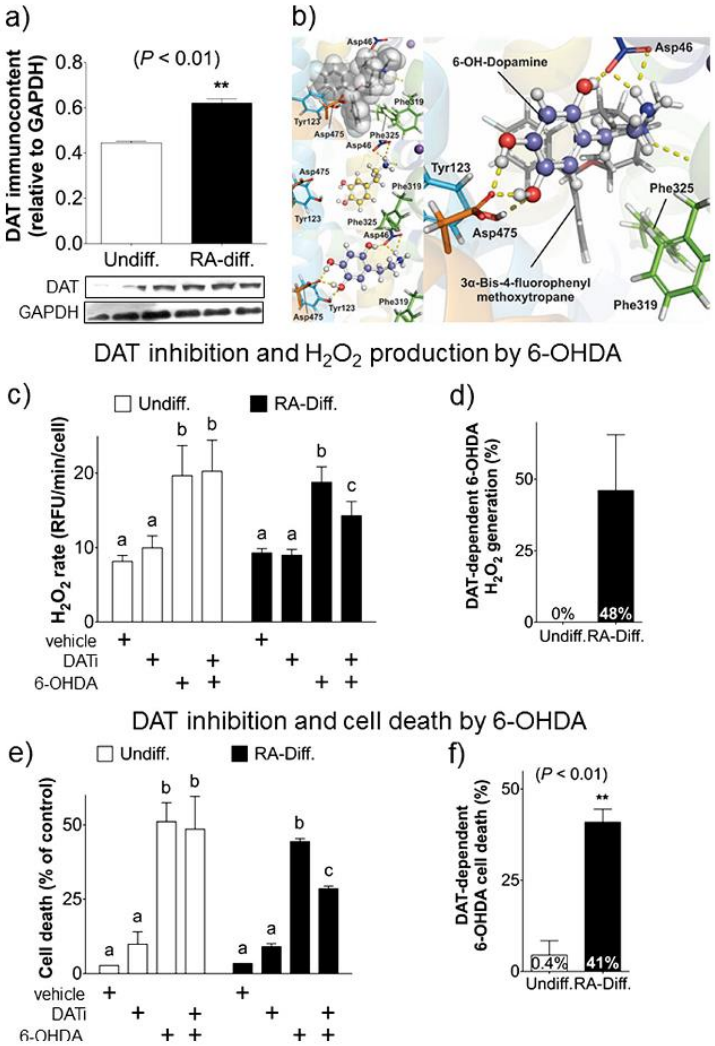


Fig. 5 Evaluation of the role of DAT in 6-OHDA-induced cell death in undifferentiated and RA-differentiated SH-SY5Y cells. **a** Changes in DAT immunocontent (dopaminergic cell marker) in response to RA differentiation was evaluated using Western blot. Representative densitometric analysis of bands and immunoblot of DAT, using GAPDH as loading control. Results were calculated and expressed as mean ± SD of densitometric units ($n = 4$). *$P < 0.01$ (Student's t test). **b** Superposition of DATi and 6-OHDA into the binding site of DAT, showing how 6-OHDA is spatially blocked from forming a salt bridge with Asp46. **c** Evaluation of DAT inhibition in the rate of H_2O_2 production, DAT-dependent H_2O_2 generation, and **e** cell death in undifferentiated and RA-differentiated SH-SY5Y cells challenged with 6-OHDA. Cells were treated for 30 min with DATi prior to incubation with TD_{50} concentration of 6-OHDA for 24 h. Cell viability was evaluated using the MTT reduction assay and results were expressed as percentage of untreated cells. Significant differences are expressed by *letters*, where *equal letters* represent no significant differences and *different letters* represent significant differences ($P < 0.05$; one-way analysis of variance). **d**, **f** DAT-dependent 6-OHDA-induced cell death in both cellular models. Data are presented as mean ± SD of four independent experiments, each carried out in triplicates ($n = 4$). *$P < 0.05$ (Student's t test)

release sites, after which vesicles are primed before being ready to undergo fusion. Genes involved in this process include *UNC13*, *RIMS1*, and syntaxin (Madison et al. 2005). The primed vesicles subsequently undergo fusion processes that are regulated by SNARE proteins, such as SNAP-25, NSF, and complexins (Hu et al. 2002). Finally, the synaptic vesicles incorporated to the plasma membrane are retrieved by endocytosis, a process which involves many proteins, e.g., dynamins and clathrins (Takei et al. 1996). Our results demonstrated that all of these genes were upregulated in RA-differentiated cells (Fig. 2e, f; Table 1), suggesting that this model has appropriate machinery to support synapses.

Our data point to highly diverse phenotypes presented by both cellular models. Undifferentiated cells exhibited characteristics typical of a tumoral phenotype, namely epithelial morphology and high proliferation rates. In contrast, RA-differentiated SH-SY5Y cells were characteristic of a neuronal phenotype, presenting low proliferation rates, a pronounced neuronal morphology, and an enrichment of the molecular machinery responsible for synaptic function.

After neuronal characterization, we aimed to verify if both cellular models have dopaminergic phenotype, since these cells are the most affected neurons in PD. Here, we demonstrated that both phenotypes of SH-SY5Y cells expressed the dopaminergic machinery. This was expected since it is well known that neuroblastoma cancers (as the primary tumor that SH-SY5Y cells were isolated from) produce catecholamines, mainly because they have low levels of dopaminergic markers (Howman-Giles et al. 2007). As such, undifferentiated cells are commonly used as PD model (Xie et al. 2010).

Previous data showed that the differentiation process does not lead to increase of dopaminergic markers in SH-SY5Y cells, which brings the discussion whether they need to be differentiated (Luchtman and Song 2010). On the other hand, many lines of evidence showed that RA-differentiated cells increase their expression of these dopaminergic markers, such as TH and DAT (Påhlman et al. 1984; Lopes et al. 2010; Filograna et al. 2015). These discrepancies in the literature might be attributable to the varying differentiation protocols used, since there are differences between them, such as duration, cell densities, serum concentration and differentiation agent (e.g., RA, staurosporine, and BDNF) (Cheung et al. 2009; Agholme et al. 2010; Lopes et al. 2010; Filograna et al. 2015).

Hence, our results show that both models have the machinery necessary to synthesize and release dopamine. Although no global statistically significant differences were observed between the two phenotypes, there are genes associated with dopamine synthesis regulation (*PKA*, *MAPK*, *CAMKII*, and *PP2A*) significantly upregulated in RA-differentiated SH-SY5Y cells (Fig. 3a; Electronic Supplementary Material Table 1) (Dunkley et al. 2004; Daubner et al. 2011). Moreover, differential expression showed significant increase in GHC1, *DRD2,* and *SLC18*, three important catecholaminergic markers. These findings demonstrated that RA differentiation potentiates the dopaminergic phenotype, which validates our protocol and its potential use as PD in vitro model.

Since dopaminergic neurons are exposed to a chronic oxidative damage, mostly attributed to the high levels of iron present in SNpc, the hydroxyl radical ($HO^{\cdot}$) produced by dopamine metabolism (Zhou et al. 2010), oxidative stress is thought to contribute to the pathogenesis of progressive neurodegeneration observed in PD (Fariello 1988). Hence, oxidative stress parameters should be investigated when establishing in vitro cell model of PD. Our in vitro validation revealed that both models have thioredoxin and glutathione antioxidant systems as the main antioxidant defense. The H_2O_2 detoxification in neuronal cells is catalyzed primarily by thioredoxin and glutathione systems, which are the most important antioxidants in the brain (Lopert et al. 2012; Garcia-Garcia et al. 2012); hence, we found that both models mimic the oxidative neuronal profile.

Moreover, we showed that RA-differentiated cells presented a higher basal antioxidant capacity and decrease of H_2O_2 production. At first, these data seem controversial because neuronal cells present low antioxidant levels (Halliwell 2006; Dexter and Jenner 2013); hence, the differentiated cells do not represent accurately the physiology of dopaminergic neurons. However, here, we are comparing the neuronal and tumoral phenotypes. The oxidative environment of the undifferentiated cells could be explained by its proliferative profile since H_2O_2 is fundamental for cellular growth (Policastro et al. 2004; Sies 2014).

Here, the most intriguing observation was that RA-differentiated SH-SY5Y cells were more resistant to H_2O_2, yet were more susceptible to 6-OHDA cytotoxicity (Table 2). Cellular resistance to H_2O_2 in the neuronal phenotype can be explained by the elevated basal antioxidant capacity. Since the RA-induced differentiation decreases levels of TrxR and GSH, this may suggest a role of these antioxidants in 6-OHDA detoxification, as previously described (Soto-Otero et al. 2000; Lopert et al. 2012). Hence, the resistance to 6-OHDA found in undifferentiated cells can be explained, at least in part, by the high GSH levels presented in the tumoral phenotype.

It is widely elucidated that 6-OHDA is taken up by dopaminergic neurons via DAT (Tranzer and Thoenen 1973) and auto-oxidation process occurs intracellularly (Glinka et al. 1997) mainly because the toxicity can be blocked by DAT inhibition (González-Hernández et al. 2004). On the other hand, previous data have shown that 6-OHDA uptake is not an essential process and the auto-oxidation occurs extracellularly in undifferentiated cells (Izumi et al. 2005). Here, we found that part of the oxidative dysfunction caused by 6-OHDA involves the uptake of the neurotoxin (or some metabolite, such as *p*-quinones) presumably followed by intracellular auto-oxidation in RA-differentiated cells.

Further investigation about intracellular oxidation demonstrated that pharmacological DAT inhibition decreases H_2O_2 production and cellular death caused by 6-OHDA only in RA-differentiated SH-SY5Y cells. Regarding undifferentiated SH-SY5YS cells, DAT inhibition did not protect the cells, possibly because these cells have low levels of DAT (Presgraves et al. 2004), which corroborates with previous results (Storch et al. 2000; Izumi et al. 2005).

These results may impact the development of new therapies and drugs for the management of the disease. To date, PD is still an incurable disease and we have failed to find

neuroprotective compounds (Olanow et al. 2008). The main reason to this issue is the lack of understanding of the initial steps underlying dopaminergic degeneration (Obeso et al. 2010). Although PD is considered a complex disorder where many mechanisms are involved (e.g., protein aggregation, mitochondria dysfunction, and oxidative stress), the common pathology found in all PD cases is the dopaminergic degeneration (Gibb 1991). Hence, the development of better dopaminergic cell models and the understanding of dopaminergic cell physiology are essential for PD research. In spite that many lines of evidence have shown that undifferentiated SH-SY5Y cells are dopaminergic-producing cells and easy to cultivate (Presgraves et al. 2004; Cheung et al. 2009; Agholme et al. 2010; Lopes et al. 2010), they do not reproduce both dopaminergic physiology and 6-OHDA-induced-cell death mechanisms of in vivo or primary cell culture studies. Thus, SH-SY5Y cells are the target of many discussions whether it should be used in PD research. Our data suggests, for the first time, the role of toxin uptake by DAT in RA-differentiated cells, showing that an easy cellular model can mimic, at least in part, 6-OHDA-induced cell death in vivo.

Conclusions

Undifferentiated and RA-differentiated SH-SY5Y cells are two unique phenotypes which can be distinguished by differences found in cell morphology, cell growth, neuronal and dopaminergic marker expression, and redox metabolism. These features may contribute towards two different mechanisms of action for 6-OHDA cytotoxicty observed in both models. In the neuronal phenotype, we demonstrated DAT dependency in 6-OHDA-induced cell death, which is likely related to their dopaminergic phenotype. Many previous studies have used undifferentiated cells as a PD model to study molecular mechanisms, to test potential drugs for the treatment of this disease, and also to evaluate 6-OHDA's mechanisms of action and cellular targets. However, our data demonstrate that undifferentiated cells does not possess neuronal properties, which can create significant bias in such studies, and may have contributed, at least in part, to the limitations in our understanding of PD pathophysiology and, consequently, the lack of potential drugs to treat the disease. Hence, our data support the use of RA-differentiated cells as an in vitro model of PD.

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Author Contributions F.M.L., L.L.M., L.F.S., D.M.V., P.S., G.F.L., and L.M. performed experiments. B.P. and B.W.A. performed the RNA extraction for the microarray analysis. G.Z. and V.N.F. performed the molecular docking. F.M.L., L.L.M., M.A.D.B., M.A.A.C., R.B.P., A.L.D., and F.K. analyzed and interpreted the data. F.M.L. and F.K. conceived and designed the experiments. F.M.L. and F.K. wrote the manuscript.

Compliance with Ethical Standards

Conflict of Interest The authors declare that they have no competing interests.

References

Abad F, Maroto R, López MG et al (1995) Pharmacological protection against the cytotoxicity induced by 6-hydroxydopamine and H_2O_2 in chromaffin cells. Eur J Pharmacol 293:55–64

Agholme L, Lindström T, Kågedal K et al (2010) An in vitro model for neuroscience: differentiation of SH-SY5Y cells into cells with morphological and biochemical characteristics of mature neurons. J Alzheimers Dis 20:1069–1082. doi:10.3233/JAD-2010-091363

ATCC (2016) http://www.atcc.org/Products/All/CRL-2266.aspx. Accessed 19 May

Bal-Price AK, Hogberg HT, Buzanska L, Coecke S (2010) Relevance of in vitro neurotoxicity testing for regulatory requirements: challenges to be considered. Neurotoxicol Teratol 32:36–41. doi:10.1016/j.ntt.2008.12.003

Beyenbach KW, Wieczorek H (2006) The V-type H+ ATPase: molecular structure and function, physiological roles and regulation. J Exp Biol 209:577–589. doi:10.1242/jeb.02014

Biedler JL, Roffler-Tarlov S, Schachner M, Freedman LS (1978) Multiple neurotransmitter synthesis by human neuroblastoma cell lines and clones. Cancer Res 38:3751–3757

Cerruti C, Walther DM, Kuhar MJ, Uhl GR (1993) Dopamine transporter mRNA expression is intense in rat midbrain neurons and modest outside midbrain. Brain Res Mol Brain Res 18:181–186

Cheung Y-T, Lau WK-W, Yu M-S et al (2009) Effects of all-trans-retinoic acid on human SH-SY5Y neuroblastoma as in vitro model in neurotoxicity research. Neurotoxicology 30:127–135. doi:10.1016/j.neuro.2008.11.001

Daubner SC, Le T, Wang S (2011) Tyrosine hydroxylase and regulation of dopamine synthesis. Arch Biochem Biophys 508:1–12. doi:10.1016/j.abb.2010.12.017

Dexter DT, Jenner P (2013) Parkinson disease: from pathology to molecular disease mechanisms. Free Radic Biol Med 62:132–144. doi:10.1016/j.freeradbiomed.2013.01.018

Dunkley PR, Bobrovskaya L, Graham ME et al (2004) Tyrosine hydroxylase phosphorylation: regulation and consequences. J Neurochem 91:1025–1043. doi:10.1111/j.1471-4159.2004.02797.x

Fariello RG (1988) Experimental support for the implication of oxidative stress in the genesis of parkinsonian syndromes. Funct Neurol 3:407–412

Filograna R, Civiero L, Ferrari V et al (2015) Analysis of the catecholaminergic phenotype in human SH-SY5Y and BE(2)-M17 neuroblastoma cell lines upon differentiation. PLoS One 10:e0136769. doi:10.1371/journal.pone.0136769

Forster JI, Köglsberger S, Trefois C et al (2016) Characterization of differentiated SH-SY5Y as neuronal screening model reveals increased oxidative vulnerability. J Biomol Screen. doi:10.1177/1087057115625190

Frade JM, Ovejero-Benito MC (2015) Neuronal cell cycle: the neuron itself and its circumstances. Cell Cycle 14:712–720. doi:10.1080/15384101.2015.1004937

Garcia-Garcia A, Zavala-Flores L, Rodriguez-Rocha H, Franco R (2012) Thiol-redox signaling, dopaminergic cell death, and Parkinson's disease. Antioxid Redox Signal 17:1764–1784. doi:10.1089/ars.2011.4501

Gibb WR (1991) Neuropathology of the substantia nigra. Eur Neurol 31(Suppl 1):48–59

Glinka Y, Gassen M, Youdim MB (1997) Mechanism of 6-hydroxydopamine neurotoxicity. J Neural Transm Suppl 50:55–66

González-Hernández T, Barroso-Chinea P, De La Cruz MI et al (2004) Expression of dopamine and vesicular monoamine transporters and differential vulnerability of mesostriatal dopaminergic neurons. J Comp Neurol 479:198–215. doi:10.1002/cne.20323

Haggarty SJ, Perlis RH (2014) Translation: screening for novel therapeutics with disease-relevant cell types derived from human stem cell models. Biol Psychiatry 75:952–960. doi:10.1016/j.biopsych.2013.05.028

Halliwell B (2006) Oxidative stress and neurodegeneration: where are we now? J Neurochem 97:1634–1658. doi:10.1111/j.1471-4159.2006.03907.x

Halperin I, Ma B, Wolfson H, Nussinov R (2002) Principles of docking: an overview of search algorithms and a guide to scoring functions. Proteins 47:409–443. doi:10.1002/prot.10115

Hämmerle B, Yañez Y, Palanca S et al (2013) Targeting neuroblastoma stem cells with retinoic acid and proteasome inhibitor. PLoS One 8:e76761. doi:10.1371/journal.pone.0076761

Hanrott K, Gudmunsen L, O'Neill MJ, Wonnacott S (2006) 6-hydroxydopamine-induced apoptosis is mediated via extracellular auto-oxidation and caspase 3-dependent activation of protein kinase Cdelta. J Biol Chem 281:5373–5382. doi:10.1074/jbc.M511560200

Hartfield EM, Yamasaki-Mann M, Ribeiro Fernandes HJ et al (2014) Physiological characterisation of human iPS-derived dopaminergic neurons. PLoS One 9:e87388. doi:10.1371/journal.pone.0087388

Herrup K, Yang Y (2007) Cell cycle regulation in the postmitotic neuron: oxymoron or new biology? Nat Rev Neurosci 8:368–378. doi:10.1038/nm2124

Howman-Giles R, Shaw PJ, Uren RF, Chung DKV (2007) Neuroblastoma and other neuroendocrine tumors. Semin Nucl Med 37:286–302. doi:10.1053/j.semnuclmed.2007.02.009

Hsu M-F, Sun S-P, Chen Y-S et al (2005) Distinct effects of N-ethylmaleimide on formyl peptide- and cyclopiazonic acid-induced Ca2+ signals through thiol modification in neutrophils. Biochem Pharmacol 70:1320–1329. doi:10.1016/j.bcp.2005.07.029

Hu K, Carroll J, Rickman C, Davletov B (2002) Action of complexin on SNARE complex. J Biol Chem 277:41652–41656. doi:10.1074/jbc.M205044200

Iglesias-González J, Sánchez-Iglesias S, Méndez-Álvarez E et al (2012) Differential toxicity of 6-hydroxydopamine in SH-SY5Y human neuroblastoma cells and rat brain mitochondria: protective role of catalase and superoxide dismutase. Neurochem Res 37:2150–2160. doi:10.1007/s11064-012-0838-6

Izumi Y, Sawada H, Sakka N et al (2005) p-Quinone mediates 6-hydroxydopamine-induced dopaminergic neuronal death and ferrous iron accelerates the conversion of p-quinone into melanin extracellularly. J Neurosci Res 79:849–860. doi:10.1002/jnr.20382

Kandel E (2013) Principles of neural science, fifth edition

KEGG Pathway Database (2016) http://www.genome.jp/kegg/pathway.html.

Korecka JA, van Kesteren RE, Blaas E et al (2013) Phenotypic characterization of retinoic acid differentiated SH-SY5Y cells by transcriptional profiling. PLoS One 8:e63862. doi:10.1371/journal.pone.0063862

Kovalevich J, Langford D (2013) Considerations for the use of SH-SY5Y neuroblastoma cells in neurobiology. Methods Mol Biol 1078:9–21. doi:10.1007/978-1-62703-640-5_2

Kunzler A, Zeidan-Chulia F, Gasparotto J et al (2016) Changes in cell cycle and up-regulation of neuronal markers during SH-SY5Y Neurodifferentiation by retinoic acid are mediated by reactive species production and oxidative stress. Mol Neurobiol. doi:10.1007/s12035-016-0189-4

Lin C-Y, Tsai C-W (2016) Carnosic acid attenuates 6-hydroxydopamine-induced neurotoxicity in SH-SY5Y cells by inducing autophagy through an enhanced interaction of Parkin and Beclin1. Mol Neurobiol. doi:10.1007/s12035-016-9873-7

Ljungdahl A, Hökfelt T, Jonsson G, Sachs C (1971) Autoradiographic demonstration of uptake and accumulation of 3H-6-hydroxydopamine in adrenergic nerves. Experientia 27:297–299

Lopert P, Day BJ, Patel M (2012) Thioredoxin reductase deficiency potentiates oxidative stress, mitochondrial dysfunction and cell death in dopaminergic cells. PLoS One 7:e50683. doi:10.1371/journal.pone.0050683

Lopes FM, Londero GF, de Medeiros LM et al (2012) Evaluation of the neurotoxic/neuroprotective role of organoselenides using differentiated human neuroblastoma SH-SY5Y cell line challenged with 6-hydroxydopamine. Neurotox Res 22:138–149. doi:10.1007/s12640-012-9311-1

Lopes FM, Schröder R, da Frota MLC et al (2010) Comparison between proliferative and neuron-like SH-SY5Y cells as an in vitro model for Parkinson disease studies. Brain Res 1337:85–94. doi:10.1016/j.brainres.2010.03.102

Luchtman DW, Song C (2010) Why SH-SY5Y cells should be differentiated. Neurotoxicology 31:164–165 . doi:10.1016/j.neuro.2009.10.015author reply 165–6

Madison JM, Nurrish S, Kaplan JM (2005) UNC-13 interaction with syntaxin is required for synaptic transmission. Curr Biol 15:2236–2242. doi:10.1016/j.cub.2005.10.049

Mohammad MK, Al-Masri IM, Taha MO et al (2008) Olanzapine inhibits glycogen synthase kinase-3beta: an investigation by docking simulation and experimental validation. Eur J Pharmacol 584:185–191. doi:10.1016/j.ejphar.2008.01.019

Mullin S, Schapira AHV (2015) Pathogenic mechanisms of neurogeneration in Parkinson Disease. Neurol Clin 33:1–17. doi:10.1016/j.ncl.2014.09.010

Obeso JA, Rodriguez-Oroz MC, Goetz CG et al (2010) Missing pieces in the Parkinson's disease puzzle. Nat Med 16:653–661. doi:10.1038/nm.2165

Olanow CW (2009) Can we achieve neuroprotection with currently available anti-parkinsonian interventions? Neurology 72:S59–S64. doi:10.1212/WNL.0b013e318199068b

Olanow CW, Kieburtz K, Schapira AHV (2008) Why have we failed to achieve neuroprotection in Parkinson's disease? Ann Neurol 64(Suppl 2):S101–S110. doi:10.1002/ana.21461

Påhlman S, Ruusala AI, Abrahamsson L et al (1984) Retinoic acid-induced differentiation of cultured human neuroblastoma cells: a comparison with phorbolester-induced differentiation. Cell Differ 14:135–144

Penmatsa A, Wang KH, Gouaux E (2013) X-ray structure of dopamine transporter elucidates antidepressant mechanism. Nature 503:85–90. doi:10.1038/nature12533

Pezzini F, Bettinetti L, Di Leva F et al (2016) Transcriptomic profiling discloses molecular and cellular events related to neuronal differentiation in SH-SY5Y neuroblastoma cells. Cell Mol Neurobiol:1–18. doi:10.1007/s10571-016-0403-y

Policastro L, Molinari B, Larcher F et al (2004) Imbalance of antioxidant enzymes in tumor cells and inhibition of proliferation and malignant features by scavenging hydrogen peroxide. Mol Carcinog 39:103–113. doi:10.1002/mc.20001

Presgraves SP, Ahmed T, Borwege S, Joyce JN (2004) Terminally differentiated SH-SY5Y cells provide a model system for studying neuroprotective effects of dopamine agonists. Neurotox Res 5:579–598

Qiao J, Paul P, Lee S et al (2012) PI3K/AKT and ERK regulate retinoic acid-induced neuroblastoma cellular differentiation. Biochem Biophys Res Commun 424:421–426. doi:10.1016/j.bbrc.2012.06.125

Radio NM, Mundy WR (2008) Developmental neurotoxicity testing in vitro: models for assessing chemical effects on neurite outgrowth. Neurotoxicology 29:361–376. doi:10.1016/j.neuro.2008.02.011

Rizo J, Xu J (2015) The synaptic vesicle release machinery. Annu Rev Biophys 44:339–367. doi:10.1146/annurev-biophys-060414-034057

Ross HJ (1996) The antiproliferative effect of trans-retinoic acid is associated with selective induction of interleukin-1 beta, a cytokine that directly inhibits growth of lung cancer cells. Oncol Res 8:171–178

Schapira AH, Mann VM, Cooper JM et al (1990) Anatomic and disease specificity of NADH CoQ1 reductase (complex I) deficiency in Parkinson's disease. J Neurochem 55:2142–2145

Schönhofen P, de Medeiros LM, Bristot IJ et al (2015) Cannabidiol exposure during neuronal differentiation sensitizes cells against redox-active neurotoxins. Mol Neurobiol 52:26–37. doi:10.1007/s12035-014-8843-1

Schüle B, Pera RAR, Langston JW (2009) Can cellular models revolutionize drug discovery in Parkinson's disease? Biochim Biophys Acta 1792:1043–1051. doi:10.1016/j.bbadis.2009.08.014

Sharow KA, Temkin B, Asson-Batres MA (2012) Retinoic acid stability in stem cell cultures. Int J Dev Biol 56:273–278. doi:10.1387/ijdb.113378ks

Sies H (2014) Role of metabolic H_2O_2 generation: redox signaling and oxidative stress. J Biol Chem 289:8735–8741. doi:10.1074/jbc.R113.544635

Soto-Otero R, Méndez-Alvarez E, Hermida-Ameijeiras A et al (2000) Autoxidation and neurotoxicity of 6-hydroxydopamine in the presence of some antioxidants: potential implication in relation to the pathogenesis of Parkinson's disease. J Neurochem 74:1605–1612

Storch A, Kaftan A, Burkhardt K, Schwarz J (2000) 6-hydroxydopamine toxicity towards human SH-SY5Y dopaminergic neuroblastoma cells: independent of mitochondrial energy metabolism. J Neural Transm 107:281–293

Subramanian A, Tamayo P, Mootha VK et al (2005) Gene set enrichment analysis: a knowledge-based approach for interpreting genome-wide expression profiles. Proc Natl Acad Sci U S A 102:15545–15550. doi:10.1073/pnas.0506580102

Takei K, Mundigl O, Daniell L, De Camilli P (1996) The synaptic vesicle cycle: a single vesicle budding step involving clathrin and dynamin. J Cell Biol 133:1237–1250

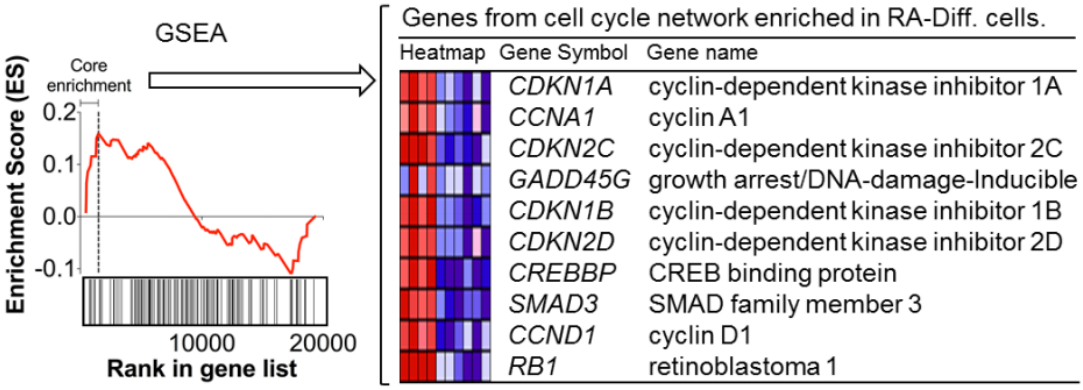
Tranzer JP, Thoenen H (1973) Selective destruction of adrenergic nerve terminals by chemical analogues of 6-hydroxydopamine. Experientia 29:314–315

Xie H, Hu L, Li G (2010) SH-SY5Y human neuroblastoma cell line: in vitro cell model of dopaminergic neurons in Parkinson's disease. Chin Med J 123:1086–1092

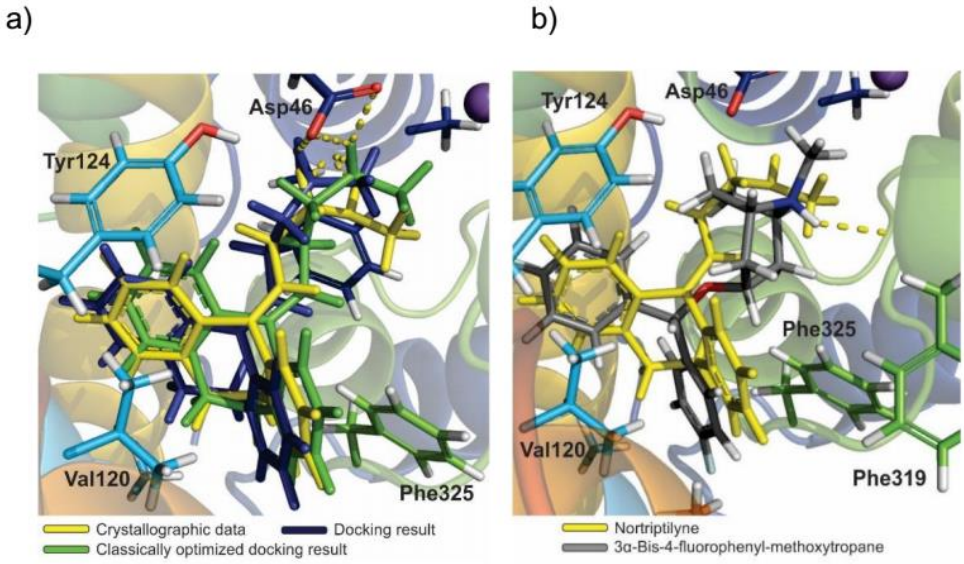
Zhou ZD, Lan YH, Tan EK, Lim TM (2010) Iron species-mediated dopamine oxidation, proteasome inhibition, and dopaminergic cell demise: implications for iron-related dopaminergic neuron degeneration. Free Radic Biol Med 49:1856–1871. doi:10.1016/j.freeradbiomed.2010.09.010

Title: RA-Differentiation Enhances Dopaminergic Features, Changes Redox Parameters and Increases Dopamine Transporter Dependency in 6-Hydroxydopamine-Induced-Neurotoxicity in SH-SY5Y cells

Authors: Fernanda M. Lopes, Leonardo L. da Motta, Marco A. De Bastiani, Bianca Pfaffenseller, Bianca W. Aguiar, Luiz F. de Souza, Geancarlo Zanatta, Daiani M. Vargas, Patrícia Schönhofen, Giovana F. Londero, Liana M. de Medeiros, Valder N. Freire, Alcir L. Dafre, Mauro A. A. Castro, Richard B. Parsons, Fabio Klamt.

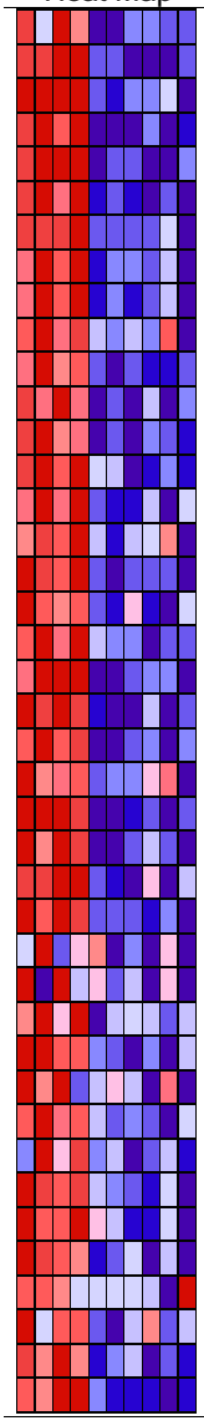


Supplementary Fig. 1: Enrichment analysis used to identify the genes that contributed individually to the global changes in expression levels observed in RA-differentiated cells in the cell cycle network. Data are presented as mean ± SD of four independent experiments (n = 4), each carried out in triplicates. Transcripts obtained as described in Materials and Methods section.



Supplementary Fig. 2. a) Redocking of nortriptilyne in the binding pocket of DAT to tune the docking machinery using the crystallographic ligand conformation. Nortriptilyne at crystallographic coordinates is represented in yellow, redocking using crystallographic conformation is represented in blue and refined redocking result using classical energy minimization procedure is shown in green. The resulting structure is representative of a cluster containing 991 out 1000 poses. Comparison with crystallographic coordinates showed RMSD value of 1.1260 Å for redocked structure and 0.7049 Å after classical refinement. Result obtained using the software Autodock 4.0. b) Superposition of 3α-Bis-4-fluorophenyl-methoxytropane and nortriptilyne in the binding pocket of DAT. Crystallographic orientation of nortriptilyne in yellow. Representative conformation of 3α-Bis-4-fluorophenyl-methoxytropane obtained through docking in gray. Important binding pocket residues are shown in stick to guide visualization.

Supplementary Table 1: Core enrichment from the dopaminergic synapse network in 7-days RA-differentiated SH-SY5Y cells compared to undifferentiated cells.

Heat Map	Gene Symbol	Gene Name
	*ITPR2*	inositol 1,4,5-triphosphate receptor, type 2
	CREB5	cAMP-responsive element binging protein 5
	MAOB	monoamine oxidase B
	SLC18A1	vesicular monoamine transporter (family 18), A1
	GNG8	G protein, gamma 8
	GNG7	G protein, gamma 7
	GNG2	G protein, gamma 2
	PPP2R2C	protein phosphatase 2A 55, reg. sub. B gamma
	PPP2R5B	protein phosphatase 2A 56, reg. sub. B, beta
	FOS	c-Fos transcription factor
	DRD2	dopamine receptor 2
	CLOCK	circadian locomotor output cycle kaput
	AKT1	RAC-alpha serine/threonine- protein kinase
	PPP2R5A	protein phosphatase 2A 56, reg. sub. B, alpha
	CREB3L2	cAMP-responsive element binding prot. 3-like 2
	MAPK10	mitogen-activating protein kinase 10
	MAPK9	mitogen-activating protein kinase 9
	ADCY5	adenylate cyclase 5
	PPP2R5C	protein phosphatase 2A 56, reg. sub. gamma
	CREB3	cAMP-responsive element binding protein 3
	GNAQ	G protein (q) subunit alpha
	PRKACA	protein kinase C, catalytic subunit alpha
	CACNA1B	calcium channel, voltage-depend., N, alpha 1B
	GNAS	GNAS complex locus
	KIF5C	kinesin heavy chain isoform 5C
	PLCB4	1-PIP-4,5 phosphodiesterase Beta 4
	CAMK2G	calcium/calmodulin-dependent PK II gamma
	GNGT1	G protein (T) subunit gamma-T1
	PLCB1	1-PIP-4,5 phosphdiesterase Beta 1
	ATF6B	activating transcription factor 6 beta
	PPP2CA	protein phosphatase 2A, cat. sub. alpha
	GNG3	G protein, subunit gamma-3
	PPP2R2D	protein phosphatase 2A 55, reg. sub. B delta
	GNB5	G protein, subunit beta-5
	PPP2CB	protein phosphatase 2A, cat. sub. beta
	MAPK8	mitogen-activating protein kinase 8 (JNK1)
	GNB1	G protein, subunit beta-1
	KCNJ3	K inward-rectifying channel, subfamily J, mem. 3
	MAPK14	mitogen-activating protein kinase 14 (p38)
	GNG12	G protein, subunit gamma-12
	GNAI3	G protein, alpha inhibiting activity 3

Supplementary Table 1: *Continued…*

Heat Map	Gene Symbol	Gene Name
	*CREB1*	cAMP-responsive element binding protein 1
	GSK3B	glycogen synthase kinase 3 beta
	PRKACB	cAMP-dependent protein kinase, cat. sub. beta

Data generated with Gene Set Enrichment Analysis (GSEA) comparing 7-days-RA-differentiated cells (n = 4) *vs.* undifferentiated SH-SY5Y cells (n = 6) transcripts obtained as described in Material and Methods section. Nominal *p* value of enrichment analysis obtained from GSEA ($p < 0.05$).

Supplementary Table 2: Raw data obtained from docking procedure

Compounds	*K*i	E_{ATD}	E_{OPT}(Kcal/mol)
Dopamine	22.64	- 6.34	- 81.82
6-Hydroxydopamine	227.72	- 4.97	- 114,74
p-Quinones	122.48	- 5.34	- 96.00
3α-Bis-4-fluorophenyl-methoxytropane	27×10^{-3}	- 10.33	- 90.21

*K*i (inhibition constant) and E_{ATD} (docking binding energy) were obtained from molecular docking using Autodock algorithm. *Ki* is expressed in μM and E in kcal/mol. E_{OPT} (optimized binding energy) was calculated after geometry improvements through classical energy minimization of docking solutions.

CHAPTER II:

EVALUATION OF PD PATHOPHYSIOLOGICAL MECHANISMS: A POSSIBLE ROLE FOR COFILIN-1?

Book chapter: *Cofilin-1 (CFL1)*

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CFL1

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Synonyms

CFL1; Cofilin-1 (non-muscle); Cofilin-1, Non-muscle isoform

Historical Background

Cofilin-1 is a major mediator of cytoskeleton dynamics as it regulates the remodeling of actin filaments (Carlier et al. 1999). It is ubiquitously expressed in all eukaryotes and has a broad tissue distribution. In mammals, for instance, it can be found in the brain, gastrointestinal tract, and lymphocytes. It was originally purified from avian and porcine brain as a 15–21 kDa globular protein with a core consisting of four or five beta-sheets surrounded by four or five alpha-helices. Its amino acid sequence and structure is highly conserved from yeast to human. In vivo and in vitro studies show that cofilin-1 protein can exist as both a monomer and oligomer, due mainly to the presence of four cysteine residues (Cys 39, Cys 80, Cys 139, and Cys 147) which are potential targets for oxidation (Fig. 1). This process can lead to disulfide bond formation, causing conformational changes in cofilin-1 (Bernstein and Banburg 2010). Another important residue of cofilin-1 is Ser-3, which upon phosphorylation results in its inactivation (Yang et al. 1998). Besides actin dynamics, cofilin-1 is also involved in pathological processes.

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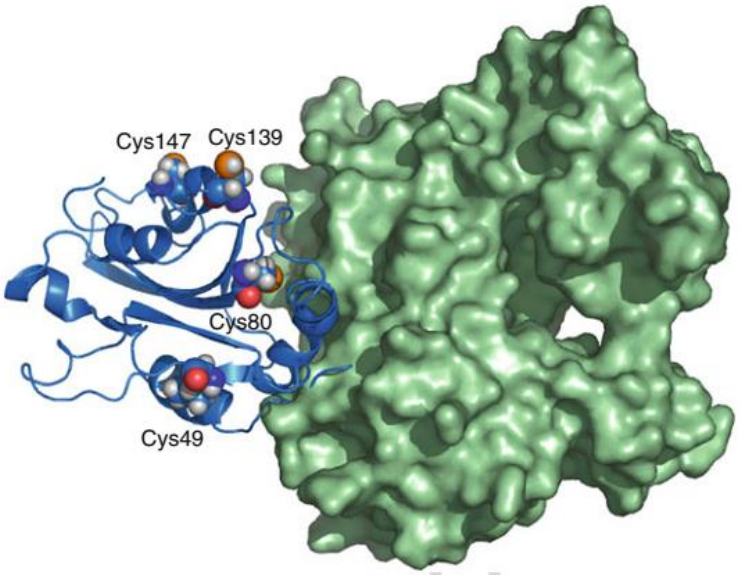
Physiology

Actin Dynamics

The major function of cofilin-1 is the regulation of actin dynamics. Actin is a family of globular proteins that form microfilaments as part of the cell cytoskeleton. Actin can be present either as a free monomer called G-actin or as part of linear polymer microfilament called F-actin (Carlier et al. 1999). At steady state, F-actin grows at one end through ATP-loaded G-actin molecule association. At the opposite end of the filament, monomers undergo dissociation via the hydrolysis of ATP bound to ADP. To enter into a new polymerization cycle, ADP-loaded actin needs to exchange ADP for ATP.

Cofilin-1 controls actin dynamics by binding to both F-actin and G-actin. Studies have suggested that, depending on its concentration, cofilin-1 can promote assembly or disassembly of actin



CFL1, Fig. 1 Cofilin-1. Representation of cofilin-1 structure (*blue*), highlighting the presence of the four cysteine residues and its interaction with monomeric actin (*green*) (Kindly generated by Prof. Dr. Hugo Verli/UFRGS)

filaments (Bernstein and Banburg 2010). When only a few cofilin-1 molecules are bound to F-actin, it leads to filament breakage. On the other hand, when the concentration of cofilin-1 is high, severing is no longer observed. In this case, there is dissociation from the pointed ends of the microfilament, which is related to cofilin-1's capacity to enhance Pi release and thus promote transformation of filaments to their ADP-loaded state. Furthermore, very high cofilin-1 concentration leads to increased nucleation and actin assembly promotion (Bernstein and Banburg 2010).

Therefore, since actin dynamics plays a role in the control of cellular morphology, cell migration, cell division, endocytosis, intracellular transport, and neuronal development, cofilin-1 is a fundamental protein in biological systems.

Regulation

Cofilin-1 has highly complex modes of regulation, which can lead to inactivation and activation, as well as changes in actin-binding affinity. Three mechanisms have been widely described: (i) phosphorylation, (ii) increase of the pH and binding of phosphatidylinositol 4,5-bisphosphate (PIP2), and (iii) oxidation. Evidence indicates that these three factors can also act in concert (Bernstein and Bamburg 2010).

The best characterized mechanism of cofilin-1 regulation is the Ser-3 phosphorylation by LIM (1, 2, and) or TES (testicles) kinases (TESK) (Arber et al. 1998) and its dephosphorylation by Slingshot phosphatase isoform 1 (SSH1) (Bernstein and Banburg 2010). Phosphorylation of CFL1 results in protein inactivation and subsequent elimination of its actin-binding function (Arber et al. 1998). The phosphorylation of CFL1 is also mediated by upstream enzymes that regulate LIMK activity, such as Rho/Rac GTPases-PAK pathways (Yang et al. 1998). On the other hand, cofilin-1 is activated by dephosphorylation (by SSH-1), which is activated by calcineurin, a Ca^{2+}/calmodulin-dependent phosphatase (Wang et al. 2005).

Another regulation mechanism of cofilin-1 is the dissociation of PIP2 binding and the increase of pH levels. A study using yeast cofilin-1 showed that the PIP2 binding site is a large positively charged surface that consists of residues in helix 3 as well as residues in other parts of the cofilin-1 molecule. Moreover, PIP2 binding overlaps F-actin binding sites, which can explain the decrease in actin activity upon PIP2 binding. The pH control of cofilin-1 activity is related to the deprotonation of His133 in the F-actin binding site. In vitro, its activity increased in neutral and alkaline pHs. However, these two mechanisms can interact with each other as when intracellular

pH increases via the influx of Na^+ and efflux of H^+, cofilin-1 is released from its PIP2 inhibitory binding.

Oxidative stress can also mediate the regulation of actin dynamics. A study using human T cells treated with H_2O_2 demonstrated the formation of an intramolecular disulfide bridge between Cys-39 and Cys-80, causing a conformational change in the molecule. Furthermore, it was found that oxidized (and dephosphorylated) cofilin-1 is unable to regulate actin dynamics. Hence, although dephosphorylated cofilin-1 is able to bind to F-actin, oxidized cofilin-1 leads to its inactivation (Klemke et al. 2008). Studies have established the key role of *CFL1* in oxidant-induced apoptosis in tumor cells (Klamt et al. 2009). Mechanistically, once oxidized, cofilin-1 translocates to the mitochondria where it induces swelling and cytochrome *c* release by mediating the opening of the permeability transition pore (PTP). Knockdown of endogenous *CFL1* using targeted siRNA inhibits oxidant-induced apoptosis, which is restored by reexpression of wildtype *CFL1* but not by *CFL1* containing Cys-to-Ala mutations (a nonoxidizable form of the protein). Thus, this data suggests that oxidized *CFL-1* mediates mitochondrial dysfunction and apoptosis induced by oxidants in tumor cells.

Pathology

Neurodegeneration

There is a link between impaired synaptic plasticity observed with both age and neurodegenerative diseases and the cofilin-1 pathway. Cofilin-1 is highly concentrated in the growth cone and dendritic spine of neurons. Overexpression of cofilin-1 and its nonphosphorylatable S3A mutant can induce more growth cone-like waves and result in longer axons. On the other hand, overexpression of LIMK-1 disrupts the growth cone structure and axon elongation (Flynn et al. 2009). Thus, cofilin-1 may play a crucial role in synaptic plasticity through the regulation of the growth cone and spine dynamics via phosphorylation/dephosphorylation. Additionally, studies have shown that LIMK-1 knockout causes an abnormal elevation of CFL1 activity. This leads to the distortion of spine morphology and may be correlated with William's syndrome (Frangiskakis et al. 1996). Lastly, hyperphosphorylation of cofilin-1 results in a reduction in dendrite number, leading to the neurodegeneration found in Alzheimer's disease. Furthermore, cofilin-1 has also been shown to be present beside amyloid plaques in human brain (Heredia et al. 2006).

Recently, a growing body of evidence suggests that actin/cofilin-1 rod formation (aggregates composed primarily of actin and cofilin-1) may be a central initiation step for neurodegeneration. Actin/cofilin-1 rods can be generated by the excessive expression of active *CFL1* and by cellular stress. Cofilin-1 oxidation may directly facilitate actin/cofilin-1 rod formation by the actin bundling activity of cofilin-1 oligomers or by the impairment of cofilin-1 phosphorylation. In neurodegenerative diseases such as Parkinson's disease, neuronal cytoplasmic rods accumulate within neurites, where they disrupt synaptic function and are a likely early cause of synaptic loss without neuronal loss (Bamburg and Bernstein 2016; Schönhofen et al. 2014).

Cancer

Cell motility is the cornerstone event of the invasion and metastasis found in aggressive cancers. It is clear in cancer models that the activation of the motility cycle is required for cell migration and invasion, tumor progression, and metastasis. In this scenario, cofilin-1 arises as a key player in cell migration, contributing in actin polymerization and in the formation of free barbed ends. The ability of cofilin-1 to interact with the actin cytoskeleton molecules suggests that it has a direct role in the processes of cell polarity, migration, and chemotaxis (Wang et al. 2007).

The importance of cofilin-1 in cancer cell motility and migration, plus its role in oxidants-induced apoptosis, suggests that this protein is a marker of an aggressive cancer phenotype (Wang et al. 2007; Müller et al. 2011). Moreover, cofilin-1 has been associated with chemotherapy resistance, especially towards alkylating drugs and as such is a possible target for cancer treatment (Castro et al. 2010; Becker et al. 2014).

Several in vitro and in vivo studies have correlated the expression of cofilin-1 with the potential for tumor cells to migrate and generate metastases. An imbalance of this pathway has been described for different tumors, including breast, lung, ovarian, head and neck, melanoma, gastrointestinal, genitourinary, and central nervous system tumors. Several studies, including metaanalysis of microarray data, cultures of human cancer cell lines, and small clinical retrospective cohorts, have demonstrated that aggressive cancer behavior correlates with high expression levels of cofilin-1, with similar results in the different tumors studied. As such, cofilin-1 expression levels may be a useful tool for discriminating between high and low aggressiveness tumors and possibly between good and bad prognoses (Castro et al. 2010).

A current challenge in the modern era of oncology is the concept of personalized medicine. In this setting, targeted treatments to driver molecules are becoming the focus of therapeutic intervention, and several drugs have already being approved for use in clinical practice. The first study of cofilin-1 as a target for cancer treatment was undertaken in human breast cancer cells and cancer metastasis xenograft models. JG6, a novel marine-derived oligosaccharide, was used to bind to cofilin-1 and inhibit cofilin-actin turnover by disrupting their interaction. JG6 was the first compound to demonstrate a positive effect in the inhibition of cell migration and prevention of cancer cell metastases. It is important to point out that JG6 effects were dependent of high levels of cofilin-1 and did not had an inherent cytotoxic effect (Huang et al. 2014).

Although tumor aggressiveness and its potential to generate metastasis are very important tumor characteristics, chemoresistance is also a major issue in the clinical setting. Two studies in lung cancer and one in an ovarian cell lines attempted to correlate cofilin-1 expression and chemotherapeutic resistance. Analysis of microarray data obtained from six human non–small cell lung cancer cell lines with different degrees of cofilin-1 expression revealed a positive correlation between high levels of *CFL1* mRNA and resistance against different anticancer drugs. When these cell lines were exposed to different concentrations of chemotherapy drugs, resistance to alkylating agents (cisplatin and carboplatin) was observed (Castro et al. 2010). This correlation was validated in another study with lung cancer adenocarcinoma cell lines where cofilin-1 was overexpressed and drug sensitivity/resistance was evaluated (Becker et al. 2014). Another study using ovarian cell lines cancer evidenced that taxol-resistant cells had a higher expression level of cofilin-1 showed an upregulation of the protein in the taxol-resistant samples (Li et al. 2013).

Studies have also shown that cofilin-1 expression confers radiation resistance in tumors. Lee and colleagues demonstrated that high cofilin-1 expression enhanced cellular radiosensitivity in H1299 cells (non–small cell lung carcinoma), which is possibly due to reduced capacity to repair double-strand breaks in DNA (Lee et al. 2005). At the same time, Wei et al. reported that cofilin-1, among other proteins, could predict multidrug resistance (MDR) and elevated radioresistance (RDR) (Wei et al. 2012). This study consisted of the irradiation of A549 lung cancer cell cultures with 6 MV photon beams of different doses, following evaluation of upregulated proteins by immunohistochemistry. The evaluation of radiation response of astrocytomas indicated that cofilin-1 might be involved in the radioresistant phenotype. All the data correlating cofilin-1 and cancer clearly demonstrate a positive link between high expression levels of the protein and a more aggressive cancer phenotype. This relationship can be observed in different tumors subtypes, both in microarray data and in culture cell. The major challenge now is to translate all these laboratory data to the clinical setting. It is imperative to further investigate cofilin-1 as a candidate for cancer treatment and also to obtain a better understanding of its role in the mechanisms of chemoresistance and radiation response.

Summary

Cofilin-1 is one of the major proteins responsible in cell migration, playing a key role in actin

filament dynamics. The regulation mechanisms of this protein are phosphorylation (inactivation)/dephosphorylation (activation), via LIM kinase/TESK and SSH, subcellular localization, pH and oxidation of its internal cysteine residues. Since actin dynamics plays a role in morphology, cell migration, cell division, endocytosis, intracellular transport, and neuronal development, cofilin-1 is a fundamental protein in biological systems. Moreover, imbalance in the physiology of this protein plays a major role in several pathological processes, such as neurodegenerative diseases and cancer.

References

Arber S, Barbayannis FA, Hanser H, Schneider C, Stanyon CA, Bernard O, et al. Regulation of actin dynamics through phosphorylation of cofilin by LIM-kinase. Nature. 1998;393(6687):805–9. Available from: http://www.ncbi.nlm.nih.gov/pubmed/9655397

Bamburg JR, Bernstein BW. Actin dynamics and cofilin-actin rods in Alzheimer disease. Cytoskeleton (Hoboken). 2016;73(9):477–97. Available from: http://www.ncbi.nlm.nih.gov/pubmed/26873625

Becker M, De Bastiani MA, Müller CB, Markoski MM, Castro MA, Klamt F. High cofilin-1 levels correlate with cisplatin resistance in lung adenocarcinomas. Tumour Biol. 2014;35(2):1233–8. Available from: http://www.ncbi.nlm.nih.gov/pubmed/24018823

Bernstein BW, Bamburg JR. ADF/cofilin: a functional node in cell biology. Trends Cell Biol. NIH Public Access; 2010;20(4):187–95. Available from: http://www.ncbi.nlm.nih.gov/pubmed/20133134

Carlier MF, Ressad F, Pantaloni D. Control of actin dynamics in cell motility. Role of ADF/cofilin. J Biol Chem. 1999;274(48):33827–30. Available from: http://www.ncbi.nlm.nih.gov/pubmed/10567336

Castro MAA, Dal-Pizzol F, Zdanov S, Soares M, Müller CB, Lopes FM, et al. CFL1 expression levels as a prognostic and drug resistance marker in nonsmall cell lung cancer. Cancer. 2010;116(15):3645–55. Available from: http://www.ncbi.nlm.nih.gov/pubmed/20564088

Flynn KC, Pak CW, Shaw AE, Bradke F, Bamburg JR. Growth cone-like waves transport actin and promote axonogenesis and neurite branching. Dev Neurobiol. 2009;69(12):761–79. Available from: http://www.pubmedcentral.nih.gov/articlerender.fcgi?artid=2845293&tool=pmcentrez&rendertype=abstract

Frangiskakis JM, Ewart AK, Morris CA, Mervis CB, Bertrand J, Robinson BF, et al. LIM-kinase1 hemizygosity implicated in impaired visuospatial constructive cognition. Cell. 1996;86(1):59–69. Available from: http://www.ncbi.nlm.nih.gov/pubmed/8689688

Heredia L, Helguera P, de Olmos S, Kedikian G, Solá Vigo F, LaFerla F, et al. Phosphorylation of actin-depolymerizing factor/cofilin by LIM-kinase mediates amyloid beta-induced degeneration: a potential mechanism of neuronal dystrophy in Alzheimer's disease. J Neurosci. 2006;26(24):6533–42. Available from: http://www.ncbi.nlm.nih.gov/pubmed/16775141

Huang X, Sun D, Pan Q, Wen W, Chen Y, Xin X, Huang M, Ding J, Geng M. JG6, a novel marine-derived oligosaccharide, suppresses breast cancer metastasis via binding to cofilin. Oncotarget. 2014;5(11):3568–78. Available from: http://www.ncbi.nlm.nih.gov/pubmed/25003327

Klamt F, Zdanov S, Levine RL, Pariser A, Zhang Y, Zhang B, et al. Oxidant-induced apoptosis is mediated by oxidation of the actin-regulatory protein cofilin. Nat Cell Biol. 2009;11(10):1241–6. Available from: http://www.pubmedcentral.nih.gov/articlerender.fcgi?artid=3393095&tool=pmcentrez&rendertype=abstract

Klemke M, Wabnitz GH, Funke F, Funk B, Kirchgessner H, Samstag Y. Oxidation of cofilin mediates T cell hyporesponsiveness under oxidative stress conditions. Immunity. 2008;29(3):404–13. Available from: http://www.ncbi.nlm.nih.gov/pubmed/18771940

Lee Y-J, Sheu T-J, Keng PC. Enhancement of radiosensitivity in H1299 cancer cells by actin-associated protein cofilin. Biochem Biophys Res Commun. 2005;335(2):286–91. Available from: http://www.ncbi.nlm.nih.gov/pubmed/16061204

Li M, Yin J, Mao N, Pan L. Upregulation of phosphorylated cofilin 1 correlates with taxol resistance in human ovarian cancer in vitro and in vivo. Oncol Rep. 2013;(1):58–66. Available from: http://www.ncbi.nlm.nih.gov/pubmed/23064469

Müller CB, de Barros RL, Castro MA, Lopes FM, Meurer RT, Roehe A, et al. Validation of cofilin-1 as a biomarker in non-small cell lung cancer: application of quantitative method in a retrospective cohort. J Cancer Res Clin Oncol. 2011;137(9):1309–16. Available from: http://www.ncbi.nlm.nih.gov/pubmed/21735353

Schönhofen P, de Medeiros LM, Catain CP, Bristot IJ, Klamt F. Cofilin/actin rod formation by dysregulation of cofilin-1 activity as a central initial step in neurodegeneration. Mini Rev Med Chem. 2014;14(5):393–400. Available from: http://www.ncbi.nlm.nih.gov/pubmed/24813767

Wang Y, Shibasaki F, Mizuno K. Calcium signal-induced cofilin dephosphorylation is mediated by Slingshot via calcineurin. J Biol Chem. 2005;280(13):12683–9. Available from: http://www.ncbi.nlm.nih.gov/pubmed/15671020

Wang W., Eddy R., Condeelis J. The cofilin pathway in breast cancer invasion and metastasis. Nat Rev Cancer. 2007;(6):429–40. Available from: http://www.ncbi.nlm.nih.gov/pubmed/17522712

Wei R, Zhang Y, Shen L, Jiang W, Li C, Zhong M, et al. Comparative proteomic and radiobiological analyses in human lung adenocarcinoma cells. Mol Cell Biochem. 2012;359(1–2):151–9. Available from: http://www.ncbi.nlm.nih.gov/pubmed/21822689

Yang N, Higuchi O, Ohashi K, Nagata K, Wada A, Kangawa K, et al. Cofilin phosphorylation by LIM-kinase 1 and its role in Rac-mediated actin reorganization. Nature. 1998;393(6687):809–12. Available from: http://www.ncbi.nlm.nih.gov/pubmed/9655398

Article: *Cofilin-1 pathway as a major contributor to Parkinson's Disease pathogenesis*

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JUSTIFICATION:

Cofilin-1 mediates mitochondrial dysfunction in oxidant-induced apoptosis, which is thought to be two major pathogenic mechanisms in PD. Although its protein expression was found to be increased in PD patient's lymphocytes, the mechanisms by which cofilin-1 may mediate PD neurogenerative processes remains to be elucidated.

GENERAL OBJECTIVE

Demonstrate that cofilin-1 has a key role in the neuronal cell death found in PD.

SPECIFIC OBJECTIVES

- Assessment of 6-OHDA-induced cell death (caspase activation, mitochondrial dysfunction, ROS production) in RA-differentiated SH-SY5Y cells;
- Investigate whether cofilin-1 translocates to the mitochondria in 6-OHDA-induced cell death in RA-differentiated SH-SY5Y cells;

- Generation of transient genetically-modified cell lines to investigate the effects of *CFL1* wild type and mutant (non-oxidizable from Cys-Ala) overexpressions;
- Evaluation of cofilin-1 immunocontent in *post mortem* PD brain;
- Investigation whether cofilin-1 pathway proteins levels can discriminate controls and PD subjects;
- Analysis of LB pathology as a covariant of cofilin-1 expression.

Title Page

Title: Cofilin-1 pathway as a major contributor to Parkinson's Disease pathogenesis

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Abstract

It is widely known that oxidation of non-phosphorylate (activated) cofilin-1 leads to its translocation to the mitochondria in tumour cells, which is an early step for oxidant-induced-apoptosis. Since Parkinson's disease (PD) is strongly related with mitochondrial dysfunction and oxidative stress, the aim of this study was to verify whether cofilin-1 has a role in the neuronal cell death found in this disorder. Using retinoic-acid-differentiated SH-SY5Y cells treated with 6-hydroxydopamine (6-OHDA) as a PD experimental model, we demonstrated that cofilin-1 mitochondrial translocation occurs before the loss of membrane mitochondria potential, indicating that this protein participates in the early stages of neuronal apoptosis. Overexpression of wild type *CFL1* resulted in increased sensitivity of SH-SY5Y to 6-OHDA-induced neuronal cell death. Furthermore, overexpression of non-oxidizable *CFL1* containing Cys-to-Ala mutations (positions 39, 80 and 139) increased neuronal resistance to 6-OHDA, suggesting that oxidation is an important step to 6-OHDA-induced-apoptosis. Analysis of *post mortem* human PD brain demonstrated a significant decrease in the p-cofilin-1/cofilin-1 ratio, which indicates an increase in the amount of activated cofilin-1 available for oxidation. The immunodetection of cofilin-1 pathway proteins allowed the discrimination of controls and PD individuals in brain areas with early-stage of neuropathological changes, demonstrating cofilin-1's early involvement in the PD pathogenic pathway. Lastly, we showed, for the first time, that cofilin-1 is deposited in Lewy bodies (LB). Ours results suggest that cofilin-1 is an early marker of neurodegeneration in PD, and may be used as a novel therapeutical target for this disorder.

Keywords: SH-SY5Y; 6-hydroxydopamine; α-synuclein; occipital cortex; Lewy Body.

Introduction

Actin depolymerizing-factor (ADF)/Cofilin family is a group of eukaryotic ubiquitous small proteins (13-19kDa) which regulate actin, and hence the cytoskeleton dynamics[1]. The remodelling and reorganization of the actin cytoskeleton is essential to many biological processes such as cytokinesis, morphogenesis, and cellular transport[2]. In the central nervous system, members of the ADF/Cofilin family are widely expressed, in particular in the growth cone during neuronal development[3,4], and it is also involved in neurotransmitter release[5], spine morphology[6], and synaptic plasticity[7].

Cofilin-1 is the major ubiquitous form of this protein class[6,8,9]. Its activity is regulated mainly via the phosphorylation of Ser-3 by LIM kinase (LIMK), resulting in protein inactivation and elimination of its actin-biding function[10]. On the other hand, slingshot phosphatase isoform 1 (SSH1) activates cofilin-1 by dephosphorylation[11]. Other sites of regulation are its 4 Cys residues (39, 80, 139 and 147), whose internal oxidations form disulphide bridges, leading to conformational changes and loss of actin binding affinity[12].

Although cofilin-1 is critical for cell physiology, it is also associated with pathological processes. In our previous studies, we established the key role of cofilin-1 in oxidant-induced-apoptosis in tumour cells[13]. Under oxidative conditions, non-phosphorylated cofilin-1 (active form) undergoes oxidation, forming intramolecular disulphide bonds between the Cys residues (39-80 and 139-147) changing its structure[12]. This leads to loss of cofilin-1 binding affinity to actin, and targets cofilin-1 to the mitochondria causing cytochrome c release and apoptosis[12–14].

This phenomenon has also been described in neuronal apoptosis. The scaffolding protein RanBP9 mediates cofilin-1 activation and translocation to the mitochondria in amyloid β (Aβ) oligomers-induced cell death in the mouse hippocampal cell line HT22[15]. Another study showed that Aβ rapidly increases cofilin-1 translocation to the mitochondria, and that there is no detection of phospho-cofilin-1 in the mitochondrial fraction, which supports the hypothesis that activation is required for cofilin translocation[16,17]. These findings suggest that cofilin-1 may play a role in the pathogenesis of Alzheimer's disease (AD).

Clinical validation studies using AD *post mortem* human brain have revealed a misbalance in the expression of proteins of the cofilin-1 pathway, however these have been somewhat contradictory. A number of studies have demonstrated an increase expression of LIMK[18] and coupled with an increase in cofilin-1 phosphorylation[19]. On the other hand, other reports have demonstrated cofilin-1 hyperactivation, which can result in the formation of cofilin-actin bundles, termed "rods"[15–17]. In AD, cytoplasmic rods accumulate within neurites, where they disrupt synaptic function and are a likely cause of synaptic loss without neuronal loss[20].

Changes in cofilin-1 levels have also been reported in Parkinson's disease (PD), in which the pathological hallmark is the degeneration of dopaminergic neurons of the *substantia nigra* (*SN*)[21]. Proteomic analysis of peripheral blood lymphocytes have demonstrated increased expression of cofilin-1 in PD patients, indicating its possible role in disease pathophysiology[22]. However, the mechanisms underlying the role of this protein in PD have not been elucidated.

Since cofilin-1 mediates mitochondrial dysfunction and oxidative-stress-induced apoptosis[13,14], thought to be important pathogenic mechanisms of PD[23–

[25], we have investigated whether cofilin-1 has a role in neuronal cell death in PD. Here, we showed that cofilin-1 mediates 6-hydroxydopamine (6-OHDA)-induced apoptosis in retinoic acid (RA)-differentiated SH-SY5Y cells. We have also demonstrated, using PD *post mortem* human brain, that the expression of cofilin-1 pathway protein is aberrant in PD patients and the co-localisation of cofilin-1 with α-synuclein (α-SYN) within Lewy bodies (LB) in PD brain.

Results

6-OHDA-induced-cell-death is caused by intrinsic apoptosis in RA-differentiated SH-SY5Y cells

Our study used RA-differentiated SH-SY5Y cells as a PD model not only because this cell line has a dopaminergic-neuronal phenotype and dopamine transporter dependency when challenged with 6-OHDA, but also due to its easy manipulation and cell culture when compared to other *in vitro* PD models[26,27]. PD pathological mechanisms in SH-SY5Y cells was induced by 6-OHDA, a dopamine metabolite that causes catecholaminergic damage[28].

Firstly, we evaluated the mechanisms of 6-OHDA neurotoxicity in RA-differentiated cells by performing control experiments to provide evidence that this toxin causes oxidant-induced-apoptosis in our PD cellular model. We first investigated the type of cell death by assessing cell morphology using Hoechst 33342/propidium iodide (PI) staining. Fig. 1b shows a representative image of nuclear staining, where control cells exhibit normal nuclear morphology with uniform blue nuclei. 6-OHDA treatment induced nuclear morphological changes in SH-SY5Y cells, as follows: (*i*) blue bright chromatin staining, with obvious DNA fragmentation were considered early apoptotic cells (indicated by the blue arrows in Fig. 1e,f); (*ii*) double-staining Hoechst/PI showing bright blue and bright red

chromatin were classified as late apoptosis (note the orange arrows indicating dual-label in Fig. 1e,f); (*iii*) necrotic cells present double-staining Hoechst/PI, with uniform enlarged blue/red nuclei (red arrows in Fig. 1e,f). Hence, Fig. 1 shows that most cells are considered early or late apoptotic after 6-OHDA treatment.

Further analysis was performed to confirm apoptosis involvement in 6-OHDA-induced cell death. We pre-incubated RA-differentiated SH-SY5Y cells with cell-permeable caspase inhibitors (Z-DEVD - 3; Z-IETD - 8; Z-LEHD - 9) and challenged them with 6-OHDA. Both caspase 3 and 9 inhibitors were able to prevent 6-OHDA neurotoxicity ($p < 0.0001$; Fig. 1g). Moreover, we measured caspase activity (3, 8 and 9) over time using cells treated with 6-OHDA for 1.5, 3, 6, 9 and 12 hours. There is a significant increase in the activities of caspases 9 and 3 ($p < 0.0001$) after 1.5 and 6 hour of 6-OHDA treatment respectively (Fig. 1h). Hence, the involvement of caspases confirms that 6-OHDA toxicity is induced by apoptosis. Moreover, the increase in caspase 9-activity and the prevention of cell death due pre-incubation with caspase 9 inhibitor indicate that SH-SY5Y cells undergo intrinsic apoptosis, which is characterized by mitochondrial dysfunction.

6-OHDA causes loss of mitochondrial membrane potential (ΔΨm) and increases ROS levels in RA-differentiated SH-SY5Y cells

To investigate mitochondrial dysfunction in response to 6-OHDA, we performed a time-course-experiment in which RA-differentiated SH-SY5Y cells were challenged with 6-OHDA for 1.5, 3, 6, 12 and 24 hours after we evaluated loss of ΔΨm, a feature of toxin-induced intrinsic-apoptosis. 6-OHDA cause a significant disruption of ΔΨm after 6 hours of treatment ($p < 0.0001$; Fig. 1i).

Next, we assessed oxidants and H_2O_2 production (Fig. 1j, k). 6-OHDA significantly increased reactive species ($p < 0.05$; Fig. 1j) and H_2O_2 ($p < 0.005$; Fig. 1k) production, indicating an increase of oxidants production in SH-SY5Y after 6-OHDA treatment. Taken together, these data show that 6-OHDA-induced cell death in our PD cellular model resulted in mitochondrial dysfunction and oxidative stress, which are processes known to be involved in cofilin-1 translocation to the mitochondria in other cell types.

6-OHDA causes endogenous cofilin-1 translocation to the mitochondria in RA-differentiated SH-SY5Y cells

To determine whether cofilin-1 mediated oxidative stress and mitochondrial dysfunction induced by 6-OHDA, we first investigated the effect of this toxin upon endogenous cofilin-1 using the same time course experiment outlined above. We evaluated whether cofilin-1 translocates to the mitochondria in 6-OHDA-induced apoptosis and if it occurred before ΔΨm collapse.

Upon challenge with 6-OHDA, cofilin-1 migrated to the mitochondria (Fig. 2a). This change in subcellular localisation was detectable 3 hours after treatment ($p < 0.0001$; Fig. 2b), demonstrating that this process occurs before a measurable decrease in ΔΨm, which indicates that this protein is involved in an early-step of 6-OHDA cell death. Hence, cofilin-1 may be involved in the initial stages of dopaminergic cell apoptosis induced by 6-OHDA.

Overexpression of non-oxidizable CFL1 reduces 6-OHDA cytotoxicity in RA-differentiated cells

In order to confirm that cofilin-1 mediates 6-OHDA toxicity, we overexpressed wild-type *CFL1* gene ($CFL1_{WT}$) in SH-SY5Y cells ($p < 0.05$; Fig. 2c) and evaluated its effect on 6-OHDA toxicity. The overexpression of $CFL1_{WT}$

in SH-SY5Y increases significantly cell death when treated with 6-OHDA toxicity ($p < 0.01$; Fig 2e).

To determine whether this was due to the direct effect of oxidized cofilin-1, SH-SY5Y cells were transfected with non-oxidizable *CFL1* gene (CFL_{NOX}) containing Cys-Ala mutations at residue positions 39, 80 and 139. Confirmation of plasmid transfection and $CFL1_{NOX}$ expression was performed by RT-PCR coupled with *BsaHI* restriction digestion (Fig. 2d). The presence of the 236 bp restriction digest product present in SH-SY5Y cells transfected with $CFL1_{NOX}$, (black arrow in Fig. 2d) indicated the expression of $CFL1_{NOX}$ mRNA. Expression of $CFL1_{NOX}$ significantly reduced 6-OHDA-induced cell death ($p < 0.001$; Fig 2f). Taken together, these data demonstrated that cofilin-1 oxidation is required for 6-OHDA toxicity and suggests a role for this protein in PD pathogenesis.

Cofilin-1 pathway immunocontent changes in PD brain

Having demonstrated evidences that cofilin-1 plays a role in mediating 6-OHDA-induced cell-death in a PD *in vitro* model, we undertook a series of experiments using *post mortem* human brain tissue to provide clinical evidence of a link between the cofilin-1 pathway and PD pathology (Experimental design is in Supplementary Figure 2).

Our results suggest that cofilin-1 is involved in early steps of apoptosis *in vitro*, hence, the use of SN as brain area of study could create bias in our study. By their very nature, all PD subjects used in this study demonstrated a high degree of dopaminergic degeneration *SN* and high Braak stage score. In addition, the *SN* is involved early in the disease, thus pathological alterations are by definition late-stage. To counter-act this, we chose brain regions with no significant degeneration process and according to the Braak hypothesis[29] We

used occipital cortex mainly because is the last area to be committed by PD. The other region chosen was cingulate gyrus, which has an earlier involvement with PD, hence the neuropathological changes are occurring for longer. We have used such an approach successfully in previous studies[30,31].

We analysed cofilin-1 pathway in both brain areas from control and PD patients by measuring cofilin-1, p-cofilin-1, LIMK, phospho-LIMK (p-LIMK) expression using quantitative immunohistochemistry. In addition, we quantified total α-SYN protein expression, a protein deposited in LB, a pathological hallmark of PD[32]. In the cingulate gyrus, cofilin-1 content was increased ($p < 0.05$; Fig. 3 a,b), and, for the occipital cortex, p-cofilin-1 content was decreased ($p < 0.01$; Fig 3 c,d) and α-SYN content was increase in ($p < 0.01$; Fig 3 e,f). Other markers tested did not show any significant differences between the groups (Supplementary Fig. 3 and 4).

Subsequently, we measured the p-cofilin-1/cofilin-1 and p-LIMK/LIMK ratios to determine whether cofilin-1 activation is altered in the PD brain (Fig 4). The p-cofilin-1/cofilin-1 ratio was reduced by 4.53 and 2.42-fold in PD patients when compared to the controls in cingulate gyrus ($p < 0.05$; Fig. 4a) and in occipital cortex ($p < 0.01$; Fig. 4c) respectively. There were no significant changes in p-LIMK/LIMK ratios in either brain areas (Fig. 4 b,d). Taken together, these data demonstrate that cofilin-1 is mainly non-phosphorylated in PD brains, thus allowing for cofilin-1 to oxidise and translocate to the mitochondria, resulting in cell death.

To further analyse this data, we performed principal component analysis (PCA), a multivariate method used to explore multiple variables. Student *t* test indicated that the scores of PC3 showed differences between control and PD

groups with and without using α-SYN (major component in LB) in the analysis in the occipital cortex (p = 0.0043, Fig. 6b). The samples included in the study were well discriminated in a three-dimensional space of the first three principal components into two groups corresponding to control and PD (Fig. 6a). In Fig. 6c, it is plotted the proteins that most contributed to each principal component, which PC3 were LIMK and p-cofilin-1, further supporting our hypothesis that cofilin-1 activation is altered in PD brain. However, when α-SYN marker is excluded from the analysis the discrimination capacity to separate the two groups decreased (Fig 6a). In the cingulate gyrus, PCA could not discriminated the individuals in control and PD (Figure 5), probably because due to the late stages of neuropathological changes, which is occurring for longer than in the occipital cortex. This provides further support to our hypothesis that cofilin-1 may have a role in the early stages of the PD degenerative process.

Cofilin-1 is associated with LB pathology

α-SYN is the major component of LB, a pathological hallmark for PD. Since the PCA analysis found strong evidences that this protein and cofilin-1 may be associated in PD degeneration process, we aimed to evaluate whether cofilin-1 may be a component of this structure as well.

For this experiment, we use *SN* from a PD patient, the brain region where LB are most commonly found. At first, we evaluate whether cofilin-1 is present in the remaining dopaminergic neuron in *SN* trough dual-label localization immunofluorescence using tyrosine hydroxylase (TH) and cofilin-1. Fig. 7a shows an intranuclear inclusion of a degenerating neuron, suggesting that cofilin-1 is deposited within LB. To further investigate this potential deposition of cofilin-1 in LB, we undertook dual-label co-localization immunofluorescence using α-SYN

and cofilin-1, which confirmed the deposition of cofilin-1 within intraneuronal deposits in dopaminergic neurons (Fig 7b).

Discussion

To date, very few studies investigated the role of cofilin-1 in PD[22,33]. Previous studies in tumour cells demonstrated that this protein mediates mitochondrial dysfunction and oxidative stress-induced apoptosis[13]. As these two pathological mechanisms are also key features of PD, we hypothesised that cofilin-1 may have a pivotal role in mediating neurodegeneration in this disease. Our study demonstrates both *in vitro* and clinically, for the first time, that cofilin-1 homeostasis is aberrant in PD.

Initially, we explored the mechanisms underlying 6-OHDA cytotoxicity in RA-differentiated SH-SY5Y cells. Although many lines of evidence have demonstrated that 6-OHDA induces apoptotic cell death[34–36], it is important to evaluate this process in our cellular PD model because there are several discrepancies among findings regarding this toxin's mechanism of action, depending on the model used[26,37,38]. Here we observed that 6-OHDA neurotoxicity induces oxidative stress, mitochondrial dysfunction and intrinsic apoptosis. This corroborates with previous evidence showing that 6-OHDA increases caspase 3 and 9 activities[36] and leads to loss of mitochondrial membrane potential[39] in undifferentiated SH-SY5Y cells. However, for the first time, we show the same phenomenon in RA-differentiated SH-SY5Y cells.

We demonstrated that cofilin-1 translocates to the mitochondria of RA-differentiated SH-SY5Y cells treated with 6-OHDA before the loss of $\Delta\Psi m$, suggesting that cofilin-1 translocation is an event upstream of mitochondrial dysfunction. This was supported by the demonstration that non-oxidizable

(*CFL1*$_{NOX}$) overexpression in SH-SY5Y significantly decreased 6-OHDA cytotoxicity. Hence, these results demonstrated that cofilin-1 mediates 6-OHDA-induced apoptosis *in vitro*.

Although this process is well characterized in tumour cells[13,14], further investigation is necessary to evaluate whether this is a common step in apoptosis pathway in other tissues, such as neuronal cell. In normal cells (*e.g.* mouse fibroblasts) treated with apoptotic inducers (but no oxidants), cofilin-1 do not translocate to the mitochondria, this data indicates a role of cofilin-1 specifically in oxidant-induced apoptosis, not in other types of apoptosis inductions[40]. Furthermore, regarding neuronal cell death, there is a strong evidence showing that cofilin-1 plays a role in AD[15–17], which is a another neurodegenerative disorder highly associated with mitochondrial dysfunction and oxidative stress. These studies demonstrated that Aβ-treated cells (an AD pathological hallmark and use as an experimental model for AD pathology) leads to cofilin-1 translocation. In spite of these previous evidences, no study has shown the role of cofilin-1 translocation in PD models.

As stated above, in our cellular model, cofilin-1 mitochondria translocation occurs before mitochondrial dysfunction, suggesting that this phenomenon may be an early step of dopaminergic cell degeneration. To provide clinical evidence for our *in vitro* findings, we verified whether cofilin-1 pathway is aberrant in PD *post mortem* brain by investigating the protein expression in two brain regions – cingulate gyrus and occipital cortex.

Our findings demonstrated changes in cofilin-1 pathway proteins expressions in PD brains compared to non-disease controls, indicating a possible link between this protein and neuropathological alterations found in this disease.

Elevated cofilin-1 protein expression in PD compared to non-disease control patients was first demonstrated in a proteomic analysis of peripheral blood lymphocytes[22], however the relationship between this elevated cofilin-1 expression and disease pathogenesis was not explored.

Our study is the first to demonstrate a significant decrease in the p-cofilin-1/cofilin-1 ratio in PD brain compared to non-disease controls, demonstrating that cofilin-1 is activated in the PD brain. This results in an increase of non-phosphorylated cofilin-1 fraction, which is prone to oxidation. It is well known that brain tissue has high physiological levels of reactive species and low levels of antioxidants[41]. In PD brain, this is exacerbated by the reduction of respiratory chain Complex I activity, a pathological hallmark of many PD patients which contributes to the generation of excessive ROS and, in turn, may lead to the induction of apoptosis[42]. Moreover, the levels of glutathione (GSH), the main antioxidant defense in the brain, are reduced in the *SN* of PD[43]. Hence, it is possible that, during oxidative stress, non-phosphorylated cofilin-1 undergoes oxidation, forming intramolecular disulfide bonds and translocates to the mitochondria, such as is observed in tumour cells[13].

Another possible explanation of cofilin-1 pathological involvement is rod formation. Cofilin-1 oxidation has been shown to directly facilitate cofilin rod formation by the actin bundling activity of cofilin-1 oligomers[44–46]. Furthermore, Lin and colleagues demonstrated that Parkin, a ubiquitin E3 ligase which ubquitinylates proteins destined for degradation by the proteasome[47–49], interacts with LIMK and attenuates its phosphorylation of cofilin-1[50]. As such, genetic mutations in the gene *PARKIN* (*PARK2*), which result in autosomal recessive

juvenile Parkinsonism, may give rise to alterations in cofilin-1 activity and thus synaptic dysfunction.

In our PCA analysis using cofilin-1, p-cofilin-1, LIMK, p-LIMK and α-SYN, we could discriminate our cohort into two groups (control and PD) only in the occipital cortex. It is likely that the immunocytochemical findings could discriminate control and PD subjects only in occipital cortex because, although it is the last area to be affected by PD according to Braak hypothesis, the pathological changes have just begun in this brain area. By virtue of their *post mortem* origin, our PD subjects with late-stage-PD. It is therefore possible that regions associated with earlier-stages of disease, such as the cingulate gyrus, could have many neuropathological alterations that occur late in the pathogenic process which confound the data. Hence, this further supports our hypothesis that cofilin-1 is an early-stage mediator of neurodegeneration in the PD brain.

As expected, we also found changes in α-SYN expression in PD brain. This presynaptic protein is involved in neuronal plasticity and is a modulator of neuronal membrane stability[51,52]. However, α-SYN can undergo misfolding leading to abnormal aggregation into LBs and Lewy neurites, which are a pathological hallmark of the disease in both genetic and sporadic PD[32]. Here we showed a significant increase in its protein expression only in occipital cortex. An increase in α-SYN mRNA expression (*SNCA* gene) in the SN of both genetic and sporadic PD brain is associated with an increase in aggregation[53,54]. On the other hand, there are conflicting data demonstrating a decrease of mRNA expression in this brain area [55,56]. Our study indicates an increase in protein expression only in occipital cortex, which may be probably due to the initial stage of the neuropathological processes in this brain region.

Our most intriguing finding is – for the first time – our demonstration of the deposition of cofilin-1 in LBs. It is unclear whether LB are a cause or a consequence of PD, but they are associated with neurodegeneration. It is possible that this deposition is a neuroprotective reaction of the neuron to excessive amounts of cofilin-1 inside the highly-oxidizing environment of the dopaminergic neuron undergoing oxidative stress. Depositing cofilin-1 in LB would be a way to preventing cofilin-1 activation and subsequently mediating oxidative stress, an effect that has been observed in tumour cells[13]. Such a cytoprotective function for LB has been proposed[57]. However, as stated above, long-term sequestration of cofilin-1 in LB may result in the disruption of axonal transport and synaptic function, thus contributing to disease pathogenesis.

Conclusions

To date, the treatment of PD aims to rebalance dopamine levels and improve motor impairment. Hence, no disease modifying therapy is available which can stop or slow disease progression, and still there are no biomarkers of PD to improve early diagnosis. Here we provide evidence that cofilin-1 plays a key role in the pathogenesis of PD. PCA analysis demonstrates that cofilin-1 pathway proteins can be useful as an early marker of neurodegeneration in PD. Furthermore, oxidized cofilin-1 can be a future therapeutic target for PD for drug discovery studies.

Material and Methods

Cell culture

The human neuroblastoma cell line SH-SY5Y (ATCC, Manassas, VA, USA) was maintained in a 1:1 mixture of Ham's F12 and Dulbeco Modified Eagle Medium (DMEM) (Thermo Fisher®, Waltham, MA, USA) supplemented with 10% heat-inactivated fetal bovine serum (FBS) (Cripion Biotecnologia Ltda®, SP,

Brazil), 2 mM of glutamine, 100 U/mL of penicillin/streptomycin and antimycotic (Thermo Fisher®, Waltham, MA, USA) in a humidified atmosphere of 5% of CO_2 at 37ºC.

To differentiate cells into a neuronal dopaminergic phenotype, 3 X 10^4 undifferentiated cells/cm^2 were seeded and, after 24 hours, the medium was replaced with media containing 1% of FBS supplemented with 10 µM of RA (Enzo Life Sciences®, Farmindale, NY, USA; all-trans-retinoic-acid , #BML_GR100,) for 7 days[26]. We have previously established the best conditions to differentiate SH-SY5Y cells into dopaminergic cells for use as an experimental model to study molecular mechanisms underlying the pathophysiology of PD[26].

PD pathological mechanisms induction

Differentiated SH-SY5Y cells were incubated with TD_{50} dose of 6-OHDA, (Sigma®, St Lois, Missouri, USA; #H116) (15 µM diluted in 0.1% of ascorbic acid), as previously described [26].

Assessment of 6-OHDA-induced-cell-death

Hoechst 33342 (Thermo Fisher®, Waltham, MA, USA; 10 mg/mL solution in water, #H3570,) was used to stain live cells with a blue fluorescence, while PI (Sigma®, St Lois, Missouri, USA; solution 1.0 mg/mL in water, #P4884), which can only permeate cells with damaged cell membranes, was used to stain dead cells with red fluorescence. According to this paradigm, cell morphology was classified as live (normal nuclei, blue chromatin with organized structure), apoptotic (early apoptotic - bright blue chromatin which is condensed, or fragmented; late apoptotic - bright blue and red chromatin which is highly condensed or fragmented) and necrotic cells (red, enlarged nuclei with normal structure) as previously described[58].

Caspase activity and inhibition

Caspase 3, 8 and 9 activities were determined by the cleavage of the fluorescent substrate Ac-DEVD-AFC, Ac-IETD-AFC and Ac-LEHD-AFC respectively (Enzo Life Sciences®, Farmindale, NY, USA; #ALX-260-032, #ALX-260-110, #ALX-260-116). Cell were treated with 6-OHDA for 1.5, 3 6 and 12 hours. Then, they were scraped out from the culture flask, collected by centrifugation at 1000 g for 5 min and the supernatant was discarded. The pellet was washed with PBS and centrifuged again. The supernatant was discarded and the cells were suspended in lysis buffer (50mM, 100 mM, 0.1% CHAPS, 1 mM DTT, 100mM EDTA, pH 7.40). Cells were then frozen at -80ºC and thawed three times. Cells extracts were centrifuged at 14000g for 15 min and the supernatant was collected. In a 96-well-black plate, 20 µg of cell extracts, assay buffer (50mM HEPES, 100mM NaCl, 0.1% CHAPS, 10 mM DTT, 100 mM EDTA, 10 % glycerol, pH7.4) and fluorescent substrates were added (final concentration 50µM). The plate was incubated at 37ºC for 2 hours before reading. Samples were assayed using SoftMax Pro fluorescence plate reader (Molecular Devices®, USA) at an excitation wavelength of 485 nm and an emission wavelength of 538 nm.

After evaluating caspase activity, differentiated SH-SY5Y cells were pre-treated for 60 min with 100 µM permeable caspase inhibitors (Z-VAD-fmk, Z-IETD-fmk, Z-LEHD-fmk- Enzo Life Sciences®, Farmindale, NY, USA; #ALX-260-020, #ALX-260-144, #ALX-260-145) after which they were incubated with TD_{50} concentration of 6-OHDA as described above for 24 hours. Cell viability was determined by 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT, Sigma®, St Lois, Missouri, USA; # M5655) reduction assay, as previously described [26].

ROS production

Differentiated SH-SY5Y cells were treated with 6-OHDA as described above in 24-well-plate. Following this, the medium was discarded and the cells were incubated with either DCFH-DA (2,7-dichlorofluorescein diacetate-DCF-DA) (Sigma®, St Lois, Missouri, USA; #D6883) or with Amplex-Red (Thermo Fisher Scientific®, Waltham, MA, USA; #a12222) to evaluate the reactive species and H_2O_2 production by SH-SY5Y cells, respectively. Reactive species were measured using an excitation wavelength of 485 nm and a fluorescence emission of 538 nm, and H_2O_2 was measured using an excitation wavelength of 485 nm and an emission wavelength of 585 nm using a SoftMax Pro fluorescence plate reader (Molecular Devices®, USA).

Mitochondrial Membrane Potential (ΔΨm)

Differentiated SH-SY5Y cells were treated with 6-OHDA as described above followed by incubation for 20 min with 10 µg/mL 5,5',6,6'-tetrachloro-1,1',3,3'-tetraethyilbenzimidazilcarbocyanine iodide (JC-1, Sigma®, St Lois, Missouri, USA; #T4069,) Subsequently, cells were centrifuged, washed once with PBS, transferred to a 96-well black-plate (10^5 cells/well), and assayed using SoftMax Pro fluorescence plate reader (Molecular Devices®, USA) using an excitation wavelength of 485 nm and emission wavelengths of 540 nm and 590 nm, plus a cut-off at 530 nm. ΔΨm was estimated using the ratio of A590 (JC-1 aggregates)/A540 (monomeric form) [58].

Mitochondria Preparation

After 6-OHDA treatment, cells were harvest by centrifugation at 600g. The cell pellet was washed in ice-cold PBS and suspended in a lysis buffer (210 mM manitol, 70 mM sucrose, 5 mM Tris-HCl at pH 7.5 and 1 mM EDTA) containing 1% protease inhibitor cocktail (Roche®, Mannheim, Germany). Cell extract was

homogenized by gentle douncing (150 strokes). Cells extracted were centrifuged at 1400 g for 5 min at 4ºC. Supernatants were collected and centrifuged twice at 14000g for 30 min at 4ºC. The final supernatant was the cytosolic fraction plus light membranes. The pellets were a crude mitochondrial fraction plus heavier membranes. All fractions were disrupted by sonication before Western blot analysis. Cytosolic and mitochondrial fractions were identified using the following antibodies: anti- neuronal specific enolase (NSE- cytosolic marker, 1:10000, Cell Signaling Technology®, Danvers, MA, USA; #871) and anti-adenine nucleotide translocator (ANT-mitochondrial marker, 1:1000, Santa Cruz Biotechnology®, Dallas, TX, USA; #sc9299). Cofilin-1 was detected using anti-cofilin-1 (1: 2000; Abcam®, Cambridge, UK; #ab42824).

Transfection efficiency in RA-differentiated SH-SY5Y cells

Undifferentiated SH-SY5Y cells were transfected with plasmid encoding green fluorescence protein (GFP) to verify transfection efficiency by facile visualization of GFP fluorescence. This signal was maintained throughout the 7 days of the differentiation process, demonstrating successful transfection and maintenance of plasmid cDNA transcription and translation in our cellular model (Supplementary Fig 1a,b,c).

Generation of plasmids

A 500 bp cDNA encoding wild-type *CFL1*, produced using SH-SY5Y mRNA as template source, was cloned into the pcDNA3.1-TOPOHIS (Thermo Fisher®, Waltham, MA, USA; #K480040) vector to create pcDNA3.1-CMV-WT ($CFL1_{WT}$). From this, a non-oxidizable form *CFL1* encoding plasmid was produced by site-directed mutagenesis (Gene Art® Sited-mutagenesis PLUS System, Thermo Fisher®, Waltham, MA, USA; #A14604) in which Cys residues 39, 80 and 139 were mutated to Ala, creating pcDNA3.1-CMV-NOX ($CFL1_{NOX}$).

Restriction digestion using BsaHI (New England Biolabs®, Ipswich, MA, USA; #R0556S,) was used to confirm successful mutagenesis, as introduction of the Cys 139 Ala mutation resulted in a further BsaHI restriction site in the plasmid containing non-oxidizable *CFL1* (Supplementary Fig 1d).

Transient Transfection for overexpression of $CFL1_{WT}$ and $CFL1_{NOX}$

Transient transfection was performed using Lipofectamine 3000 (ThermoFisher®, Waltham, MA, USA; #L3000) in accordance with the manufacturer's instructions. Briefly, exponentially growing SH-SY5Y cells were seeded in 24-well-plate overnight before transfection with 0.75 µg of each construct for 48 hours. Transfection efficiency was evaluated by dot blot for $CFL1_{WT}$ and PCR using the following primers: 5' CACCATGGCCTCCGG 3'- 5' TCACAAAGGCTTGCCCTC 3' followed by restricted digestion using BSaHI for $CFL1_{NOX}$. Transiently-transfected cells were subsequently treated with 6-OHDA as described above to evaluate changes in cellular response to 6-OHDA.

In vitro statistical analysis

Data are expressed as mean ± SD of at least 3 independent experiments Statistical analysis of significance was assessed using Student's *t* test or one- or two-way ANOVA as appropriate (GraphPad® Software 6.0). $P < 0.05$ was taken as significant.

Cohort Analysis

A cohort of forty patients - 20 PD and 20 non-PD - was obtained from the Queen Square Brain Bank, University College London, UK. A retrospective review of patient information was conducted independently by two researchers according to the neuropathological diagnosis provided and the availability of reliable clinical data. The evaluation of the cohort aimed to identify any disorder that could cause bias in our research. Here we considered as exclusion factors:

Braak and Braak stage > 2 (neurofibrilar tangle-NFT stage), dementias, moderate to severe β-amyloid deposits, brain metastasis, other neurodegenerative diseases (*e.g.* AD and argyrophilic grain disease), inconclusive diagnosis, vascular dementia and cerebral infarction. After the patient selection, 21 patients - 10 controls and 11 PD individuals - remained in our study*.

Control cases were from individuals who had no clinical diagnosis of a neurological or psychiatric disorder during life, nor any neuropathological abnormality evidence other than normal age-related changes. Regarding PD patients, the neuropathology analysis of the brain bank revealed that all of them were staged at Braak VI with severe loss of dopaminergic neurons in SN and presented similar motor and non-motor symptoms (Supplementary Fig. 2 and Supplementary Table 1).

Immunohistochemistry

Formalin-fixed paraffin-embedded 12 µm occipital cortex and cingulate gyrus tissue sections were used to evaluate changes in coflin-1 pathway. Ethical permission for their use was obtained from NHS Research Authority NRES Committee London - Central (REC# 08/H0718/54+5). Brain tissue sections were subjected to DAB immunohistochemistry as previously described[30,31] using the following antibodies (all obtained from Abcam®, Cambridge, UK): anti-cofilin-1 (1:700- #ab42824), anti-p-cofilin-1 (1:100- #ab131274), anti-LIMK (1:300 - #ab810446), anti-p-LIMK (1:200 – ab 38508) and anti-α-SYN (1:1000,

* For cingulate gyrus, just 9 controls were considered since the brain bank did not provide sections for this area from one control patient- P45/04).

ab138501). Typical IHC for each antibody for each brain area analysed are in Supplementary Figure 3.

Image analysis

Digital images were obtained at 40X magnification using a Leica Microscope (Leica Microsytem®, Wetzlar, Germany). Stained sections were digitalized using the same illuminations and image capture parameters for each slide. For image analysis, the freeware Image J v.33 was downloaded from the NIH website (http://rsb.info.nih.gov/ij). The threshold operation converted foreground pixels into white colour and background pixels into black. Thus, the binary image represented the analysed DAB reaction. The area of positive DAB reaction was estimated by the number of pixels per area (integrated density).

Immunofluorescence

Co-localisation of LB pathology with cofilin-1 expression was assessed in SN by immunohistochemistry, using TH (1:1000, Abcam®, Cambridge, UK; #ab76442) or α-SYN (1:1000, Abcam®, Cambridge, UK; #ab138501) or /cofilin-1 (1:700, Abcam®, Cambridge, UK; #ab42824) antibodies followed by incubation with both AlexaFluor® 488 goat anti-rabbit IgG (H+ L) (green fluoresence-cofilin-1) (Thermo Fisher®, Waltham, MA, USA; #A11008) and Alexa Fluor® 594 goat anti-mouse IgG (H+ L) (red fluoresce- α-SYN or TH) (Thermo Fisher®, Waltham, MA, USA; #A11005).

Clinical data Statistical analysis

Data were analysed using SPSS™ statistics software v.19 (IBM) and were presented as mean ± S.D. Initially, integral density values were BoxCox transformed and tested for homoscedasticity using Levine's tests. Afterwards Student's *t*-test with or without Welch correction was used to analyse differences between control and PD groups.

For the PCA, transformed data was centered and scaled prior the computation, which was performed using FactoMineR package[59]. Visualization of the PCA results were obtained using factoextra[60], ggplot2[61] and rgl[62], rgl: 3D[63] packages.

Author's Contributions Statement

F.M.L., C.B.M. and I.J.B. performed the caspase activity, mitochondria isolation and western blot. F.M.L. and S.S. generate the plasmids and performed the transfections. F.M.L. and J.P. read patient records and performed IHC. F.M.L., M.A.D.B. and G.Z. performed the bioinformatic analysis of the IHC. M.Z. and C.M.E. performed the immunofluorescence. F.M.L., R.B.P., and F.K. conceived the experiments and analyzed the data. F.M.L wrote the manuscript.

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References

1. Carlier, M. F., Ressad, F. & Pantaloni, D. Control of actin dynamics in cell motility. Role of ADF/cofilin. *J. Biol. Chem.* **274,** 33827–30 (1999).
2. Kanellos, G. & Frame, M. C. Cellular functions of the ADF/cofilin family at a glance. *J. Cell Sci.* **129,** (2016).

3. Flynn, K. C., Pak, C. W., Shaw, A. E., Bradke, F. & Bamburg, J. R. Growth cone-like waves transport actin and promote axonogenesis and neurite branching. *Dev. Neurobiol.* **69,** 761–79 (2009).

4. Racz, B. & Weinberg, R. J. Spatial organization of cofilin in dendritic spines. *Neuroscience* **138,** 447–456 (2006).

5. Wolf, M. *et al.* ADF/Cofilin Controls Synaptic Actin Dynamics and Regulates Synaptic Vesicle Mobilization and Exocytosis. *Cereb. Cortex* **25,** 2863–75 (2015).

6. Hotulainen, P., Paunola, E., Vartiainen, M. K. & Lappalainen, P. Actin-depolymerizing factor and cofilin-1 play overlapping roles in promoting rapid F-actin depolymerization in mammalian nonmuscle cells. *Mol. Biol. Cell* **16,** 649–64 (2005).

7. Gu, J. *et al.* ADF/cofilin-mediated actin dynamics regulate AMPA receptor trafficking during synaptic plasticity. *Nat. Neurosci.* **13,** 1208–1215 (2010).

8. Nishida, E., Maekawa, S. & Sakai, H. Cofilin, a protein in porcine brain that binds to actin filaments and inhibits their interactions with myosin and tropomyosin. *Biochemistry* **23,** 5307–13 (1984).

9. Bamburg, J. R. & Bernstein, B. W. Roles of ADF/cofilin in actin polymerization and beyond. *F1000 Biol. Rep.* **2,** 62 (2010).

10. Arber, S. *et al.* Regulation of actin dynamics through phosphorylation of cofilin by LIM-kinase. *Nature* **393,** 805–9 (1998).

11. Niwa, R., Nagata-Ohashi, K., Takeichi, M., Mizuno, K. & Uemura, T. Control of actin reorganization by Slingshot, a family of phosphatases that dephosphorylate ADF/cofilin. *Cell* **108,** 233–46 (2002).

12. Klemke, M. *et al.* Oxidation of cofilin mediates T cell hyporesponsiveness under oxidative stress conditions. *Immunity* **29,** 404–13 (2008).

13. Klamt, F. *et al.* Oxidant-induced apoptosis is mediated by oxidation of the actin-regulatory protein cofilin. *Nat. Cell Biol.* **11,** 1241–6 (2009).

14. Chua, B. T. *et al.* Mitochondrial translocation of cofilin is an early step in apoptosis induction. *Nat. Cell Biol.* **5,** 1083–9 (2003).

15. Roh, S.-E. *et al.* Mitochondrial dysfunction and calcium deregulation by the RanBP9-cofilin pathway. *FASEB J. Off. Publ. Fed. Am. Soc. Exp. Biol.* **27,** 4776–4789 (2013).

16. Woo, J. A. *et al.* Slingshot-Cofilin activation mediates mitochondrial and synaptic dysfunction via Abeta ligation to beta1-integrin conformers. *Cell Death Differ.* **22,** 921–934 (2015).

17. Woo, J. A. *et al.* RanBP9 at the intersection between cofilin and Abeta pathologies: rescue of neurodegenerative changes by RanBP9 reduction. *Cell Death Dis.* **6,** 1676 (2015).

18. Heredia, L. *et al.* Phosphorylation of actin-depolymerizing factor/cofilin by LIM-kinase mediates amyloid beta-induced degeneration: a potential mechanism of neuronal dystrophy in Alzheimer's disease. *J. Neurosci.* **26,**

6533–42 (2006).

19. Barone, E., Mosser, S. & Fraering, P. C. Inactivation of brain Cofilin-1 by age, Alzheimer's disease and γ-secretase. *Biochim. Biophys. Acta* **1842,** 2500–9 (2014).

20. Bamburg, J. R. & Bernstein, B. W. Actin dynamics and cofilin-actin rods in Alzheimer disease. *Cytoskeleton (Hoboken).* (2016). doi:10.1002/cm.21282

21. Gibb, W. R. Neuropathology of the substantia nigra. *Eur. Neurol.* **31 Suppl 1,** 48–59 (1991).

22. Mila, S. *et al.* Lymphocyte proteomics of Parkinson's disease patients reveals cytoskeletal protein dysregulation and oxidative stress. *Biomark. Med.* **3,** 117–28 (2009).

23. Dias, V., Junn, E. & Mouradian, M. M. The role of oxidative stress in Parkinson's disease. *J. Parkinsons. Dis.* **3,** 461–91 (2013).

24. Franco-Iborra, S., Vila, M. & Perier, C. The Parkinson Disease Mitochondrial Hypothesis: Where Are We at? *Neuroscientist* 1073858415574600- (2015). doi:10.1177/1073858415574600

25. Schapira, A. H. *et al.* Anatomic and disease specificity of NADH CoQ1 reductase (complex I) deficiency in Parkinson's disease. *J. Neurochem.* **55,** 2142–5 (1990).

26. Lopes, F. M. *et al.* Comparison between proliferative and neuron-like SH-SY5Y cells as an in vitro model for Parkinson disease studies. *Brain Res.* **1337,** 85–94 (2010).

27. Lopes, F. M. *et al.* RA Differentiation Enhances Dopaminergic Features, Changes Redox Parameters, and Increases Dopamine Transporter Dependency in 6-Hydroxydopamine-Induced Neurotoxicity in SH-SY5Y Cells. *Neurotox. Res.* (2017). doi:10.1007/s12640-016-9699-0

28. Ljungdahl, A., Hökfelt, T., Jonsson, G. & Sachs, C. Autoradiographic demonstration of uptake and accumulation of 3H-6-hydroxydopamine in adrenergic nerves. *Experientia* **27,** 297–9 (1971).

29. Braak, H. *et al.* Staging of brain pathology related to sporadic Parkinson's disease. *Neurobiol. Aging* **24,** 197–211 (2003).

30. Parsons, R. B., Smith, M.-L., Williams, A. C., Waring, R. H. & Ramsden, D. B. Expression of nicotinamide N-methyltransferase (E.C. 2.1.1.1) in the Parkinsonian brain. *J. Neuropathol. Exp. Neurol.* **61,** 111–24 (2002).

31. Parsons, R. B., Smith, S. W., Waring, R. H., Williams, A. C. & Ramsden, D. B. High expression of nicotinamide N-methyltransferase in patients with idiopathic Parkinson's disease. *Neurosci. Lett.* **342,** 13–6 (2003).

32. Spillantini, M. G. *et al.* [alpha]-Synuclein in Lewy bodies. **388,** 839–840 (1997).

33. Lim, M. K. *et al.* Parkin interacts with LIM Kinase 1 and reduces its cofilin-phosphorylation activity via ubiquitination. *Exp. Cell Res.* **313,** 2858–74

(2007).

34. Gomez-Lazaro, M. *et al.* 6-Hydroxydopamine activates the mitochondrial apoptosis pathway through p38 MAPK-mediated, p53-independent activation of Bax and PUMA. *J. Neurochem.* **104,** 1599–612 (2008).

35. Bernstein, A. I., Garrison, S. P., Zambetti, G. P. & O'Malley, K. L. 6-OHDA generated ROS induces DNA damage and p53- and PUMA-dependent cell death. *Mol. Neurodegener.* **6,** 2 (2011).

36. Hanrott, K., Gudmunsen, L., O'Neill, M. J. & Wonnacott, S. 6-hydroxydopamine-induced apoptosis is mediated via extracellular auto-oxidation and caspase 3-dependent activation of protein kinase Cdelta. *J. Biol. Chem.* **281,** 5373–82 (2006).

37. Cheung, Y.-T. *et al.* Effects of all-trans-retinoic acid on human SH-SY5Y neuroblastoma as in vitro model in neurotoxicity research. *Neurotoxicology* **30,** 127–35 (2009).

38. Izumi, Y. *et al.* p-Quinone mediates 6-hydroxydopamine-induced dopaminergic neuronal death and ferrous iron accelerates the conversion of p-quinone into melanin extracellularly. *J. Neurosci. Res.* **79,** 849–60 (2005).

39. Wei, L. *et al.* Wnt3a protects SH-SY5Y cells against 6-hydroxydopamine toxicity by restoration of mitochondria function. *Transl. Neurodegener.* **4,** 11 (2015).

40. Rehklau, K. *et al.* ADF/cofilin proteins translocate to mitochondria during apoptosis but are not generally required for cell death signaling. *Cell Death Differ.* **19,** 958–67 (2012).

41. Halliwell, B. Oxidative stress and neurodegeneration: where are we now? *J. Neurochem.* **97,** 1634–58 (2006).

42. Schapira, A. H. *et al.* Mitochondrial complex I deficiency in Parkinson's disease. *J. Neurochem.* **54,** 823–7 (1990).

43. Sian, J. *et al.* Alterations in glutathione levels in Parkinson's disease and other neurodegenerative disorders affecting basal ganglia. *Ann. Neurol.* **36,** 348–55 (1994).

44. Dahl, J.-U., Gray, M. J. & Jakob, U. Protein quality control under oxidative stress conditions. *J. Mol. Biol.* **427,** 1549–63 (2015).

45. Minamide, L. S., Striegl, A. M., Boyle, J. A., Meberg, P. J. & Bamburg, J. R. Neurodegenerative stimuli induce persistent ADF/cofilin-actin rods that disrupt distal neurite function. *Nat. Cell Biol.* **2,** 628–36 (2000).

46. Pfannstiel, J. *et al.* Human cofilin forms oligomers exhibiting actin bundling activity. *J. Biol. Chem.* **276,** 49476–84 (2001).

47. Shimura, H. *et al.* Familial Parkinson disease gene product, parkin, is a ubiquitin-protein ligase. *Nat. Genet.* **25,** 302–5 (2000).

48. Imai, Y., Soda, M. & Takahashi, R. Parkin suppresses unfolded protein stress-induced cell death through its E3 ubiquitin-protein ligase activity. *J.*

Biol. Chem. **275,** 35661–4 (2000).

49. Zhang, Y. *et al.* Parkin functions as an E2-dependent ubiquitin- protein ligase and promotes the degradation of the synaptic vesicle-associated protein, CDCrel-1. *Proc. Natl. Acad. Sci. U. S. A.* **97,** 13354–9 (2000).

50. Lim, M. K. *et al.* Parkin interacts with LIM Kinase 1 and reduces its cofilin-phosphorylation activity via ubiquitination. *Exp. Cell Res.* **313,** 2858–74 (2007).

51. Iwai, A. *et al.* The precursor protein of non-A beta component of Alzheimer's disease amyloid is a presynaptic protein of the central nervous system. *Neuron* **14,** 467–75 (1995).

52. Wang, T. & Hay, J. C. Alpha-synuclein Toxicity in the Early Secretory Pathway: How It Drives Neurodegeneration in Parkinsons Disease. *Front. Neurosci.* **9,** 433 (2015).

53. Kingsbury, A. E. *et al.* Alteration in α-synuclein mRNA expression in Parkinson's disease. *Mov. Disord.* **19,** 162–170 (2004).

54. Xu, J. *et al.* Dopamine-dependent neurotoxicity of α-synuclein: A mechanism for selective neurodegeneration in Parkinson disease. *Nat. Med.* **8,** 600–606 (2002).

55. Wirdefeldt, K. *et al.* Expression of α-synuclein in the human brain: relation to Lewy body disease. *Mol. Brain Res.* **92,** 58–65 (2001).

56. Jowaed, A., Schmitt, I., Kaut, O. & Wullner, U. Methylation Regulates Alpha-Synuclein Expression and Is Decreased in Parkinson's Disease Patients' Brains. *J. Neurosci.* **30,** 6355–6359 (2010).

57. Tanaka, M. *et al.* Aggresomes formed by alpha-synuclein and synphilin-1 are cytoprotective. *J. Biol. Chem.* **279,** 4625–31 (2004).

58. Klamt, F. & Shacter, E. Taurine chloramine, an oxidant derived from neutrophils, induces apoptosis in human B lymphoma cells through mitochondrial damage. *J. Biol. Chem.* **280,** 21346–21352 (2005).

59. Husson, F., Julie, A., Agrocampus, J. & Es Agrocampus, J. Technical Report – Agrocampus Principal component methods -hierarchical clustering -partitional clustering: why would we need to choose for visualizing data?

60. Extract and Visualize the Results of Multivariate Data Analyses [R package factoextra version 1.0.3].

61. Wickham, H. *ggplot2*. (Springer New York, 2009). doi:10.1007/978-0-387-98141-3

62. CRAN - Package rgl. Available at: https://cran.r-project.org/web/packages/rgl/index.html.

63. R: 3D Visualization Using OpenGL. Available at: http://finzi.psych.upenn.edu/library/rgl/html/00Index.html.

Figure Legends

Figure 1: 6-OHDA-induced-cell-death assessment. (a,d) Representative phase-contrast and (b,e) Hoechst 33342/(c,f) PI staining fluorescence images in RA-differentiated SH-SY5Y cells. 6-OHDA treatment induced nuclear morphological changes. The blue arrows indicate round shape and nuclear condensation typical in cell labeled with Hoechst 33342 (early apoptosis). Orange arrows shows cells with bright blue and red fluorescence (late apoptosis). Red arrows exhibiting uniform enlarged blue/red nuclei were necrotic cells (necrosis). (g) Effect of caspase inhibition in 6-OHDA-neurotoxicity. Cells were pre-incubated with cell-permeable caspases inhibitors (Z-DVED – 3; Z-IETD – 8; Z-LEHD – 9) and treated with 6-OHDA for 24h and cell death was determined by MTT assay. All experiments were performed in at least triplicates (n = 3). The results are expressed as percentage of untreated cells. Significant differences are expressed by *letters*, where *equal letters* represent no significant differences and *different letters* represent significant differences ($p < 0.0001$) (one-way-analysis of variance) (h) Time course experiments of caspases activities in RA-differentiated SH-SY5Y cells treated with 6-OHDA. Cell were treated, harvest and incubated with Caspases-3, -8 and -9 substrates. All experiments were performed in at least triplicates (n = 4). The results are expressed in fold-increase to untreated cells *Statistically different from the corresponding control values*** $p < 0.0001$ (one-way-analysis of variance). (i) Evaluation of mitochondrial dysfunction in 6-OHDA-treated-RA-differentiated-SH-SY5Y-cells. Cells were treated during 1.5, 3, 6, 12 and 24 hours, harvest and incubated with 10 µg/ML of JC-1. All experiments were performed in at least triplicates (n = 3). *Statistically different from the corresponding control values *** $p < 0.0001$ (two-way-analysis

of variance). (j) Oxidants production by DCF assay (k) and H_2O_2 production by Amplex Red. Cells were treated with 6-OHDA for 24 hours and then incubate with fluorescence probe (DCF or AmplexRed). All experiments were performed in at least triplicates (n = 3). *Statistically different from the corresponding control values * p<0.05, ** p<0.005 (Student *t* test).

Figure 2: The role of cofilin-1 is apoptosis-induced by 6-OHDA (a,b) Cofilin-1 mitochondrial translocation. Cells were treated with 6-OHDA for 1.5, 3 and 6 hours and the mitochondria were isolated. In (a) a representative immunoblot and (b) densitometry analysis showing increase of cofilin-1 in mitochondrial fraction when compared to untreated cells after 3 hours of treatment. ANT and NSE were used as mitochondrial and cytosolic markers respectively. All experiments were performed in at least triplicates (n = 3). Significant differences are expressed by *letters*, where *equal letters* represent no significant differences and *different letters* represent significant differences ($p < 0.0001$) (one-way-analysis of variance). (c, d, e, f) Transient transfection and overexpression of *CFL1WT and CFL1-NOX.* SH-SYSY were transfected with mock (empty vector) or plasmid containing each construct, and the differentiation protocol were performed. c) Representative *dot blot* of *CFL1-WT* and densitometry analysis showing an increase in the protein immunocontent in transfected cells when compared to mock cells. *Statistically different from the corresponding control values **p < 0.05 (Student t test). (h) Cell death (percentage of control) of mock and transfected cells treated with 6OHDA TD_{50} value. Significant differences are expressed by *letters*, where *equal letters* represent no significant differences and *different letters* represent significant differences ($p < 0.01$) (two-way-analysis of variance). (g) Representative gel of restricted digestion of PCR product. Black

arrow indicates the diagnostic band in 236 bp for *CFL1*$_{NOX}$. (h) Effect of non-oxidizable *CFL1* transfection in 6-OHDA-indiced-neurotoxicity. All experiments were performed in at least triplicates (n = 3). Significant differences are expressed by *letters*, where *equal letters* represent no significant differences and *different letters* represent significant differences ($p < 0.001$) (two-way analysis of variance).

Figure 3: Immunohistochemistry of cofilin-1 pathway in control and PD brains. Cingulate gyrus and occipital cortex 12 µm sections were subjected to immunohistochemistry using the following antibodies anti-cofilin-1, anti-p-cofilin-1, and anti-α-SYN (scale bar = 50µm). Quantification of protein expression was assessed by Image J by threshold operation, forming a binary image. The area of positive reaction was estimated by the number of pixels/area (integrated density). These values were BoxCox transformed and tested for homoscedasticity using Levine's tests. (a, c, e). Representative images of the immunochemical reactions and (b, d, f) quantitative analysis. *Statistically different from the corresponding control values * $p < 0.05$; ** $p < 0.01$ (Student *t* test with or without Welch's correction).

Figure 4: Cofilin-1 and LIMK phosphorylation status. The integrated density values from the immunohistochemistry reactions were used to calculate both ratios of p-cofilin-1/cofilin-1 and p-LIMK/LIMK to infer cofilin-1 activation in (a, b) cingulate gyrus and (c,d) occipital cortex. . *Statistically different from the corresponding control values * p 0.05; ** p<0.01 (Student *t* test with or without Welch's correction).

Figure 5: Principal Component Analysis of Immunohistochemistry Markers in cingulate gyrus. (a) 3D and 2D scatter plots of the three first principal components with (upper figure) and without (lower figure) α-SYN as input for the PCA computation. (b) Boxplot showing the group difference between PD and control individuals for each PC. (c) Percentage contribution of each marker to the variance of the PC.

Figure 6: Principal Component Analysis of Immunohistochemistry Markers in occipital cortex. (a) 3D and 2D scatter plots of the three first principal components with (upper figure) and without (lower figure) α-SYN as input for the PCA computation. (b) Boxplot showing the group difference between PD and control individuals for each PC. (c) Percentage contribution of each marker to the variance of the PC. Note that control and PD subjects can be distinguish in 3D scatter plot.

Figure 7: (a) Cofilin-1 and TH co-localisation in SNpc of PD brains. Representative immunofluorescent staining of cofilin-1 (green) and TH (red). The white arrow indicates a Lewy Body. (b) Cofilin-1 and α-SYN co-localisation in SNpc of PD brains. Representative immunofluorescent staining of coflin-1 (green) and α-SYN (red). The white arrow indicates cofilin-1 deposition in a LB.

Supplementary Information

Supplementary Figure 1: (a,b) Transient transfection and overexpression of GFP. SH-SY5Y cells were transfected with plasmid containing GFP cDNA and

the differentiation protocol were performed. (c) Note that differentiated cells for 7 days present the stellate morphology with intense neurites and are still positive for GFP (200X magnification). (d) Generation of plasmids containing *CFL1*$_{WT}$ and *CFL1*$_{NOX}$. Representative gel showing the PCR product of the plasmids in 500 bp. (e) Representative gel showing the PCR product of the plasmids followed by restricted digestion by BsaHI. Introduction of the Cys139Ala mutation resulted in the introduction of a further BsaHI restriction site in the plasmid (black arrow).

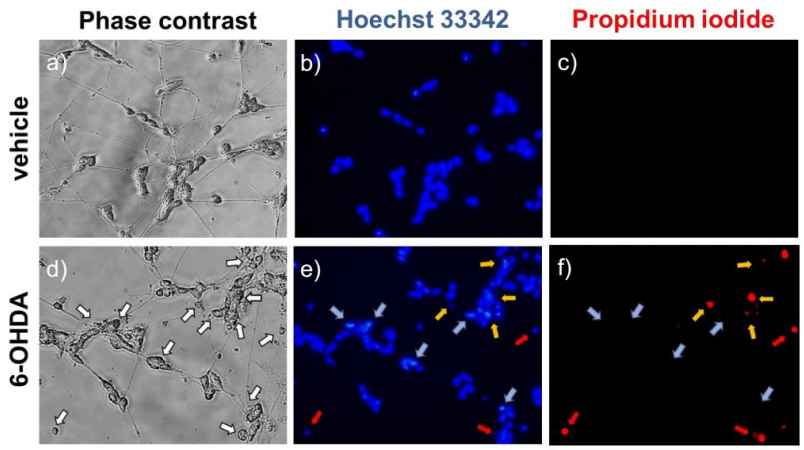
Supplementary Figure 2: Experimental design of the quantification of the cofilin-1 pathway proteins immunocontent. A cohort of forty patients - 20 PD and 20 non-PD - was obtained from the Queen Square Brain Bank, University College London, UK. The cohort was evaluated by two researchers to identify any disorder that could cause bias in our research. *Exclusion factors: (*i*) Braak and Braak stage > 2 (neurofibrilar tangle-NFT stage), (*ii*) dementias, (*iii*) moderate to severe β-amyloid deposits, (*iv*) brain metastasis, (*v*) other neurodegenerative diseases (*e.g.* AD and argyrophilic grain disease), (*vi*) inconclusive diagnosis, (*vii*) vascular dementia and (*viii*) cerebral infarction. After the patient selection, 21 patients - 10 controls and 11 PD individuals. The brains areas were chosen according to Braak hypothesis: cingulate gyrus and occipital cortex as highlighted at the image.

Supplementary Figure 3: Representative images of typical immunochemical staining of the following antibodies: cofilin-1, phosphor-cofilin-1 -, LIM kinase

(LIMK), phospho-LIMK (p-LIMK) and α-synuclein (α-SYN) in cingulate gyrus and occipital cortex.

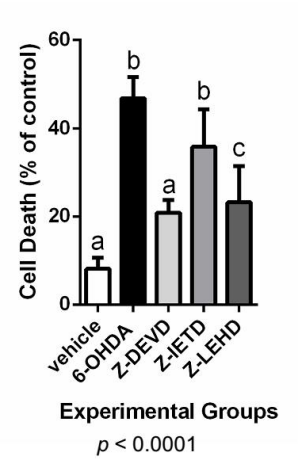
Supplementary Figure 4: Quantification of the immunohistochemistry of cofilin-1 pathway proteins in control and PD brains. Cingulate gyrus and occipital cortex 12 µm sections were subjected to immunohistochemistry using the following antibodies anti-cofilin-1, anti-p-cofilin-1, LIM kinase (LIMK), phospho-LIMK (p-LIMK) and anti-α-SYN (scale bar = 50µm). Quantification of protein expression was assessed by Image J by threshold operation, forming a binary image. The area of positive reaction was estimated by the number of pixels/area (integrated density). These values were BoxCox transformed and tested for homoscedasticity using Levine's tests.

Morphological Assessment of 6-OHDA-induced-cell-death



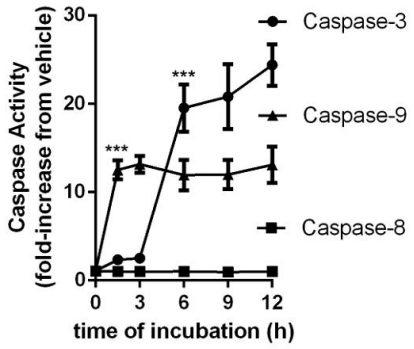
The role of caspases in 6-OHDA-induced-cell-death

g)



$p < 0.0001$

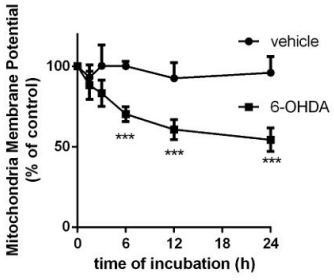
h)



$p < 0.0001$

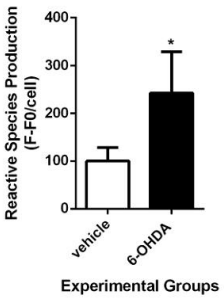
Mitochondria Dysfunction and ROS production in 6-OHDA-induced-cell death

i)



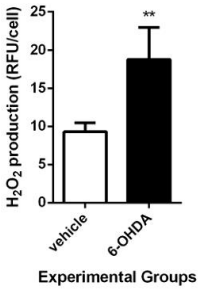
$p < 0.0001$

j)



$p < 0.05$

k)



$p < 0.005$

Figure 1

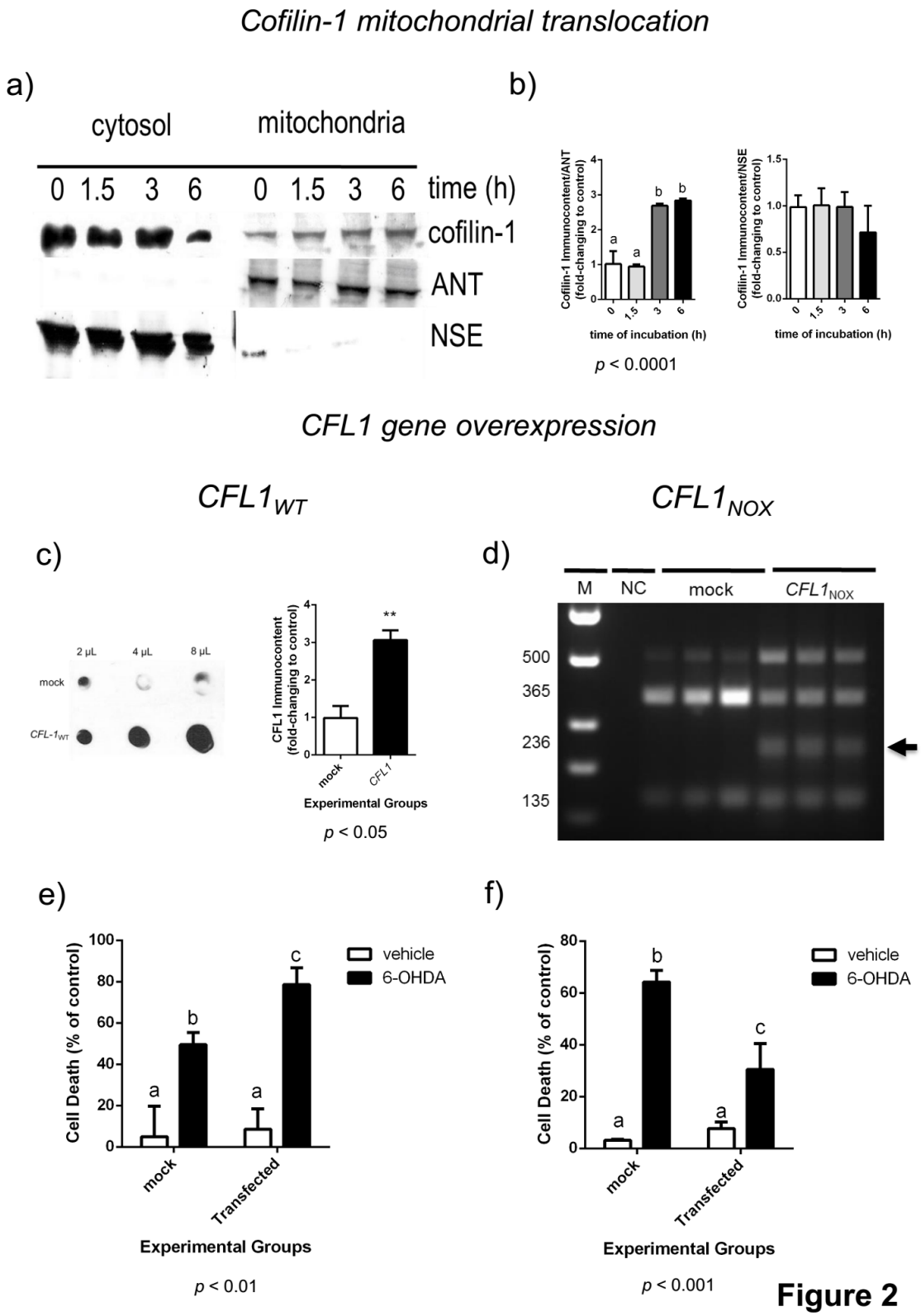
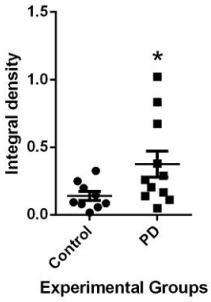

Cofilin-1 mitochondrial translocation
a)
cytosol
mitochondria
0 1.5 3 6 0 1.5 3 6 time (h)
cofilin-1
ANT
NSE
b)
Cofilin-1 Immunocontent/ANT (fold-changing to control)
time of incubation (h)
Cofilin-1 Immunocontent/NSE (fold-changing to control)
time of incubation (h)
p < 0.0001
CFL1 gene overexpression
CFL1WT
CFL1NOX
c)
2 µL
4 µL
8 µL
mock
CFL-1WT
CFL1 Immunocontent (fold-changing to control)
mock
CFL1
Experimental Groups
p < 0.05
d)
M
NC
mock
CFL1NOX
500
365
236
135
e)
Cell Death (% of control)
vehicle
6-OHDA
mock
Transfected
Experimental Groups
p < 0.01
f)
Cell Death (% of control)
vehicle
6-OHDA
mock
Transfected
Experimental Groups
p < 0.001

Figure 2

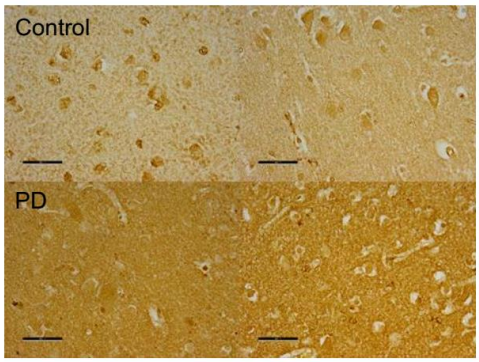
Cingulate Gyrus

a) Cofilin-1



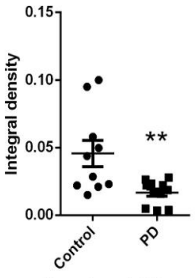
$p < 0.05$

b)



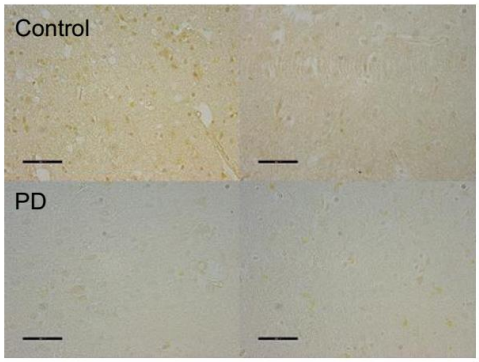
Occipital Cortex

c) p-cofilin-1

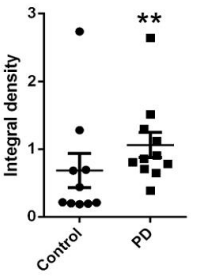


$p < 0.01$

d)



e) α-SYN



$p < 0.01$

f)

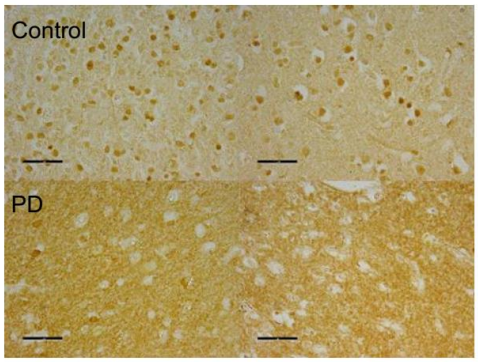
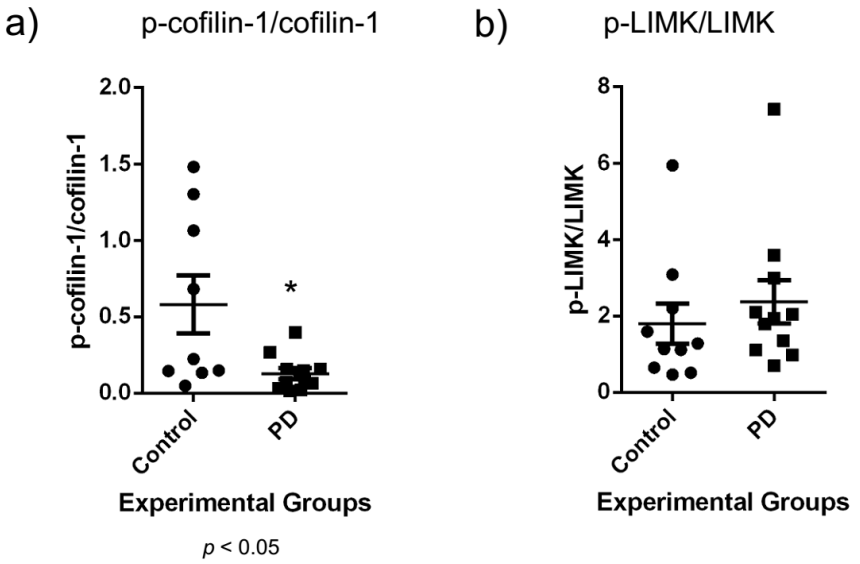


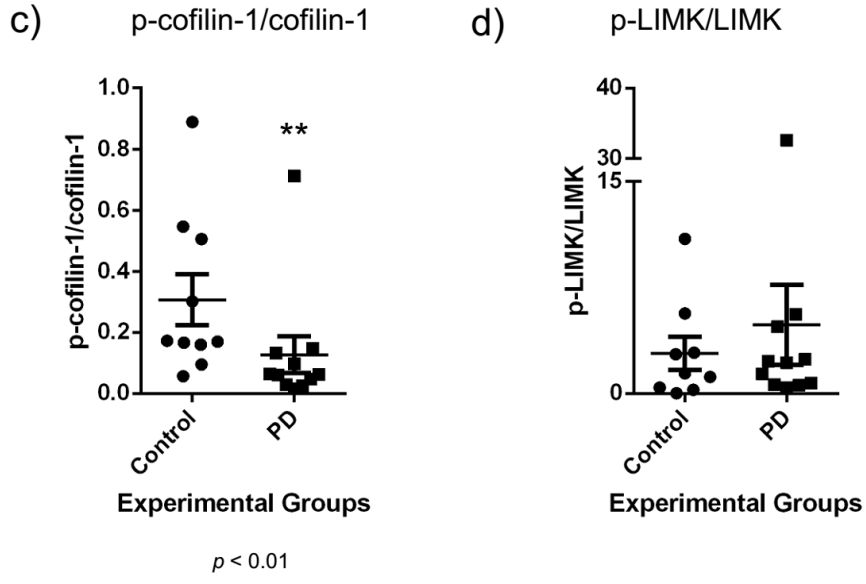
Figure 3

Cingulate Gyrus



$p < 0.05$

Occipital Cortex



$p < 0.01$

Figure 4

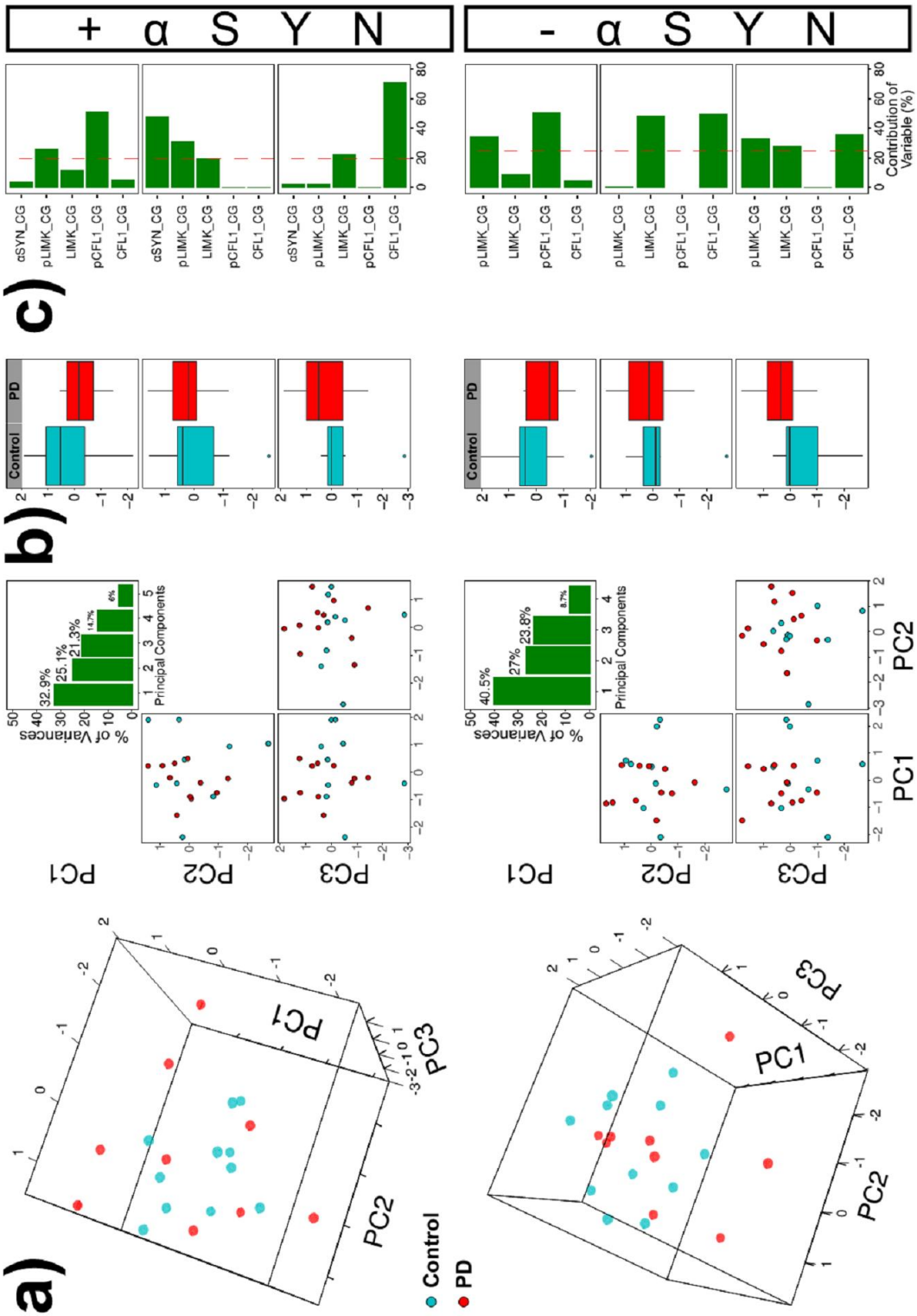

+ α S Y N
- α S Y N
a)
b)
c)
Control
PD
αSYN_CG
pLIMK_CG
LIMK_CG
pCFL1_CG
CFL1_CG
% of Variances
Principal Components
Contribution of Variable (%)
PC1
PC2
PC3

Figure 5

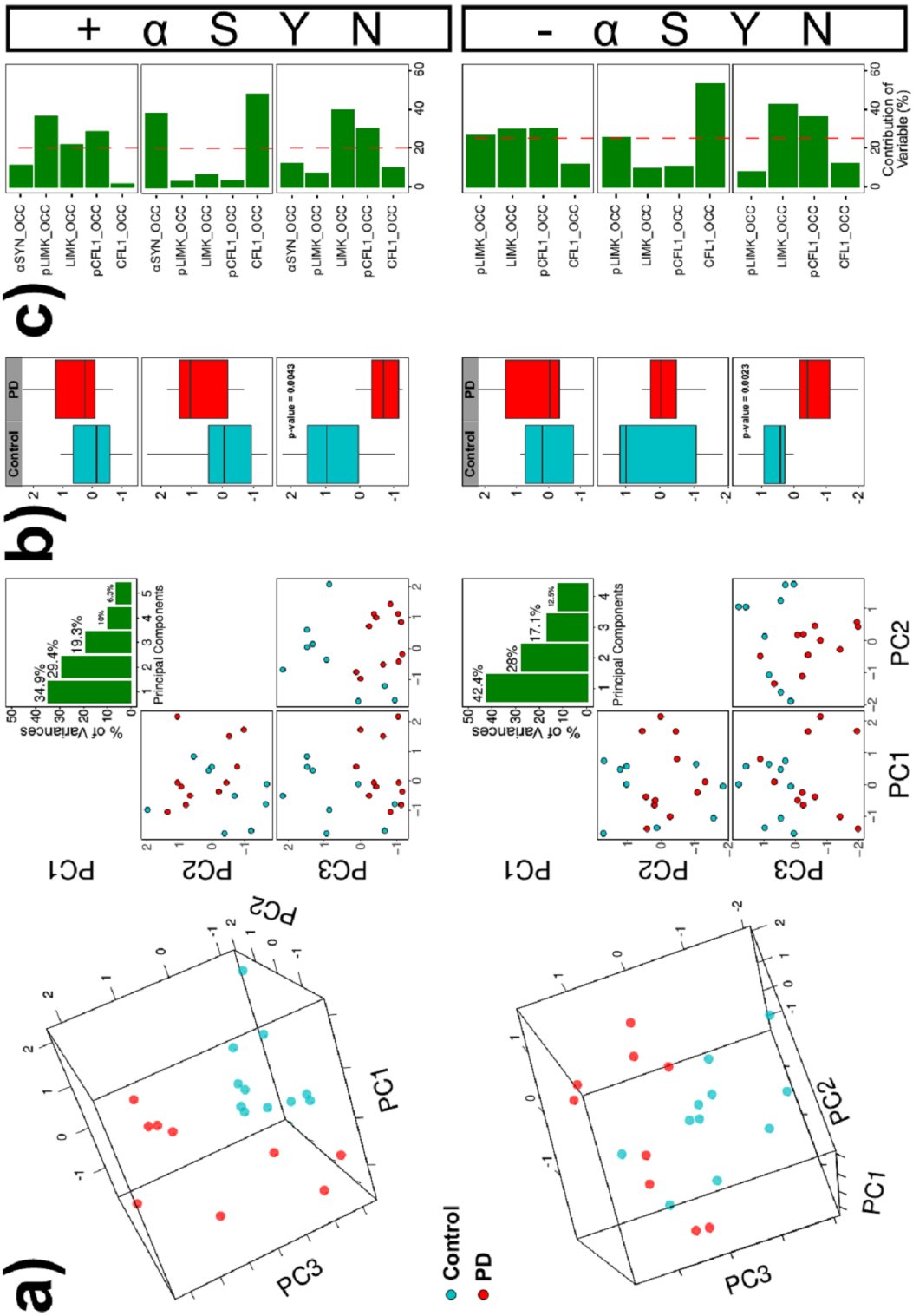

+ α S Y N
- α S Y N
a)
b)
c)
Control
PD
p-value = 0.0043
p-value = 0.0023
αSYN_OCC
pLIMK_OCC
LIMK_OCC
pCFL1_OCC
CFL1_OCC
Contribution of Variable (%)
% of Variances
Principal Components
34.9%
29.4%
19.3%
42.4%
28%
17.1%
PC1
PC2
PC3
Control
PD

Figure 6

Cofilin-1 in a Lewy body from a PD patient

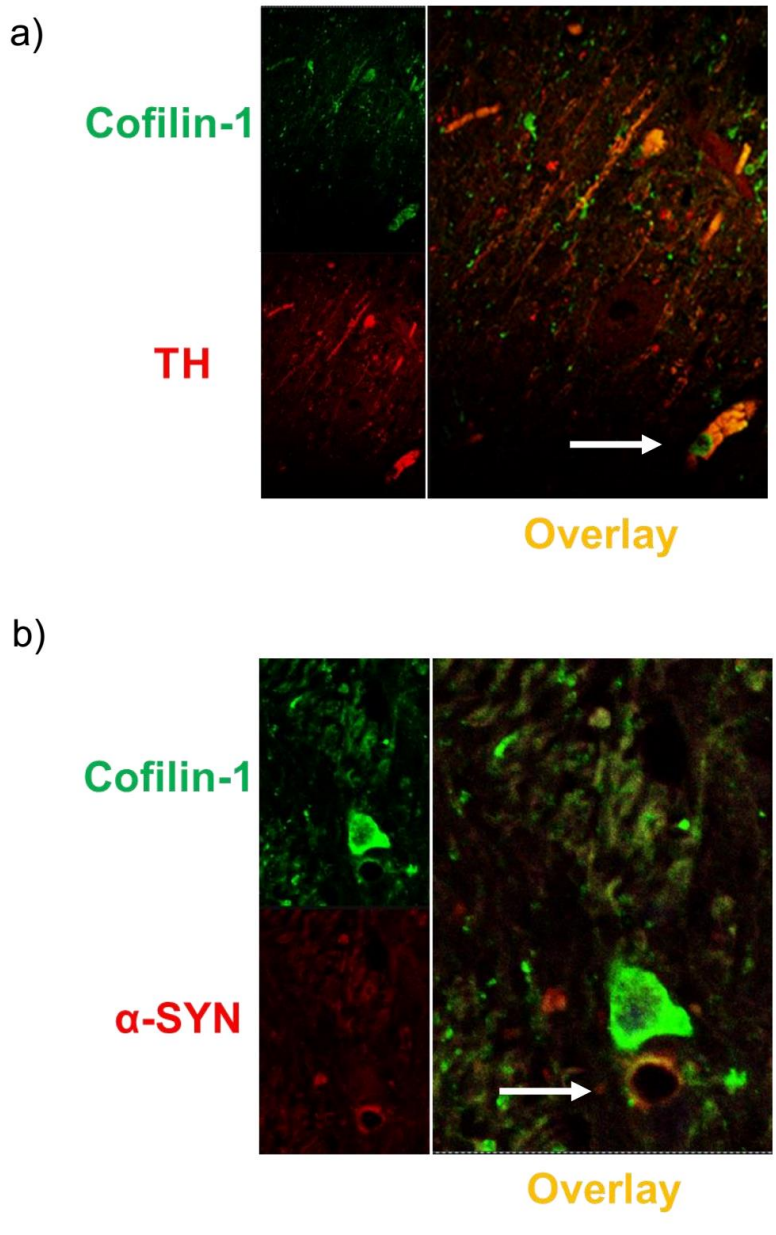
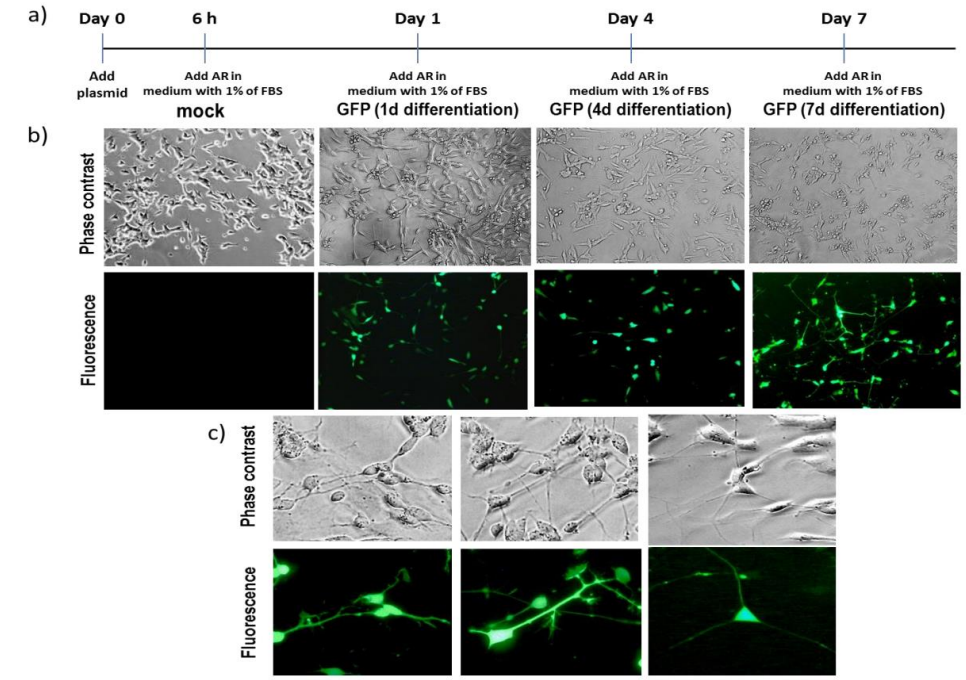
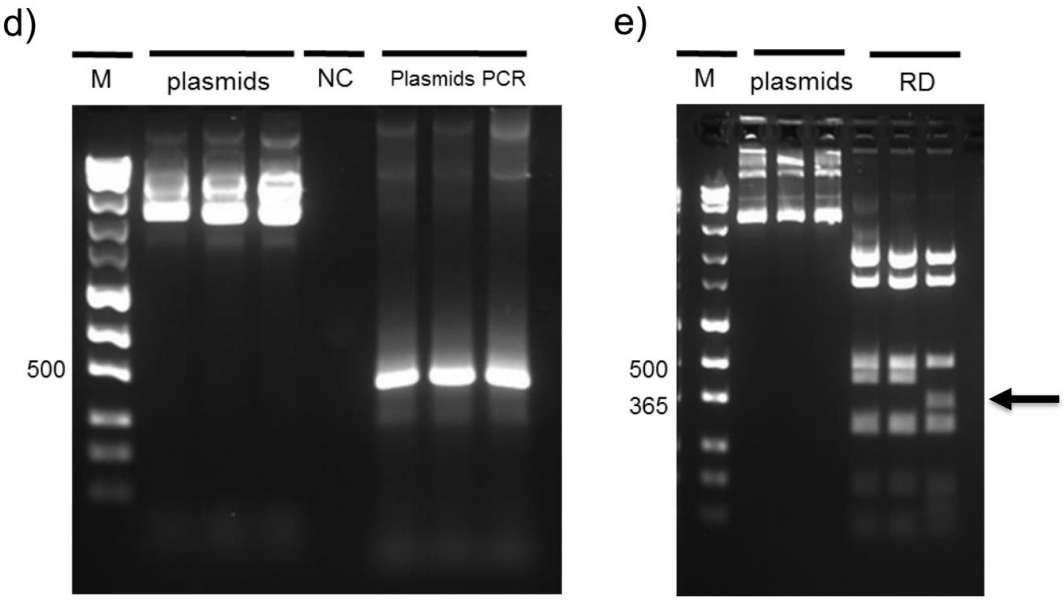


Figure 7

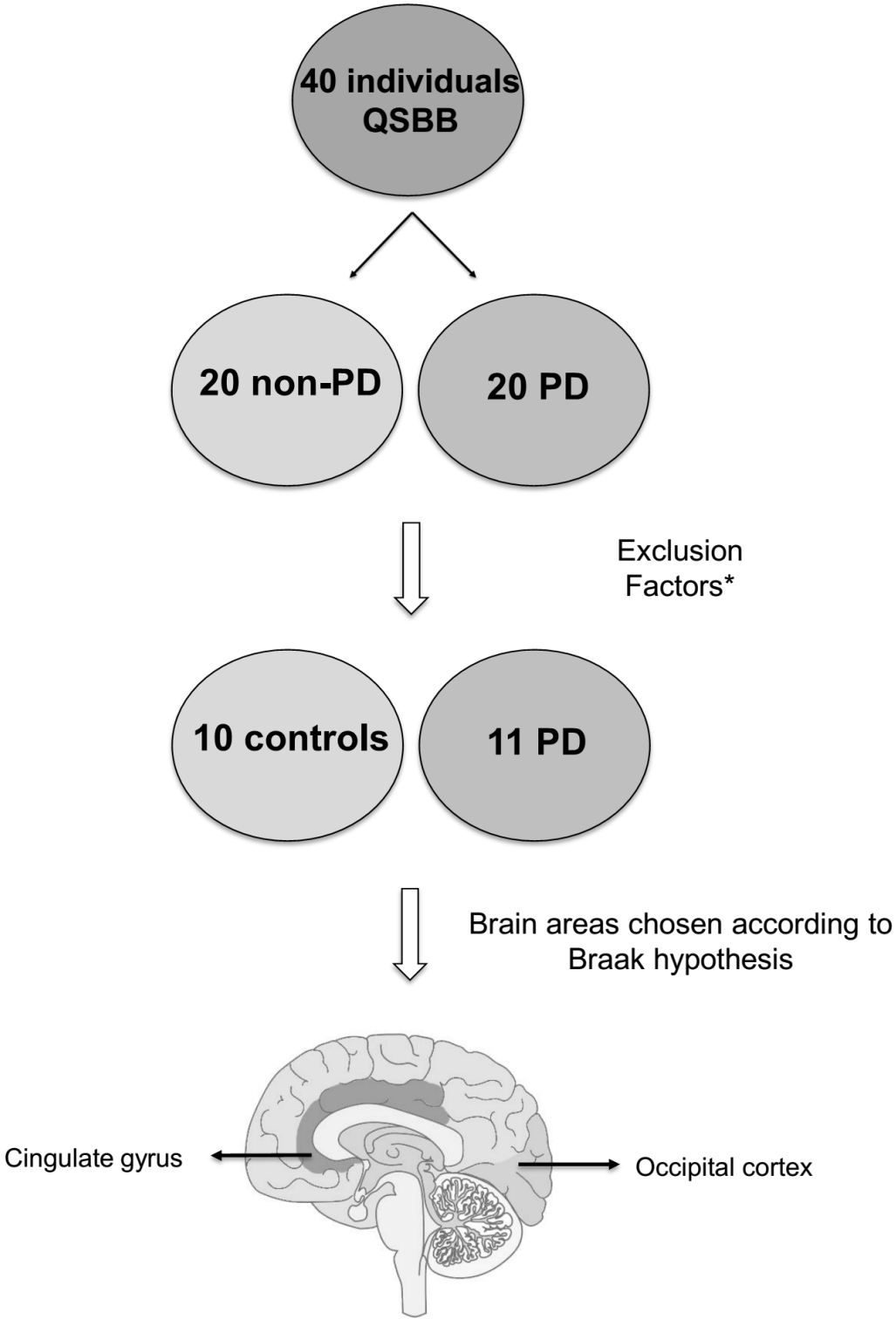
Transfection efficiency of GFP plasmid in RA-differentiated SH-SY5Y cells



Generation of plasmid $CFL1_{WT}$ and $CFL1_{NOX}$

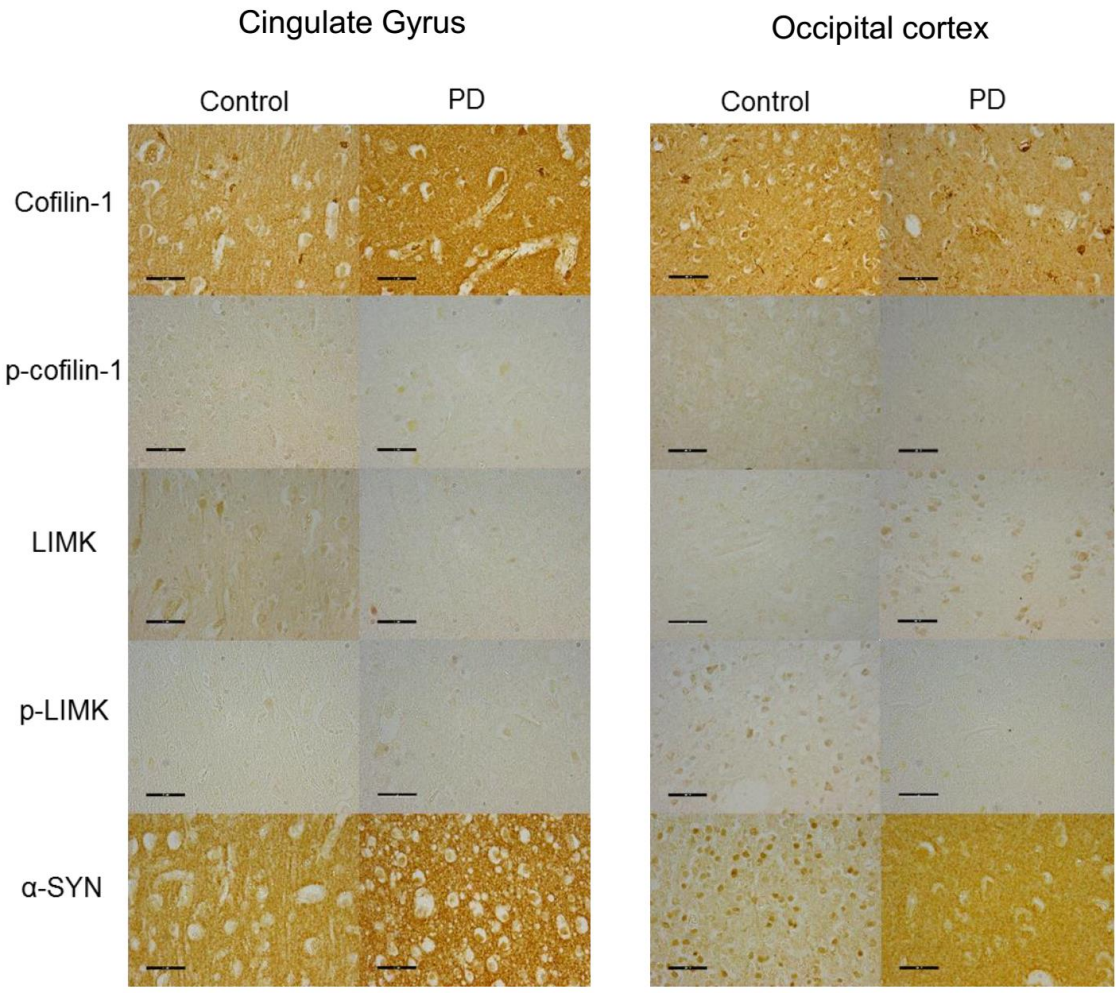


Supplementary Figure 1

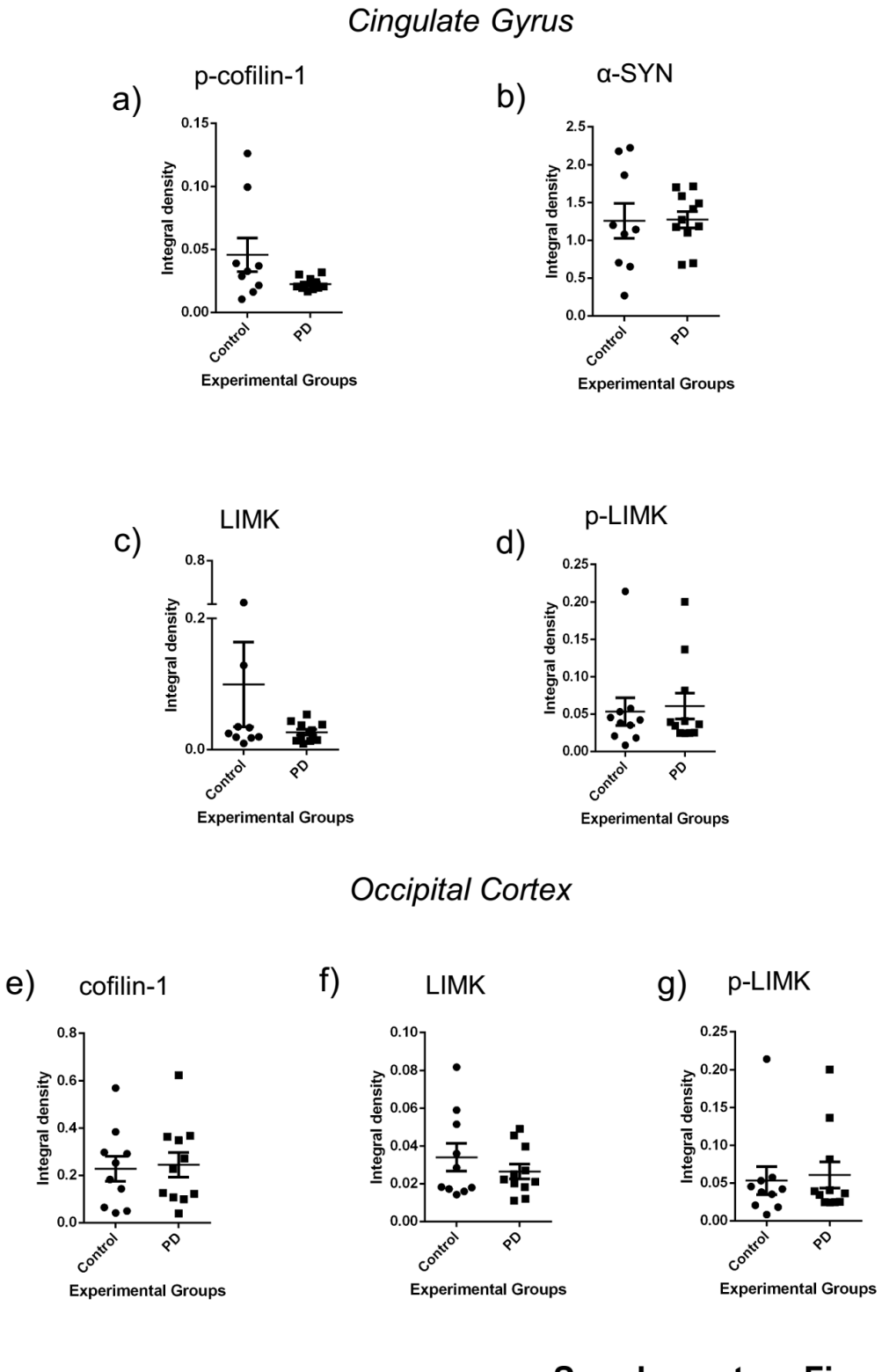


Supplementary Figure 2

Typical immunohistochemistry staning



Supplementary Figure 3



Supplementary Figure 4

Supplementary Table 1: Patient information of PD and control subjects

Case	Age	Gender	PMI	α-SYN score	NFT stage	Clinical Diagnosis
31-11	88	M	15	NO	II	Control
32-09	86	F	41	NO	I	Control
44-02	78	F	24	NO	II	Control
45-04	78	F	29	NO	II	Control
47-11	79	F	72	NO	I	Control
58-10	87	F	48	NO	I	Control
66-10	87	M	48	NO	II	Control
75-08	87	M	48	NO	I	Control
77-06	82	F	72	NO	II	Control
94-05	71	M	24	NO	II	Control
04-11	63	M	15	VI	I	PD
20-10	88	M	24	VI	II	PD
22-08	80	F	48	VI	II	PD
25-08	80	F	72	VI	II	PD
30-09	77	F	72	VI	I	PD
38-09	81	M	48	VI	II	PD
55-09	78	F	72	VI	II	PD
63-09	70	M	72	VI	II	PD
67-08	77	F	24	VI	I	PD
79-10	92	M	24	VI	II	PD
85-10	76	F	48	VI	II	PD

PMI = post-mortem interval (hours); α-SYN score = α-synuclein; NFT= Neurofibrillry tangle stage; PD = Parkinson's disease.

Appendix

Title: Use of structural differences between oxidized and native cofilin-1 isoforms to screen specific inhibitors of the cofilin-1 -dependent neuronal death pathway new potential disease-modifying therapy for Parkinson's Disease

Authors: Fernanda M. Lopes[1,2], Geancarlo Zanatta[1,3,4], Richard Parsons[2], Bonnie Ann Wallace[3], Fabio Klamt[1].

Filiation: [1]Laboratory of Cellular Biochemistry, Department of Biochemistry, ICBS/UFRGS, 90035-003 Porto Alegre (RS), Brazil; [2]Institute of Pharmaceutical Science, King's College London, 150 Stamford Street, London SE1 9NH, UK; [3]Department of Physics at Federal University of Ceará (UFC), 60455-760 Fortaleza (CE), Brazil; [4]Birkbeck College, University of London, London WC1E 7HX, UK.

The results of this chapter provided evidence that cofilin-1 plays a key role in neurodegeneration found in Parkinson's disease (PD), which it can be used as a therapeutical target for this disorder. However, since cofilin-1 is an ubiquitous protein involved in several biological processes, it makes difficult to place it as suitable new drug target for treating PD without potentially disturbing many important physiological functions.

Hence, using the crucial information that neuronal degeneration is caused by oxidized cofilin-1 in PD, it is possible to target a specific ligand with inhibitory properties over the oxidized cofilin-1. The consequences of these effects will be to break the pathogenic cycle of PD by keeping those neurons that are remaining functional.

Based on crystallographic structure and Molecular Dynamics Simulations, we found some consistent structural differences between both cofilin-1 isoforms native and oxidized (Fig.1a). The resulting structures were used as input for the search binding sites using FTMap and FTSite algorithms. This allowed the identification of distinct binding site, which was used for Virtual Screening to search for selective ligands (Fig.1b). Blind Docking was performed to test the selectivity of the binding site to the oxidized isoform (Fig.1c).

Afterwards, we performed Virtual Screening based on our findings using a set of ligands (~3000 compounds) to search for selective ligands to oxidized cofilin-1, with low or none profile for the native cofilin-1, followed by a Blind Docking procedure using the top virtual screening results in order to identify their specificity for the potential binding site in the oxidized cofilin-1. The most significant drug candidates (with higher binding affinity differences between native and oxidized cofilin-1) are Cinacalcet (Senipar™, Mimpara™), Eletriptan

(Relpax™, Relert™), Nebivolol (a bisoprolol derivative), Fenoterol (Berotec N™) and Dydrogesterone (Duphaston™, ispregnenone) (Table 1). All of them are commercial drugs in use in the clinics, critical information regarding bioavailability, and safety issues are already available.

These data reinforce and validate the clinical relevance of our preliminary findings and support the additional investigation of the role played by these drugs and targeting the cofilin-1-dependent neuronal cell death as a new therapeutic venue for the management of PD.

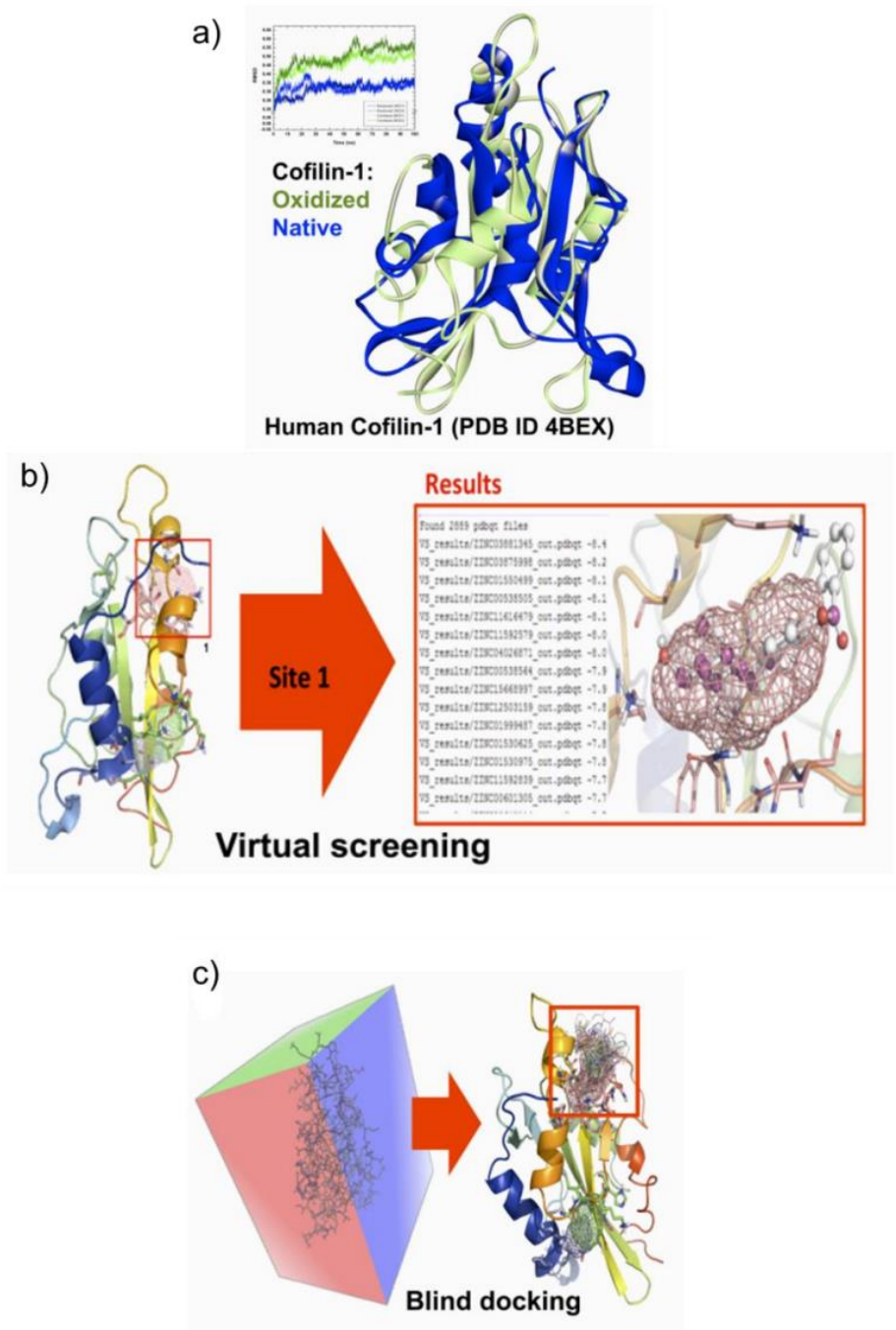
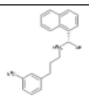
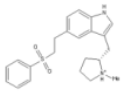
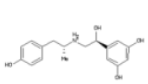
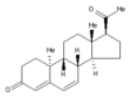


Figure 1: a) Superposition of native and oxidized cofilin-1 structures, highlighting structural differences, after 100 ns of molecular dynamics simulations based on crystallographic structure of human cofilin-1 . b) Representative data of the Virtual Screening procedure performed using predicted binding sites in the oxidized and native cofilin-1 structures and a ligand dataset (~3000 compounds) from ZINC Database. c) Blind docking using top compounds from Virtual Screening to test the selectivity of the binding site to the oxidized isoform.

Table 1: Selective ligands to oxidized cofilin-1

Code	Name	Structure	E_0- cofilin-1 native form (kcal/mol)	E_0 -cofilin-1 oxidized form (kcal/mol)	Difference E_0 native- E_0 oxidized (kcal/mol)
ZINC 01550499	Cinacalcet		-5.1	-7.8	-2.7
ZINC0382 3475	Eleptriptan		-6.0	-8.5	-2.5
ZINC0421 3946	Nebivolol		-8.1	-5.7	-2.4
ZINC0002 0252	Fenoterol Hydrobromide		-5.1	-7.3	-2.2
ZINC0387 5998	Dydrogesterone		-6.2	-8.3	-2.1

PART III

4. DISCUSSION

PD can be considered a heterogeneous syndrome, because it comprises variable symptoms, which manifests differently among patients (MA et al., 2015). The multiple and not well elucidated pathogenic mechanisms hamper drug discovery of curative therapies. To date, dopaminergic degeneration and LB pathology are the only common hallmarks in PD (GIBB, 1991; SPILLANTINI et al., 1997), thus there are many studies investigating the upstream mechanisms that mediates these two processes (CHU et al., 2014; DEL HOYO et al., 2010; YE et al., 2015).

Many lines of evidence have attributed the absence of reliable experimental PD models as one of the causes of the lack of understanding PD etiology (BEAL, 2010; DAUER; PRZEDBORSKI, 2003; JAGMAG et al., 2015b; OLANOW; KIEBURTZ; SCHAPIRA, 2009; SCHÜLE; PERA; LANGSTON, 2009). Although experimental models are essential for PD research because, at least in part, they helped the elucidation of PD pathology, all of them failed to entirely replicate the disease. Moreover, the models available are only acute, consequently they do not represent accurately the features of PD, a chronic and progressive disorder (JAGMAG et al., 2015a).

Regarding animal models, none of them is able to recreate dopaminergic degeneration, LB pathology and disease symptomatology (both motor and non-motor dysfunctions) (PAN-MONTOJO et al., 2010). Even the gold-standard model -non-human primates treated with MPTP- failed to reproduce the whole spectrum of the disease. Although they developed motor impairment and dopaminergic degeneration (LANGSTON; LANGSTON; IRWIN, 1984), they do not reproduce the LB pathology and non-motor symptomology (HALLIDAY et al.,

2009). Genetic models are even more problematic. The majority of genetic mutations associated with PD (*e.g LRKK2, PINK1, DJ-1*) do not present any of the features mentioned above, hence it makes it very difficult to measure outcomes and disease progression (ANDRES-MATEOS et al., 2009; HINKLE et al., 2012; SANCHEZ et al., 2014). Table 1 summarizes the features of the most used animal models and describes the PD features found in each of them.

Table 1: Animal models of Parkinson's disease – Table reproduced from (BLESA; PRZEDBORSKI, 2014)

	Animal model	Motor behavior	SNc neuron loss	Striatal DA loss	Lewy body/Syn pathology
Toxin-based	MPTP Mice	Reduced locomotion, bradykinesia	↑↑↑	↑↑↑	NO
	MPTP Monkeys	Reduced locomotion, altered behavior, tremor, and rigidity	↑↑↑	↑↑↑	NO
	6-OHDA rat	Reduced locomotion, altered behavior	↑↑↑	↑↑↑	NO
	Rotenone	Reduced locomotion	↑↑	↑↑↑	YES
	Paraquat/maneb	Reduced locomotion	↑↑	↑↑↑	YES
	MET/MDMA	Reduced locomotion	↑↑	↑↑↑	NO
Genetic mutations*	α-Synuclein	Altered behavior, reduced or increased motor activity	↑ Not consistent	↑	↑ (in old animals)
	LRKK2	Mild behavioral alteration	NO	NO	NO
	PINK1	No obvious alterations or reduced locomotion	NO	NO	NO
	PARKIN	No obvious locomotion or reduced locomotion	NO	↑	NO
	DJ-1	Decreased locomotor activity	NO	NO	NO
	ATP13A2	Late onset sensorimotor deficits	NO	NO	NO
Others	SHH	Reduced locomotion	↑↑	↑↑	NO
	Nurr1	Reduced locomotion	↑↑	↑↑	NO
	Engrailed 1	Reduced locomotion	↑↑	↑	NO
	Pitx3	Reduced locomotion	↑↑↑	↑↑↑	NO
	C-Rel-NFKB	Gait, bradykinesia, rigidity	↑↑	↑↑	YES
	MitoPark	Reduced locomotion, tremor, and rigidity	↑↑	↑↑	YES
	Atg7	Late onset locomotor deficits	↑↑	↑↑	YES
	VMAT2	Reduced locomotion and altered behavior	↑↑	↑↑	YES

↑↑↑, Severe loss; ↑↑, Moderate loss; ↑, Mild loss.
**This table summarizes general observations for each model. See the main text for full and specific description of different animal models for each genetic mutation.*

The other disadvantage of using animal models are ethical issues, the high costs of the animal's maintenance and the difficulty in translating the findings regarding molecular mechanisms to humans. Lastly, its major drawback is related

to difficulty in perform drug screening. For these type of studies, a great amount of animals is necessary to test a large number drugs and concentrations, which increases significantly the costs for this research. (BEZARD et al., 2013; BLANDINI; ARMENTERO, 2012).

To counter-act this, *in vitro* models are commonly used in PD research. A significant merit of this type of model is the controlled environment (ALBERIO; LOPIANO; FASANO, 2012). Moreover, it is possible to perform high-through-put drug screening by testing several molecules at a variety of concentrations at the same time in contrast to animal models (AVIOR; SAGI; BENVENISTY, 2016; BEZARD et al., 2013).

To perform drug screening, firstly, the cytotoxicity of the compound is evaluated by treating the cells with a range of concentrations. The main objective of this is to find a non-toxic dose for future experiments. Afterwards, it is verified whether these drugs may change cellular physiology (*e.g.* analysis of cellular morphology, enzymatic activity) (CLEMEDSON; KOLMAN; FORSBY, 2007; LOPES et al., 2012). Once the effects of the drug are characterized, neuroprotection assays are performed. There are many compounds that possess a significant neuroprotective effects in PD *in vitro* models. However, translation of these findings into *in vivo* (pre-clinical) and clinical trials have failed to demonstrated the same results.

One example of a promising compound for PD therapy was CEP-1347, a mixed-lineage kinase inhibitor. This drug was neuroprotective towards dopaminergic neurons *in vitro* (LOTHARIUS et al., 2005; MATHIASEN et al., 2004) and *in vivo* (BOLL et al., 2004). As a consequence, reviews regarding PD therapy at that time point it out CEP-1347 as a possible candidate for clinical trials

(JOHNSTON; BROTCHIE, 2004; WU; FRUCHT, 2005). In spite of the positive findings in experimental models, this drug failed to delay disease progression in early-stage PD patients (PARKINSON STUDY GROUP PRECEPT INVESTIGATORS, 2007).

Subsequently, other molecules were tested, such as creatine (ELM; NINDS NET-PD INVESTIGATORS, 2012), PYM50028 (promotes BDNF and GDNF release) ("Investigation of Cogane (PYM50028) in Early-stage Parkinson's Disease (CONFIDENT-PD) - Full Text View - ClinicalTrials.gov", [s.d.]) and MitoQ (SNOW et al., 2010). These compounds covered a range of neuroprotective mechanisms namely antioxidant properties, mitochondrial bioenergetic enhancers and neurotrophin functions, respectively. They were also able to protect dopaminergic cells from damage *in vitro* and *in vivo*, yet they failed to achieve neuroprotection in clinical trials. In fact, the majority of these studies were stopped due to low efficacy when compared to placebo.

As many clinical trials failed to achieve neuroprotection, reviews about this subject aimed to investigate the reasons regarding this issue, one such reason is the lack of reliable PD models (ATHAUDA; FOLTYNIE, 2014; OHLOW et al., 2017; OLANOW, 2009; OLANOW; KIEBURTZ; SCHAPIRA, 2008; RADIO; MUNDY, 2008). Hence, the development of more suitable models is necessary, especially *in vitro* models because they are the first step of the drug discovery research pipeline. Additionally, the understanding of the advantages and disadvantages of models already established would also be beneficial for PD research, which our research group addressed by reviewing this subject in article 1 of Chapter I. Since the common pathological pathway of PD is dopaminergic degeneration, the focus of the experimental models is mimicking dopaminergic

cell environment. There are many ways to obtain DA-producing cells, the most used ones are from cell lines, primary and organotypic cultures and iPSC. Hence, we listed these 4 most used PD *in vitro* models and describe their features, protocols and accessibility.

By reviewing these topics, we chose SH-SY5Y cell line as a model for the following reasons. Although, the primary and organotypic cultures are excellent models of PD because they are not single-cell-cultures, and can recreate, at least in part, the neuronal environment (DAVIAUD et al., 2014; GAVEN; MARIN; CLAEYSEN, 2014), they have at least three important pitfalls: (*i*) they are non-human in origin; (*ii*) the difficulty in isolating dopaminergic cells in these cultures, which can affect the reproducibility of the experiments; and (*iii*) the inability or significantly difficulty in performing genetic manipulation (WEINERT et al., 2015).

To counter-act this, there is an increase of interest in the use of iPSC cells models. Their main advantages are their the human origin and the ability to obtain these cells directly from a PD patient (JIANG et al., 2012; PU et al., 2012; SÁNCHEZ-DANÉS et al., 2012). Phenotypic characterization of terminally-differentiated neurons derived from iPSCs revealed that they possess high levels of DA and high immunocontent of dopaminergic markers (*e.g.* TH and DAT). Moreover, these cells can be genetically modified (HARTFIELD et al., 2014). Even though iPSC-derived neurons seem the best way to mimic dopaminergic cell features *in vitro*, there are several ethical issues involving human tissue acquisition. Furthermore, the technique to perform dopaminergic differentiation is time-consuming (35 days) and requires significant expertise.

Due to these facts, DA-producing cell lines with human origin, such as neuroblastoma cell line SH-SY5Y are widely used in PD research (XICOY; WIERINGA; MARTENS, 2017b; XIE; HU; LI, 2010), especially because they are easy to cultivate when compared to other models. Furthermore, they are excellent models for studies that use molecular biology techniques (STUCHBURY; MÜNCH, 2010).

Nevertheless, there is a major drawback of using this cell line as model, because they are immortalized tumor cells, derived from a bone marrow metastatic site (GILANY et al., 2008). Although they possess low levels of TH and betahydroxylase, they are epithelial cells with high levels of proliferation ("www.atcc.org/Products/All/CRL-2266.aspx", [s.d.]). Hence, the use of cell lines such as SH-SY5Y cells in PD research lacks one important feature: neuronal physiology and morphology (Figure 9).

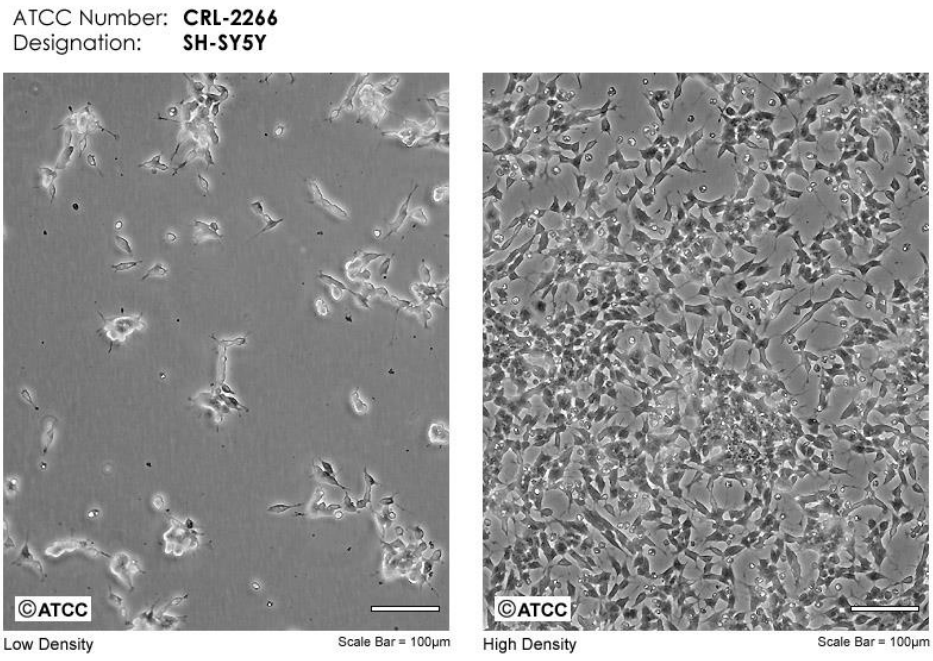


Figure 9: Undifferentiated SH-SY5Y cells. Image reproduced from ATCC website.

In mitigation, these drawbacks can be circumvented by differentiation protocols (AGHOLME et al., 2010; ENCINAS et al., 2000; PÅHLMAN et al., 1984). In this context, many studies have tested a myriad of differentiation protocols and evaluated whether this process enhances neuronal and dopaminergic phenotypes. The results are conflicting and range from those who have found no differences between these two models to those which showing that one of the models enhances dopaminergic phenotype (CHEUNG et al., 2009; FILOGRANA et al., 2015; FORSTER et al., 2016; KORECKA et al., 2013; LOPES et al., 2010).

A systematic review of the use of SH-SY5Y in PD research provided an overview about culture conditions and differentiation protocols. Surprisingly, this review demonstrated that the majority of the studies use undifferentiated cells as PD model. Additionally, this review verified a great range of differentiation protocols, which may contribute to the different findings regarding dopaminergic phenotype (XICOY; WIERINGA; MARTENS, 2017b). This reinforces the need of standardization of the use of SH-SY5Y cells (Figure 10).

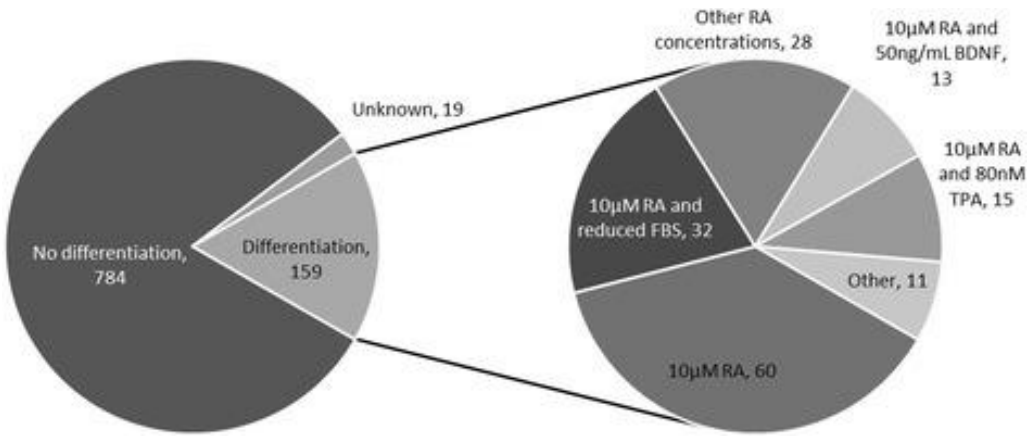


Figure 10: Studies reporting the differentiation of the SH-SY5Y cell line for PD-research. Left: Proportion of studies that do not use differentiation protocols (no differentiation), those that do not specify the differentiation status (unknown) and those that include a differentiation regime (differentiation). Among the papers in which differentiated cells were used, the main differentiation treatments used are depicted in the right chart, including 10 μM RA, 10 μM RA and reduced fetal bovine serum (FBS), other concentrations of RA, 10 μM RA and 50 ng/ml BDNF and 10 μM RA and 80nM 12-O-Tetradecanoylphorbol-13-acetate (TPA). Other includes 10 μM RA, 1%FBS and 0.3 mM dibutyryl-cAMP; 10 μM RA or 10 μg/mL BDNF; 10 μM RA and 80nM TPA or 50 ng/mL BDNF; 100 ng/mL of GDF5 or recombinant BMP2; neurobasal media with 6-10nM staurosporine or B27 supplement, 2 mM L-glutamine and 10 μM RA; 10 μM RA and 5 μM cAMP; 50 ng/ml GDNF; 10 μM RA and 80nM tissue plasminogen activator. Image reproduced from (XICOY; WIERINGA; MARTENS, 2017a)

Considering this, we chose the human neuroblastoma cell line SH-SY5Y as a PD model to be used for the studies described in this thesis. We aimed to validate a differentiation protocol using RA as previously described by our research group (LOPES et al., 2010) comparing undifferentiated and RA-differentiated SH-SY5Y cells regarding neuronal features.

There are many studies focusing upon the evaluation of dopaminergic phenotype after the differentiation process and the best conditions to acquire this feature (CHEUNG et al., 2009; FILOGRANA et al., 2015; FORSTER et al., 2016; KORECKA et al., 2013; LOPES et al., 2010). However, a DA-producing cell, it does not necessarily need to be a neuron, which is the case of the neuroblastomas (*e.g.* undifferentiated SH-SY5Y cells) (BIEDLER et al., 1978). In PD, the most affected cell population is the dopaminergic neurons of the *SNpc*, hence the evaluation of neuronal properties in any *in vitro* model is fundamental. Hence, the second article of Chapter I aimed to evaluate not only the dopaminergic markers, but also characterize the neuronal features of the two SH-SY5Y models.

We found that RA-induced-differentiation induced neuronal features by increasing expression of genes associated with the synaptic vesicle cycle, decreased proliferation rates and increased the number of neurites. This resulted

in an enhancement of fundamental neuronal features, namely the molecular machinery that supports synapses, cellular growth reduction and stellate morphology.

Regarding the proliferation status and evaluation of cellular morphology, the majority of studies in the literature have reported similar results. Once differentiation begins, SH-SY5Y cells decrease their growth and increases the number of neurites (CHEUNG et al., 2009; FILOGRANA et al., 2015; KUNZLER et al., 2016; LOPES et al., 2010; MILOSO et al., 2004; PRESGRAVES et al., 2004; ROSS, 1996). The major differences among these studies are those regarding the capability of these cells to support synapses.

Previous work has demonstrated that RA-differentiated cells have increased the number of synaptic vesicles after depolarizing cell via high K^+ solution (TEPPOLA et al., 2016). Furthermore, it was demonstrated that only RA-differentiated cells were excitable in cell culture (HALITZCHI; JIANU; AMUZESCU, 2015). On the other hand, there are many studies showing that RA-differentiation has no effect upon neuronal markers and synapse machinery (CHEUNG et al., 2009; KORECKA et al., 2013). Here we reported that only RA-differentiated SH-SY5Y cells have increased the expression of genes related to synapse machinery.

Moreover, the presence of neurites, which refers to axonal and dendrict extensions in neuronal cell lines, are another advantage of this cell model (BAL-PRICE et al., 2010; RADIO; MUNDY, 2008) (Figure 11). In studies of neuroprotection/neurotoxicity screening drug therapies, cell lines are commonly used to evaluate cytotoxicity of compounds. By using RA-differentiated SH-SY5Y

cells rather the undifferentiated ones, it is possible to analyze one extra feature: cellular morphology by neurite quantification.

In one example of the importance of evaluating such parameter, a previous study from our research group evaluated 9 organoselenide compounds regarding neuroprotective features. By assessing drug cytotoxicity through MTT assay, we found sublethal doses of each compound to determine whether they were neuroprotective against 6-OHDA-induced-cell-death. Even though these compounds could protect RA-differentiated-cells from 6-OHDA, some of them changed cellular morphology assessed as a reduction in the number of neurites. This means that these compounds may be modifying other complex cellular processes and ultimately, cellular physiology. Taking these findings in consideration, perhaps the failure of previous drug screening approaches using cell lines is due to the fact that this physiological feature is not accessible when using undifferentiated models. This may be a key contributor to further validate candidate therapeutic in animal and clinical trials (LOPES et al., 2012).

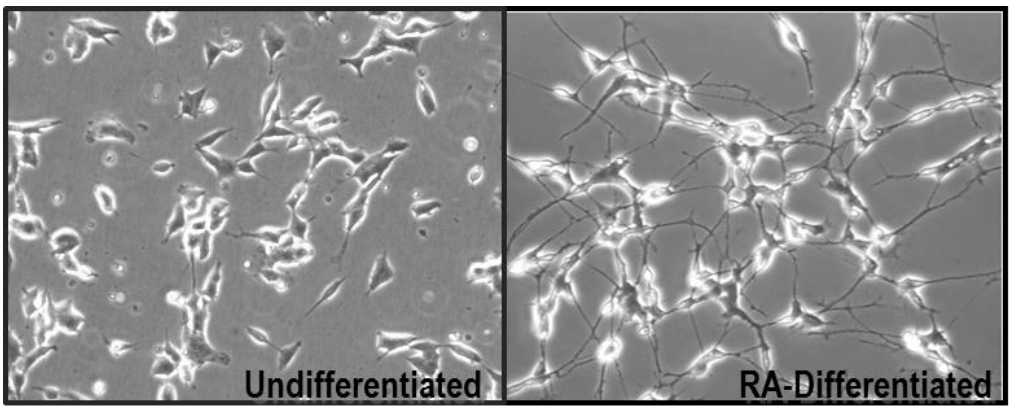


Figure 11: Morphological changes of undifferentiated and RA-differentiated SH-SY5Y cells. Image reproduced from (LOPES et al., 2010).

Since one of the most criticized features of cell lines in PD research is the lack of neuronal features, the use of RA-differentiated cells can circumvent this issue. The cellular model established by our research group not only demonstrates neuronal and dopaminergic features, but also is easy to culture and has low costs in terms of finance and time for maintenance compared to other cell models. Using our cellular model, we can obtain a terminally-differentiated human cell within 7 days (Figure 12).

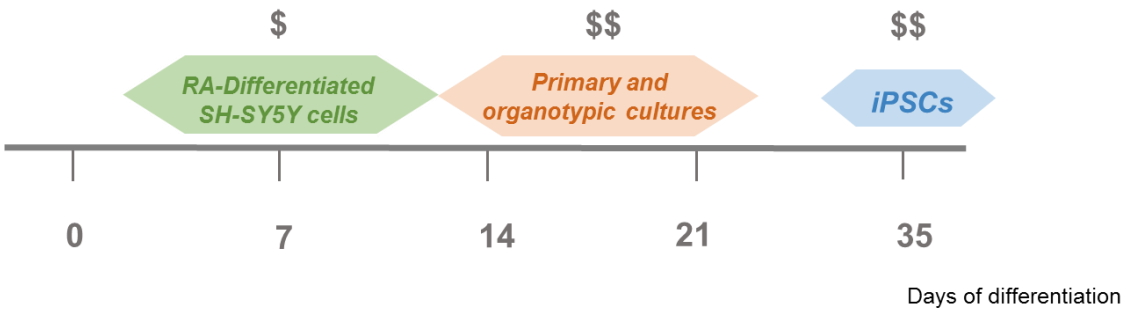


Figure 12: Time line showing how many days are necessary to obtain terminally-differentiated dopaminergic neurons and the costs of research in each PD cellular model.

To fully establish a PD model, it is necessary to induce the disease's pathological evidences. In our study, we choose a toxin-based-model by using 6-OHA. Hence, after exploring the neuronal features, we investigated how undifferentiated and RA-differentiated respond to 6-OHDA toxicity. This compound is produced during DA oxidative metabolism and it is present in tissues and body fluids in contrast to other toxin-based-models, which use non-physiologically relevant toxins (*e.g.* MPTP, rotenone). Previous data has shown an increase of 6-OHDA levels in caudate nucleus (CURTIUS et al., 1974) and urine (ANDREW et al., 1993) of PD patients, suggesting that this compound may

contribute to disease pathogenesis. However, the mechanisms underlying its toxicity remain to be elucidated.

Even though the undifferentiated cells have major drawbacks as described above, papers published in high-impact journals have used these cells to investigate 6-OHDA molecular mechanisms (GOMEZ-LAZARO et al., 2008; KARUNAKARAN et al., 2008; SAITO et al., 2007). Although it is well known that 6-OHDA is taken up by DAT *in vivo* and in primary cultures (ABAD et al., 1995; CERRUTI et al., 1993), this has yet to be shown in SH-SY5Y cells. The mechanism of toxicity attributed to this cellular model was extracellular auto-oxidation followed a massive increase in oxidative stress (IZUMI et al., 2005; SOTO-OTERO et al., 2000).

However, here we demonstrated that differentiation induces DAT dependency in 6-OHDA susceptibility. Our data suggests, for the first time, the role of toxin uptake by DAT in RA-differentiated cells, showing that an easy cellular model may mimic an important aspect of the 6-OHDA-induced cell death observed *in vivo*.

The data of Chapter I validated the use of RA-different-SH-SY5Y as a useful model for PD research. Hence, in Chapter II, it was used to investigate disease molecular mechanisms, focusing in cofilin-1, a major regulator of actin dynamics (CARLIER; RESSAD; PANTALONI, 1999), that is also involved in neurodegeneration process found in AD.

Here we demonstrated that cofilin-1 played a role in early stages of neuronal apoptosis induced by 6-OHDA in our cellular model because cofilin-1 mitochondrial translocation precedes mitochondrial dysfunction. Moreover, by overexpressing mutant *CFL1* gene (Cys - Ala 39, 80, 139), which unables to

undergo Cys residue oxidation, the cell death induced by 6-OHDA was prevented. This data suggests that cofilin-1 oxidation is an essential step for 6-OHDA-induced-cell-death as summarized in Figure 13.

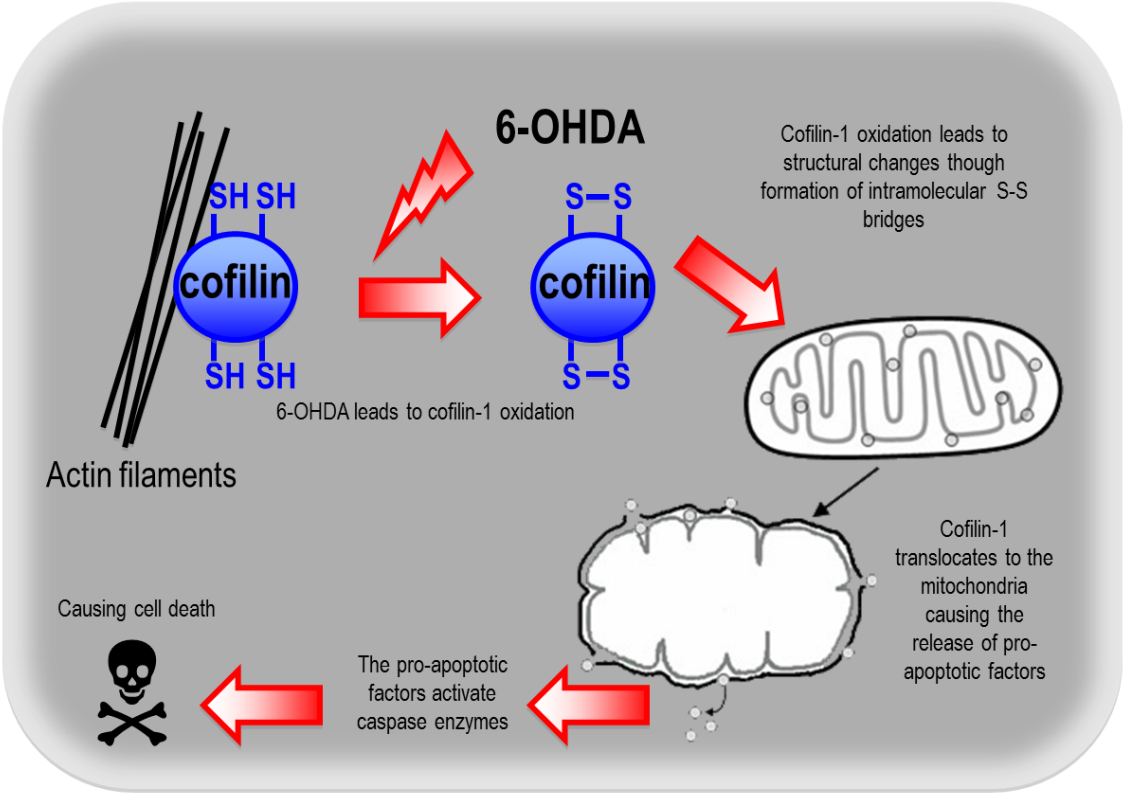


Figure 13: Proposal mechanism of cofilin-1 in 6-OHDA-induced-cell death

These findings corroborate with previous data from our research group which demonstrated that cofilin-1 oxidation was responsible for oxidant-induced apoptosis in tumour cells (KLAMT et al., 2009). Additionally, our results correlate with those observed in AD (WALSH et al., 2002). Aβ is able to induce cofilin-1 activation and consequently translocate to the mitochondria (WOO et al., 2015a). This data were validated by measuring cofilin-1 immunocontent in AD brains,

which showed an increase of the that total and oxidized cofilin-1 levels (WOO et al., 2015b).

We also have demonstrated the role of cofilin-1 clinically by investigating whether there are changes in cofilin-1 pathway proteins immunocontent in *post mortem* PD brain tissues. Since we found strong evidence that cofilin-1 plays a role in early steps of apoptosis in our *in vitro* model as showed in Figure 13, this hampered the use of *SNpc* as brain region of study. Since we are dealing with *post mortem* tissue, all of PD subjects are end-stage-disease with a high degree of dopaminergic degeneration in this area. Thus, considering Braak hypothesis, we chose two brain regions without a significant degree of degeneration process. Occipital cortex was used mainly because is the last area to be committed by PD with pathological changes. The other region chosen was cingulate gyrus, which has an earlier involvement with PD and the neuropathological process is occurring for longer.

To evaluate cofilin-1 pathway immunocontent, a cohort of forty patients - 20 PD and 20 non-PD - was obtained from the Queen Square Brain Bank, University College London, UK. Cases considered as controls were from individuals who had no clinical diagnosis of a neurological or psychiatric disorder during life, nor any neuropathological abnormality evidence other than normal age-related changes. After assessment to patient records, we listed the following exclusion factors to avoid any bias in our study:(*i*) Braak and Braak stage > 2 (neurofibrilar tangle-NFT stage), (*ii*) moderate to severe β-amyloid deposits, (*iii*) brain metastasis, (*iv*) other neurodegenerative diseases (*e.g.* AD and argyrophilic grain disease), (*v*) inconclusive diagnosis, (*vi*) vascular dementia and (*vii)* cerebral infarction. Regarding PD patients, we used the same paradigm as the

controls. Moreover, all the PD patients were staged at Braak VI with severe loss of dopaminergic neurons in SN and presented similar disease severity. Hence, here we attempted to obtain a homogenous cohort because the heterogeneous feature of this illness with multiple pathogenic mechanisms and different progressions are also considered one of the causes of failure in clinical trials (ATHAUDA; FOLTYNIE, 2016).

We demonstrated that cofilin-1 is possibly overactivated in PD brain as evidenced by a due to a reduction of in the ratio p-cofilin-1/cofilin-1 (inactive:active) ratio. Since the CNS presents a highly an oxidative environment, non-phosphorylate cofilin-1, can undergo to oxidation resulting in mitochondrial dysfunction, which as the ratio is skewed towards an increase in non-phosphorylated in PD brain, may occur to a greater degree than in non-disease controls. The clinical data provided in this thesis reinforces that cofilin-1 may have a role in PD pathogenesis.

Afterwards, we also analyzed all the markers tested (cofilin-1 pathway proteins) simultaneously by using principal component analysis. This data revealed we could discriminate our cohort's in two groups (control and PD) only in the occipital cortex. According to Braak hypothesis, this area is the last part of the brain to be affected by PD.

The occipital lobe is strongly related to visual symptoms of PD, such as hallucinations and vivid dreams (ARMSTRONG et al., 2011; ZHAO et al., 2014). Furthermore, it has been described that PD can cause hypoperfusion (ABE et al., 2003), atrophy (BURTON et al., 2004) and decrease of cerebral glucose metabolism in the occipital cortex (TANG et al., 2016). Regarding the neuropathological alterations caused by the disease, although this brain area has

a late-involvement with PD, the LB pathology may be in its the early stages. Hence, this may explain why it was the only region which cofilin-1 pathway proteins content could discriminate controls and PD subjects. This evidence also suggests that cofilin-1 may play a role in early steps of degeneration. Furthermore, as we could distinguish controls and PD subjects, it is likely that cofilin-1 pathway proteins are another target for biomarkers research.

Regarding it use as potential biomarker, it has been already reported high levels of cofilin-1 in lymphocytes of PD patients, suggesting that the immunocontent of this protein can be also misbalanced in peripherical tissues. However, more studies using other tissues and body fluids (*e.g* cerebrospinal fluid) are needed to verify whether this protein can be a reliable biomarker.

The discovery of new biomarkers that could specifically discriminate healthy and PD patients would improve PD diagnosis and management, since this disorder is mainly diagnosed once the motor impairments onset. By this time, 50-70 % of the dopaminergic neurons in the *SNpc* have already been degenerated (STOESSL, 2007).

As mentioned before, PD diagnosis is basically clinical and its confirmation occurs only after the patient's death via neuropathological examination. Besides, in spite of neurologists' efforts, it is still very difficult to diagnose accurately. New additional complications are the non-motor symptoms, which can precede the motor impairment, and have become a novel subject of investigation to perform an early PD diagnosis. Some of these symptoms comprise constipation, sleep disorder and anosmia. None of them, however, are pathognomonic for PD. The clinical description below shows a PD case in prodromal phase and describes the problematic issue of diagnosing this disease:

"*A 78-year-old man presented with a 15-year history of chronic constipation. At age 68, he experienced a near complete loss of his sense of smell. Recently, he has developed an unusual sleep disorder, characterized by abrupt and at times combative behavior during the night that has resulted in injury to his spouse on two separate occasions; he is referred to a sleep disorders clinic"*

Quote reproduced by (LANGSTON, 2006)

Because of the inherent difficulties in using clinical outcomes to assess early diagnosis, the identification of biomarkers for PD is of paramount importance. Furthermore, these studies would also be beneficial for PD management since URPDS scale has inter and intra-rater variability and it is difficult to distinguish small changes in disease progression. In fact, much research has been started in attempt to find biomarker to improve diagnosis for this disorder, such as PD Marker's Initiative from Michael J Fox Foundation and PD Biomarker Program from National Institutes of Neuropathological Disorders and Stroke.

One of the most promising biomarkers is α-SYN, since it is a major component of LB. Firstly, it was demonstrated that α-SYN is secreted in extracellular fluids, as a consequence, it may be detected in peripherical tissues. Regarding its capability to distinguish control and PD subjects, it was verified that total α-SYN levels in plasma is 3.5-fold higher in PD patients (EL-AGNAF et al., 2006). In contrast, another study was not able to replicate these findings (LI et al., 2007). Using another biological fluid as sample, such as cerebrospinal fluid, the outcomes were contradictory as well. (BORGHI et al., 2000; RIZZO et al., 2016).

As total levels of α-SYN were not able to distinguish controls and PD subjects, current research has been developed the use of oligomeric forms (which are the toxic forms) of this protein to increase the specificity in plasma and cerebrospinal fluid. The outcomes of several trials showed that by combining measurements of different forms of α-SYN, it is possible to observe remarkable differences between control and PD subjects. (EL-AGNAF et al., 2006; LI et al., 2002; MAJBOUR et al., 2016). To date, in spite of the many studies, α-SYN is still not considered as PD biomarker since it needs to be validated in a longitudinal and well-controlled study and be compared with other neurological illness. Hence, the search for other biomarkers is still a hot topic of investigation in the field, which cofilin-1 may be a promising candidate mainly because our results indicates an early involvement with the disease.

Our most intriguing finding was hints, for the first time, that cofilin-1 is co-localised with a-SYN in the LBs. Up to the present, although several proteins have been identified in the LBs, its precise composition has not been confirmed yet. The discovery of α-SYN as major component of LB was a breakthrough in PD research. Afterwards, several studies aim to search proteins that could be co-localised with α-SYN in the LBs, such as Tau, LRRK2, tubulin and Aβ (GUERREIRO et al., 2013; ISHIZAWA et al., 2003; LEVERENZ et al., 2007). Even though there is no evidence showing the co-localisation of cofilin-1 and α-SYN, 14-3-3 (KAWAMOTO et al., 2002; UBL et al., 2002), a protein involved in cofilin-1 regulation is deposited in LBs (GOHLA; BOKOCH, 2002).

14-3-3 proteins are a superfamily homologous proteins (AITKEN, 1995), which can exist as monomers or heterodimer (LIU et al., 1995). It is also ubiquitously expressed, especially in the human brain (BAXTER et al., 2002). Its

physiological function is not well understood, but its neuroprotective effect has been shown in previous studies (DING et al., 2015; YACOUBIAN et al., 2010) . Besides its localization in LB's, the levels of phosphorylated isoform were found increased in PD brains and PD models (SLONE et al., 2015; WERNER et al., 2008), suggesting a possible role in this disorder.

Regarding its association with cofilin-1 pathway protein, 14-3-3 can stabilize phosphorylate cofilin-1 isoform, increase the levels of p-cofilin-1 (GOHLA; BOKOCH, 2002). Furthermore, Slingshot-1L (SSH-1L), a protein which dephosphorylate cofilin-1, can also bind to 14-3-3, hampering SSH-1L activation. Additionally, under oxidative stress conditions, 14-3-3 can undergo to oxidation and dissociates SSH1-L (KIM; HUANG; BOKOCH, 2009). Taking this into account and our results showing a possible activation of cofilin-1 in PD brains, it is possible that 14-3-3 may be mediating this process. However, more studies are necessary to elucidated these hypotheses.

The presence of cofilin-1 in the LB reinforce a possible role for this protein in PD pathogenic mechanisms. Besides its potential use as a biomarker, cofilin-1 may also be a target for drug discovery, which could impact PD management since no disease modifying therapies are available.

However, there is a major drawback in using cofilin-1 as drug target to treat PD as this protein is involved in essential physiological processes (GURNIAK; PERLAS; WITKE, 2005; RACZ; WEINBERG, 2006). On the other hand, since our mechanistic data indicates that oxidized cofilin-1 may be responsible for neuronal cell death, perhaps targeting this isoform would circumvent this problem.

Using Virtual Screening, we found selective ligands to oxidized cofilin-1, with low or none specificity for the native form, which the most significant drug candidates (with higher binding affinity differences between native and oxidized cofilin-1) are Cinacalcet (Senipar™, Mimpara™), Eletriptan (Relpax™, Relert™), Nebivolol (a bisoprolol derivative), Fenoterol (Berotec N™) and Dydrogesterone (Duphaston™, ispregnenone).

Further analysis aimed to verify whether these drugs have been used in neuroprotective assays or present any interaction with standard PD therapy (*e.g* *L*-DOPA, DA agonists and MAO and COMT inhibitors) in order to select the most promising ones for further studies.

Regarding the use of these compounds in neuroprotective studies, only Cinacalcet has been tested as therapeutical approach for PD and also have been patented as New therapeutic approach for treating this disorder (WO 2013127918 A1 Deposit form). This drug is calciumimetic widely used in hyperparathyroidism (POON, 2005). In PD studies, the drug is able to decrease glutamate toxicity (excitotoxicity) *in vitro* (WO 2013127918 A1 Deposit form), which is one of the hypothetical mechanisms underlying dopaminergic degeneration (IZUMI et al., 2009). However, other studies demonstrated that glutamate-mediate-neurotoxicity is not responsible for the initial steps, but is a secondary effect (AMBROSI; CERRI; BLANDINI, 2014). In addition to this, since no other further study has shown any protective effect of Cinacalcet, it is possible that this drug has no defensive effect against neurodegeneration. Many lines of research have indicated the calcium antagonists, rather the agonists or calcium mimetic have a protective effect in PD (SCHUSTER et al., 2009).

Eleptripan is a serotonin agonist (5HT1 family) (GUPTA et al., 1999), used clinically to treat migraine headache since it decreases the swelling of brain blood vessels (WILLEMS et al., 1998). Although this compounds has never been used in PD therapy or neuroprotective studies, other drugs from the class of serotonin agonists (*e.g* buspirone) are able to decrease L-DOPA-induced-dyskinesia (LINDENBACH et al., 2015). To date, no study attempt to evaluate the mechanism underlying this process and also there is no research regarding the use of this class of drug as a disease-modifying-therapy.

Nebivolol is a β_1 receptor blocker use for hypertension treatment since it has a vasodilatory effect (DE BOER; VOORS; VAN VELDHUISEN, 2007). Its effect in neuronal cells was demonstrated by one study using neuronal *in vitro* models (SK-N-MC cells) (MANTHEY; GAMERDINGER; BEHL, 2010). However, looking at drug interactions with standard PD therapies, it is described that this compound interacts with dopamine agonists. This class of drugs can cause sleeping disorder, fatigue and impotence. Hence, to decrease these effects discontinuation of β_1 receptor blocker is recommended (BITNER et al., 2015).

Fenoterol hydrobromide is a β_2 adrenoreceptor agonist used as a bronchodilator. There is no study evaluating its neuroprotective effect, however other agonists (*e.g* salmeterol) showed an decrease of neurotoxical effect *in vitro* by inhibition of lipopolysaccharid-induced microglial activation (PETERSON et al., 2014; QIAN et al., 2011). Moreover, no drug interactions with dopamine replace therapies have been described.

Dydrogesterone is a synthetic progestogen, which is used for control of ovarian and endometrium functions. Its neuroprotective effects has not been investigate yet, but other progesterone group compounds leads to a decrease in

toxicity in PD models (BOURQUE et al., 2015; LITIM; MORISSETTE; DI PAOLO, 2016). Additionally, it is widely known that PD is more prevalent in men, hence female sexual hormones may have protective effect (SMITH; DAHODWALA, 2014).

After evaluate this information regarding these 5 compounds from virtual screening data, 3 drugs (Eleptripan, Fenoterol Hydrobromide, dydrogesterone) were selected to be used in future projects. Hence, these data validate the relevance of our findings and support the investigation of these compounds as possible disease-modifying therapies.

5. CONCLUSIONS

Our data showed that RA-differentiated SH-SY5Y cells present terminally-differentiated neuronal features, such as low proliferation rates, stellate morphology and the machinery to support synapses, being a suitable model for PD research regarding its pathogenic mechanisms. By using this cellular model in concert with *post mortem* brain tissue, we demonstrated a role for cofilin-1 in the early stages of the neurodegeneration process of PD, which it can impact biomarker and drug discovery studies.

6. PROSPECTS

Since we found that cofilin-1 pathway proteins' immunocontent were able to distinguish control and PD subjects (using PCA) during the initial steps of degeneration, cofilin-1 may be a potential biomarker for early-stage PD diagnosis. Hence, as prospect of this study, we aimed to evaluate the protein expression of cofilin-1 pathway proteins in cerebrospinal fluid and peripheral tissues in early-diagnosis PD patients.

Moreover, we also will explore the potential neuroprotective actions of the drugs that target oxidized cofilin-1 using RA-differentiated SH-SY5Y as a PD cellular model. If we have promising results *in vitro*, we will also evaluate neuroprotection in animal models.

7. REFERENCES

ABAD, F. et al. Pharmacological protection against the cytotoxicity induced by 6-hydroxydopamine and H2O2 in chromaffin cells. **European journal of pharmacology**, v. 293, n. 1, p. 55–64, 26 maio 1995.

ABE, H. et al. Xenopus laevis actin-depolymerizing factor/cofilin: a phosphorylation-regulated protein essential for development. **The Journal of cell biology**, v. 132, n. 5, p. 871–85, mar. 1996.

ABE, Y. et al. Occipital hypoperfusion in Parkinson's disease without dementia: correlation to impaired cortical visual processing. **Journal of neurology, neurosurgery, and psychiatry**, v. 74, n. 4, p. 419–22, abr. 2003.

AGHOLME, L. et al. An in vitro model for neuroscience: differentiation of SH-SY5Y cells into cells with morphological and biochemical characteristics of mature neurons. **Journal of Alzheimer's disease : JAD**, v. 20, n. 4, p. 1069–82, jan. 2010.

AITKEN, A. 14-3-3 proteins on the MAP. **Trends in biochemical sciences**, v. 20, n. 3, p. 95–7, mar. 1995.

ALAM, Z. I. et al. Oxidative DNA damage in the parkinsonian brain: an apparent selective increase in 8-hydroxyguanine levels in substantia nigra. **Journal of neurochemistry**, v. 69, n. 3, p. 1196–203, set. 1997.

ALBERIO, T.; LOPIANO, L.; FASANO, M. Cellular models to investigate biochemical pathways in Parkinson's disease. **FEBS Journal**, v. 279, n. 7, p. 1146–1155, abr. 2012.

ALLEN REISH, H. E.; STANDAERT, D. G. Role of α-synuclein in inducing innate and adaptive immunity in Parkinson disease. **Journal of Parkinson's disease**, v. 5, n. 1, p. 1–19, 2015.

AMBROSI, G.; CERRI, S.; BLANDINI, F. A further update on the role of excitotoxicity in the pathogenesis of Parkinson's disease. **Journal of Neural Transmission**, v. 121, n. 8, p. 849–859, 1 ago. 2014.

ANDRES-MATEOS, E. et al. Unexpected Lack of Hypersensitivity in LRRK2 Knock-Out Mice to MPTP (1-Methyl-4-Phenyl-1,2,3,6-Tetrahydropyridine). **Journal of Neuroscience**, v. 29, n. 50, p. 15846–15850, 16 dez. 2009.

ANDREW, R. et al. The determination of hydroxydopamines and other trace amines in the urine of parkinsonian patients and normal controls. **Neurochemical research**, v. 18, n. 11, p. 1175–7, nov. 1993.

ARMSTRONG, R. A. et al. Visual symptoms in Parkinson's disease. **Parkinson's disease**, v. 2011, p. 908306, 2011.

ASCHERIO, A.; SCHWARZSCHILD, M. A. The epidemiology of Parkinson's disease: risk factors and prevention. **The Lancet Neurology**, v. 15, n. 12, p.

1257–1272, 2016.

ATHAUDA, D.; FOLTYNIE, T. The ongoing pursuit of neuroprotective therapies in Parkinson disease. **Nature Reviews Neurology**, v. 11, n. 1, p. 25–40, 2 dez. 2014.

ATHAUDA, D.; FOLTYNIE, T. Challenges in detecting disease modification in Parkinson's disease clinical trials. **Parkinsonism & Related Disorders**, v. 32, p. 1–11, 2016.

AVIOR, Y.; SAGI, I.; BENVENISTY, N. Pluripotent stem cells in disease modelling and drug discovery. **Nature Reviews Molecular Cell Biology**, v. 17, n. 3, p. 170–182, 28 jan. 2016.

BAL-PRICE, A. K. et al. Relevance of in vitro neurotoxicity testing for regulatory requirements: challenges to be considered. **Neurotoxicology and teratology**, v. 32, n. 1, p. 36–41, jan. 2010.

BALDWIN, A. J. et al. Metastability of Native Proteins and the Phenomenon of Amyloid Formation. **Journal of the American Chemical Society**, v. 133, n. 36, p. 14160–14163, 14 set. 2011.

BAMBURG, J. R.; BERNSTEIN, B. W. Actin dynamics and cofilin-actin rods in Alzheimer disease. **Cytoskeleton (Hoboken, N.J.)**, 13 fev. 2016.

BARBOSA, M. T. et al. Parkinsonism and Parkinson's disease in the elderly: a community-based survey in Brazil (the Bambuí study). **Movement disorders : official journal of the Movement Disorder Society**, v. 21, n. 6, p. 800–8, jun. 2006.

BAXTER, H. C. et al. Specific 14-3-3 isoform detection and immunolocalization in prion diseases. **Biochemical Society transactions**, v. 30, n. 4, p. 387–91, ago. 2002.

BEAL, M. F. Parkinson's disease: a model dilemma. **Nature**, v. 466, n. 7310, p. S8–S10, 26 ago. 2010.

BERNHEIMER, H. et al. Brain dopamine and the syndromes of Parkinson and Huntington. Clinical, morphological and neurochemical correlations. **Journal of the neurological sciences**, v. 20, n. 4, p. 415–55, dez. 1973.

BERNSTEIN, B. W. et al. Formation of actin-ADF/cofilin rods transiently retards decline of mitochondrial potential and ATP in stressed neurons. **American Journal of Physiology - Cell Physiology**, v. 291, n. 5, 2006.

BERNSTEIN, B. W. et al. Incorporation of cofilin into rods depends on disulfide intermolecular bonds: implications for actin regulation and neurodegenerative disease. **The Journal of neuroscience : the official journal of the Society for Neuroscience**, v. 32, n. 19, p. 6670–81, 9 maio 2012.

BETHLEM, J.; HARTOG, D.; W A. The incidence and characteristics of Lewy bodies in idiopathic paralysis agitans (Parkinson's disease). **Journal of**

neurology, neurosurgery, and psychiatry, v. 23, p. 74–80, fev. 1960.

BEZARD, E. et al. Animal models of Parkinson's disease: limits and relevance to neuroprotection studies. **Movement disorders : official journal of the Movement Disorder Society**, v. 28, n. 1, p. 61–70, jan. 2013.

BIEDLER, J. L. et al. Multiple neurotransmitter synthesis by human neuroblastoma cell lines and clones. **Cancer research**, v. 38, n. 11 Pt 1, p. 3751–7, nov. 1978.

BITNER, A. et al. Drug Interactions in Parkinson's Disease: Safety of Pharmacotherapy for Arterial Hypertension. **Drugs - Real World Outcomes**, v. 2, n. 1, p. 1–12, 27 mar. 2015.

BLANDINI, F.; ARMENTERO, M.-T. Animal models of Parkinson's disease. **The FEBS journal**, v. 279, n. 7, p. 1156–66, abr. 2012.

BLESA, J.; PRZEDBORSKI, S. Parkinsonâ€™s disease: animal models and dopaminergic cell vulnerability. **Frontiers in Neuroanatomy**, v. 8, p. 155, 15 dez. 2014.

BOLL, J. B. et al. Improvement of embryonic dopaminergic neurone survival in culture and after grafting into the striatum of hemiparkinsonian rats by CEP-1347. **Journal of neurochemistry**, v. 88, n. 3, p. 698–707, fev. 2004.

BONIFATI, V. et al. Mutations in the DJ-1 gene associated with autosomal recessive early-onset parkinsonism. **Science (New York, N.Y.)**, v. 299, n. 5604, p. 256–9, 10 jan. 2003.

BORGHI, R. et al. Full length alpha-synuclein is present in cerebrospinal fluid from Parkinson's disease and normal subjects. **Neuroscience letters**, v. 287, n. 1, p. 65–7, 16 jun. 2000.

BOURQUE, M. et al. Neuroprotective Effect of Progesterone in MPTP-Treated Male Mice. **Neuroendocrinology**, v. 103, n. 3–4, p. 300–314, 24 jul. 2015.

BOVOLENTA, T. M. et al. Parkinson's patients in the Brazilian Public Health Policy context. **Einstein (São Paulo)**, v. 14, n. 3, p. 7–9, set. 2016.

BRAAK, H. et al. Staging of brain pathology related to sporadic Parkinson's disease. **Neurobiology of aging**, v. 24, n. 2, p. 197–211, jan. 2003.

BURRÉ, J. et al. Properties of native brain α-synuclein. **Nature**, v. 498, n. 7453, p. E4–E6, 12 jun. 2013.

BURRÉ, J.; SHARMA, M.; SÜDHOF, T. C. α-Synuclein assembles into higher-order multimers upon membrane binding to promote SNARE complex formation. **Proceedings of the National Academy of Sciences of the United States of America**, v. 111, n. 40, p. E4274-83, 7 out. 2014.

BURTON, E. J. et al. Cerebral atrophy in Parkinson's disease with and without dementia: a comparison with Alzheimer's disease, dementia with Lewy bodies

and controls. **Brain**, v. 127, n. 4, 2004.

CARLIER, M. F.; RESSAD, F.; PANTALONI, D. Control of actin dynamics in cell motility. Role of ADF/cofilin. **The Journal of biological chemistry**, v. 274, n. 48, p. 33827–30, 26 nov. 1999.

CELARDO, I.; MARTINS, L. M.; GANDHI, S. Unravelling mitochondrial pathways to Parkinson's disease. **British journal of pharmacology**, v. 171, n. 8, p. 1943–57, abr. 2014.

CERRUTI, C. et al. Dopamine transporter mRNA expression is intense in rat midbrain neurons and modest outside midbrain. **Brain research. Molecular brain research**, v. 18, n. 1–2, p. 181–6, abr. 1993.

CHAUDHURI, K. R.; SCHAPIRA, A. H. V. Non-motor symptoms of Parkinson's disease: dopaminergic pathophysiology and treatment. **Lancet neurology**, v. 8, n. 5, p. 464–74, maio 2009.

CHEUNG, Y.-T. et al. Effects of all-trans-retinoic acid on human SH-SY5Y neuroblastoma as in vitro model in neurotoxicity research. **Neurotoxicology**, v. 30, n. 1, p. 127–35, jan. 2009.

CHOI, J. et al. Oxidative modifications and aggregation of Cu,Zn-superoxide dismutase associated with Alzheimer and Parkinson diseases. **The Journal of biological chemistry**, v. 280, n. 12, p. 11648–55, 25 mar. 2005.

CHU, Y. et al. Abnormal alpha-synuclein reduces nigral voltage-dependent anion channel 1 in sporadic and experimental Parkinson's disease. **Neurobiology of disease**, v. 69, p. 1–14, set. 2014.

CLEMEDSON, C.; KOLMAN, A.; FORSBY, A. The integrated acute systemic toxicity project (ACuteTox) for the optimisation and validation of alternative in vitro tests. **Alternatives to laboratory animals : ATLA**, v. 35, n. 1, p. 33–8, mar. 2007.

COLLA, E. et al. Accumulation of toxic α-synuclein oligomer within endoplasmic reticulum occurs in α-synucleinopathy in vivo. **The Journal of neuroscience : the official journal of the Society for Neuroscience**, v. 32, n. 10, p. 3301–5, 7 mar. 2012.

CONSTANTINESCU, R. et al. Neuronal differentiation and long-term culture of the human neuroblastoma line SH-SY5Y. **Journal of neural transmission. Supplementum**, n. 72, p. 17–28, jan. 2007.

CURTIUS, H. C. et al. Mass fragmentography of dopamine and 6-hydroxydopamine. Application to the determination of dopamine in human brain biopsies from the caudate nucleus. **Journal of chromatography**, v. 99, p. 529–40, 6 nov. 1974.

DAUER, W.; PRZEDBORSKI, S. Parkinson's disease: mechanisms and models. **Neuron**, v. 39, n. 6, p. 889–909, 11 set. 2003.

DAVIAUD, N. et al. Modeling nigrostriatal degeneration in organotypic cultures, a new ex vivo model of Parkinson's disease. **Neuroscience**, v. 256, p. 10–22, 3 jan. 2014.

DAVIE, C. A. A review of Parkinson's disease. **British medical bulletin**, v. 86, p. 109–27, jan. 2008.

DE BOER, R. A.; VOORS, A. A.; VAN VELDHUISEN, D. J. Nebivolol: third-generation β-blockade. **Expert Opinion on Pharmacotherapy**, v. 8, n. 10, p. 1539–1550, 30 jul. 2007.

DEL HOYO, P. et al. Oxidative stress in skin fibroblasts cultures from patients with Parkinson's disease. **BMC neurology**, v. 10, p. 95, jan. 2010.

DEXTER, D. T. et al. Basal lipid peroxidation in substantia nigra is increased in Parkinson's disease. **Journal of neurochemistry**, v. 52, n. 2, p. 381–9, fev. 1989a.

DEXTER, D. T. et al. Increased nigral iron content and alterations in other metal ions occurring in brain in Parkinson's disease. **Journal of neurochemistry**, v. 52, n. 6, p. 1830–6, jun. 1989b.

DING, H. et al. 14-3-3 inhibition promotes dopaminergic neuron loss and 14-3-3θ overexpression promotes recovery in the MPTP mouse model of Parkinson's disease. **Neuroscience**, v. 307, p. 73–82, 29 out. 2015.

DORSEY, E. R. et al. Projected number of people with Parkinson disease in the most populous nations, 2005 through 2030. **Neurology**, v. 68, n. 5, p. 384–6, 30 jan. 2007.

EL-AGNAF, O. M. A. et al. Detection of oligomeric forms of -synuclein protein in human plasma as a potential biomarker for Parkinson's disease. **The FASEB Journal**, v. 20, n. 3, p. 419–425, 1 mar. 2006.

ELM, J. J.; NINDS NET-PD INVESTIGATORS. Design innovations and baseline findings in a long-term Parkinson's trial: the National Institute of Neurological Disorders and Stroke Exploratory Trials in Parkinson's Disease Long-Term Study-1. **Movement disorders : official journal of the Movement Disorder Society**, v. 27, n. 12, p. 1513–21, out. 2012.

ENCINAS, M. et al. Sequential treatment of SH-SY5Y cells with retinoic acid and brain-derived neurotrophic factor gives rise to fully differentiated, neurotrophic factor-dependent, human neuron-like cells. **Journal of neurochemistry**, v. 75, n. 3, p. 991–1003, set. 2000.

FALKENBURGER, B. H.; SCHULZ, J. B. Limitations of cellular models in Parkinson's disease research. **Journal of neural transmission. Supplementum**, n. 70, p. 261–8, jan. 2006.

FERRER, I. et al. Abnormal levels of prohibitin and ATP synthase in the substantia nigra and frontal cortex in Parkinson's disease. **Neuroscience letters**, v. 415, n. 3, p. 205–9, 30 mar. 2007.

FILOGRANA, R. et al. Analysis of the Catecholaminergic Phenotype in Human SH-SY5Y and BE(2)-M17 Neuroblastoma Cell Lines upon Differentiation. **PLOS ONE**, v. 10, n. 8, p. e0136769, 28 ago. 2015.

FORSTER, J. I. et al. Characterization of Differentiated SH-SY5Y as Neuronal Screening Model Reveals Increased Oxidative Vulnerability. **Journal of biomolecular screening**, 6 jan. 2016.

FRANCO-IBORRA, S.; VILA, M.; PERIER, C. The Parkinson Disease Mitochondrial Hypothesis: Where Are We at? **The Neuroscientist : a review journal bringing neurobiology, neurology and psychiatry**, p. 1073858415574600-, 11 mar. 2015.

GAENSLEN, A. et al. The patients' perception of prodromal symptoms before the initial diagnosis of Parkinson's disease. **Movement disorders : official journal of the Movement Disorder Society**, v. 26, n. 4, p. 653–8, mar. 2011.

GAVEN, F.; MARIN, P.; CLAEYSEN, S. Primary culture of mouse dopaminergic neurons. **Journal of visualized experiments : JoVE**, n. 91, p. e51751, 8 jan. 2014.

GELB, D. J.; OLIVER, E.; GILMAN, S. Diagnostic criteria for Parkinson disease. **Archives of neurology**, v. 56, n. 1, p. 33–9, jan. 1999.

GIASSON, B. I. et al. A Hydrophobic Stretch of 12 Amino Acid Residues in the Middle of -Synuclein Is Essential for Filament Assembly. **Journal of Biological Chemistry**, v. 276, n. 4, p. 2380–2386, 26 jan. 2001.

GIBB, W. R. Neuropathology of the substantia nigra. **European neurology**, v. 31 Suppl 1, p. 48–59, jan. 1991.

GILANY, K. et al. The proteome of the human neuroblastoma cell line SH-SY5Y: an enlarged proteome. **Biochimica et biophysica acta**, v. 1784, n. 7–8, p. 983–5, jan. 2008.

GOEDERT, M. et al. 100 years of Lewy pathology. **Nature Reviews Neurology**, v. 9, n. 1, p. 13–24, 27 nov. 2012.

GOETZ, C. G. et al. Movement Disorder Society-sponsored revision of the Unified Parkinson's Disease Rating Scale (MDS-UPDRS): scale presentation and clinimetric testing results. **Movement disorders : official journal of the Movement Disorder Society**, v. 23, n. 15, p. 2129–70, 15 nov. 2008.

GOETZ, C. G. The History of Parkinson's Disease: Early Clinical Descriptions and Neurological Therapies. **Cold Spring Harbor Perspectives in Medicine**, v. 1, n. 1, p. a008862–a008862, 1 set. 2011.

GOHLA, A.; BOKOCH, G. M. **14-3-3 Regulates Actin Dynamics by Stabilizing Phosphorylated CofilinCurrent Biology**. [s.l: s.n.].

GOMEZ-LAZARO, M. et al. 6-Hydroxydopamine activates the mitochondrial apoptosis pathway through p38 MAPK-mediated, p53-independent activation of

Bax and PUMA. **Journal of neurochemistry**, v. 104, n. 6, p. 1599–612, mar. 2008.

GREFFARD, S. et al. Motor score of the Unified Parkinson Disease Rating Scale as a good predictor of Lewy body-associated neuronal loss in the substantia nigra. **Archives of neurology**, v. 63, n. 4, p. 584–8, abr. 2006.

GUERREIRO, P. S. et al. LRRK2 interactions with α-synuclein in Parkinson's disease brains and in cell models. **Journal of Molecular Medicine**, v. 91, n. 4, p. 513–522, 27 abr. 2013.

GUPTA, P. et al. Characterisation of the contractile activity of eletriptan at the canine vascular 5-HT1B receptor. **European journal of pharmacology**, v. 367, n. 2–3, p. 283–90, 19 fev. 1999.

GURNIAK, C. B.; PERLAS, E.; WITKE, W. The actin depolymerizing factor n-cofilin is essential for neural tube morphogenesis and neural crest cell migration. **Developmental biology**, v. 278, n. 1, p. 231–41, 1 fev. 2005.

HALITZCHI, F.; JIANU, L.; AMUZESCU, B. ELECTROPHYSIOLOGY AND PHARMACOLOGY STUDY OF A HUMAN NEUROBLASTOMA CELL LINE. **Romanian Reports in Physics**, v. 67, n. 2, p. 439–451, 2015.

HALLIDAY, G. et al. No Lewy pathology in monkeys with over 10 years of severe MPTP Parkinsonism. **Movement disorders : official journal of the Movement Disorder Society**, v. 24, n. 10, p. 1519–23, 30 jul. 2009.

HALLIWELL, B. Oxidative stress and neurodegeneration: where are we now? **Journal of neurochemistry**, v. 97, n. 6, p. 1634–58, jun. 2006.

HARTFIELD, E. M. et al. Physiological characterisation of human iPS-derived dopaminergic neurons. **PloS one**, v. 9, n. 2, p. e87388, 2014.

HATTORI, N. et al. Immunohistochemical studies on complexes I, II, III, and IV of mitochondria in Parkinson's disease. **Annals of neurology**, v. 30, n. 4, p. 563–71, out. 1991.

HINKLE, K. M. et al. LRRK2 knockout mice have an intact dopaminergic system but display alterations in exploratory and motor co-ordination behaviors. **Molecular Neurodegeneration**, v. 7, n. 1, p. 25, 2012.

HIRTZ, D. et al. How common are the "common" neurologic disorders? **Neurology**, v. 68, n. 5, p. 326–337, 30 jan. 2007.

HOTULAINEN, P. et al. Actin-depolymerizing factor and cofilin-1 play overlapping roles in promoting rapid F-actin depolymerization in mammalian nonmuscle cells. **Molecular biology of the cell**, v. 16, n. 2, p. 649–64, fev. 2005.

HUGHES, A. J. et al. Accuracy of clinical diagnosis of idiopathic Parkinson's disease: a clinico-pathological study of 100 cases. **Journal of neurology, neurosurgery, and psychiatry**, v. 55, n. 3, p. 181–4, mar. 1992.

Investigation of Cogane (PYM50028) in Early-stage Parkinson's Disease (CONFIDENT-PD) - Full Text View - ClinicalTrials.gov. Disponível em: <https://clinicaltrials.gov/ct2/show/NCT01060878?term=pym50028&rank=2>. Acesso em: 31 jan. 2017.

ISHIZAWA, T. et al. Colocalization of tau and alpha-synuclein epitopes in Lewy bodies. **Journal of neuropathology and experimental neurology**, v. 62, n. 4, p. 389–97, abr. 2003.

IWAI, A. et al. The precursor protein of non-A beta component of Alzheimer's disease amyloid is a presynaptic protein of the central nervous system. **Neuron**, v. 14, n. 2, p. 467–75, fev. 1995.

IZUMI, Y. et al. p-Quinone mediates 6-hydroxydopamine-induced dopaminergic neuronal death and ferrous iron accelerates the conversion of p-quinone into melanin extracellularly. **Journal of neuroscience research**, v. 79, n. 6, p. 849–60, 15 mar. 2005.

IZUMI, Y. et al. Vulnerability to glutamate toxicity of dopaminergic neurons is dependent on endogenous dopamine and MAPK activation. **Journal of Neurochemistry**, v. 110, n. 2, p. 745–755, jul. 2009.

JAGMAG, S. A. et al. Evaluation of Models of Parkinson's Disease. **Frontiers in neuroscience**, v. 9, p. 503, 2015a.

JAGMAG, S. A. et al. Evaluation of Models of Parkinson's Disease. **Frontiers in neuroscience**, v. 9, p. 503, jan. 2015b.

JAKES, R.; SPILLANTINI, M. G.; GOEDERT, M. Identification of two distinct synucleins from human brain. **FEBS letters**, v. 345, n. 1, p. 27–32, 23 maio 1994.

JANKOVIC, J.; AGUILAR, L. G. Current approaches to the treatment of Parkinson's disease. **Neuropsychiatric disease and treatment**, v. 4, n. 4, p. 743–57, ago. 2008.

JANKOVIC, J.; POEWE, W. Therapies in Parkinson's disease. **Current opinion in neurology**, v. 25, n. 4, p. 433–47, ago. 2012.

JIANG, H. et al. Parkin controls dopamine utilization in human midbrain dopaminergic neurons derived from induced pluripotent stem cells. **Nature communications**, v. 3, p. 668, jan. 2012.

JOHNSTON, T. H.; BROTCHIE, J. M. Drugs in development for Parkinson's disease. **Current opinion in investigational drugs (London, England : 2000)**, v. 5, n. 7, p. 720–6, jul. 2004.

KALIA, L. V.; LANG, A. E. Parkinson disease in 2015: Evolving basic, pathological and clinical concepts in PD. **Nature Reviews Neurology**, v. 12, n. 2, p. 65–66, 18 jan. 2016.

KANAZAWA, T. et al. Pale Neurites, Premature α-Synuclein Aggregates with Centripetal Extension from Axon Collaterals. **Brain Pathology**, v. 22, n. 1, p. 67–

78, jan. 2012.

KARUNAKARAN, S. et al. Selective activation of p38 mitogen-activated protein kinase in dopaminergic neurons of substantia nigra leads to nuclear translocation of p53 in 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine-treated mice. **The Journal of neuroscience : the official journal of the Society for Neuroscience**, v. 28, n. 47, p. 12500–9, 19 nov. 2008.

KAWAMOTO, Y. et al. 14-3-3 proteins in Lewy bodies in Parkinson disease and diffuse Lewy body disease brains. **Journal of neuropathology and experimental neurology**, v. 61, n. 3, p. 245–53, mar. 2002.

KEENEY, P. M. et al. Parkinson's disease brain mitochondrial complex I has oxidatively damaged subunits and is functionally impaired and misassembled. **The Journal of neuroscience : the official journal of the Society for Neuroscience**, v. 26, n. 19, p. 5256–64, 10 maio 2006.

KHOO, T. K. et al. The spectrum of nonmotor symptoms in early Parkinson disease. **Neurology**, v. 80, n. 3, p. 276–81, 15 jan. 2013.

KIEBURTZ, K.; OLANOW, C. W. Advances in clinical trials for movement disorders. **Movement disorders : official journal of the Movement Disorder Society**, v. 30, n. 11, p. 1580–7, 15 set. 2015.

KIM, G. H. et al. The Role of Oxidative Stress in Neurodegenerative Diseases. **Experimental Neurobiology**, v. 24, n. 4, p. 325, 2015.

KIM, J.-S.; HUANG, T. Y.; BOKOCH, G. M. Reactive oxygen species regulate a slingshot-cofilin activation pathway. **Molecular biology of the cell**, v. 20, n. 11, p. 2650–60, jun. 2009.

KIUCHI, T. et al. Cofilin promotes stimulus-induced lamellipodium formation by generating an abundant supply of actin monomers. **The Journal of cell biology**, v. 177, n. 3, p. 465–76, 7 maio 2007.

KLAMT, F. et al. Oxidant-induced apoptosis is mediated by oxidation of the actin-regulatory protein cofilin. **Nature cell biology**, v. 11, n. 10, p. 1241–6, out. 2009.

KORECKA, J. A. et al. Phenotypic Characterization of Retinoic Acid Differentiated SH-SY5Y Cells by Transcriptional Profiling. **PLoS ONE**, v. 8, n. 5, p. e63862, 28 maio 2013.

KRACK, P. et al. Five-year follow-up of bilateral stimulation of the subthalamic nucleus in advanced Parkinson's disease. **The New England journal of medicine**, v. 349, n. 20, p. 1925–34, 13 nov. 2003.

KUME, T. et al. Dibutyryl cyclic AMP induces differentiation of human neuroblastoma SH-SY5Y cells into a noradrenergic phenotype. **Neuroscience letters**, v. 443, n. 3, p. 199–203, 10 out. 2008.

KUNZLER, A. et al. Changes in Cell Cycle and Up-Regulation of Neuronal Markers During SH-SY5Y Neurodifferentiation by Retinoic Acid are Mediated by

Reactive Species Production and Oxidative Stress. **Molecular neurobiology**, out. 2016.

LANG, A. E. et al. Trial designs used to study neuroprotective therapy in Parkinson's disease. **Movement Disorders**, v. 28, n. 1, p. 86–95, jan. 2013.

LANGSTON, J. W. The parkinson's complex: Parkinsonism is just the tip of the iceberg. **Annals of Neurology**, v. 59, n. 4, p. 591–596, abr. 2006.

LANGSTON, J. W.; LANGSTON, E. B.; IRWIN, I. MPTP-induced parkinsonism in human and non-human primates--clinical and experimental aspects. **Acta neurologica Scandinavica. Supplementum**, v. 100, p. 49–54, 1984.

LEE, A.; GILBERT, R. M. Epidemiology of Parkinson Disease. **Neurologic Clinics**, v. 34, n. 4, p. 955–965, nov. 2016.

LEVERENZ, J. B. et al. Proteomic Identification of Novel Proteins in Cortical Lewy Bodies. **Brain Pathology**, v. 17, n. 2, p. 139–145, abr. 2007.

LI, Q.-X. et al. Platelet alpha- and gamma-synucleins in Parkinson's disease and normal control subjects. **Journal of Alzheimer's disease : JAD**, v. 4, n. 4, p. 309–15, ago. 2002.

LI, Q.-X. et al. Plasma α-synuclein is decreased in subjects with Parkinson's disease. **Experimental Neurology**, v. 204, n. 2, p. 583–588, abr. 2007.

LINDENBACH, D. et al. Side effect profile of 5-HT treatments for Parkinson's disease and L-DOPA-induced dyskinesia in rats. **British Journal of Pharmacology**, v. 172, n. 1, p. 119–130, jan. 2015.

LITIM, N.; MORISSETTE, M.; DI PAOLO, T. Neuroactive gonadal drugs for neuroprotection in male and female models of Parkinson's disease. **Neuroscience & Biobehavioral Reviews**, v. 67, p. 79–88, ago. 2016.

LIU, D. et al. Crystal structure of the zeta isoform of the 14-3-3 protein. **Nature**, v. 376, n. 6536, p. 191–4, 13 jul. 1995.

LÖHLE, M. Clinical neuroprotection in Parkinson's disease — Still waiting for the breakthrough. **Journal of the Neurological Sciences**, v. 289, n. 1, p. 104–114, 2010.

LOPES, F. M. et al. Comparison between proliferative and neuron-like SH-SY5Y cells as an in vitro model for Parkinson disease studies. **Brain research**, v. 1337, p. 85–94, 14 jun. 2010.

LOPES, F. M. et al. Evaluation of the neurotoxic/neuroprotective role of organoselenides using differentiated human neuroblastoma SH-SY5Y cell line challenged with 6-hydroxydopamine. **Neurotoxicity research**, v. 22, n. 2, p. 138–49, ago. 2012.

LOTHARIUS, J. et al. Progressive degeneration of human mesencephalic neuron-derived cells triggered by dopamine-dependent oxidative stress is

dependent on the mixed-lineage kinase pathway. **The Journal of neuroscience : the official journal of the Society for Neuroscience**, v. 25, n. 27, p. 6329–42, 6 jul. 2005.

LUCHTMAN, D. W.; SONG, C. Why SH-SY5Y cells should be differentiated. **Neurotoxicology**, v. 31, n. 1, p. 164-5-6, jan. 2010.

MA, L.-Y. et al. Heterogeneity among patients with Parkinson's disease: Cluster analysis and genetic association. **Journal of the Neurological Sciences**, v. 351, n. 1–2, p. 41–45, abr. 2015.

MAJBOUR, N. K. et al. Oligomeric and phosphorylated alpha-synuclein as potential CSF biomarkers for Parkinson's disease. **Molecular neurodegeneration**, v. 11, p. 7, 19 jan. 2016.

MANTHEY, D.; GAMERDINGER, M.; BEHL, C. The selective beta1-adrenoceptor antagonist nebivolol is a potential oestrogen receptor agonist with neuroprotective abilities. **British journal of pharmacology**, v. 159, n. 6, p. 1264–73, mar. 2010.

MARKLUND, S. L. Superoxide dismutase, catalase and glutathione peroxidase in degenerative diseases. **Bulletin europeen de physiopathologie respiratoire**, v. 17 Suppl, p. 259–65, 1981.

MAROTEAUX, L.; CAMPANELLI, J. T.; SCHELLER, R. H. Synuclein: a neuron-specific protein localized to the nucleus and presynaptic nerve terminal. **The Journal of neuroscience : the official journal of the Society for Neuroscience**, v. 8, n. 8, p. 2804–15, ago. 1988.

MARQUES, O.; OUTEIRO, T. F. Alpha-synuclein: from secretion to dysfunction and death. **Cell Death and Disease**, v. 3, n. 7, p. e350, jul. 2012.

MATHIASEN, J. R. et al. Inhibition of mixed lineage kinase 3 attenuates MPP+-induced neurotoxicity in SH-SY5Y cells. **Brain Research**, v. 1003, n. 1–2, p. 86–97, 2 abr. 2004.

MCNAUGHT, K. S.; JENNER, P. Proteasomal function is impaired in substantia nigra in Parkinson's disease. **Neuroscience letters**, v. 297, n. 3, p. 191–4, 19 jan. 2001.

MILLER WC, D. M. Parkinsonian symptomatology. An anatomical and physiological analysis. - PubMed - NCBI. **Annals of the New York Academy of Sciences**, v. 515, p. 287–302, 1988.

MILOSO, M. et al. Retinoic acid-induced neuritogenesis of human neuroblastoma SH-SY5Y cells is ERK independent and PKC dependent. **Journal of neuroscience research**, v. 75, n. 2, p. 241–52, 15 jan. 2004.

MINAMIDE, L. S. et al. Neurodegenerative stimuli induce persistent ADF/cofilin-actin rods that disrupt distal neurite function. **Nature cell biology**, v. 2, n. 9, p. 628–36, set. 2000.

Ministério da Saúde – Portal da Saúde - www.saude.gov.br - Principal. Disponível em: <http://portalsaude.saude.gov.br/>. Acesso em: 13 dez. 2016.

MOISOI, N. et al. Mitochondrial dysfunction triggered by loss of HtrA2 results in the activation of a brain-specific transcriptional stress response. **Cell death and differentiation**, v. 16, n. 3, p. 449–64, 21 mar. 2009.

MORGEN, K. et al. Structural brain abnormalities in patients with Parkinson disease: a comparative voxel-based analysis using T1-weighted MR imaging and magnetization transfer imaging. **AJNR. American journal of neuroradiology**, v. 32, n. 11, p. 2080–6, dez. 2011.

MYTHRI, R. B. et al. Evaluation of markers of oxidative stress, antioxidant function and astrocytic proliferation in the striatum and frontal cortex of Parkinson's disease brains. **Neurochemical research**, v. 36, n. 8, p. 1452–63, ago. 2011.

NALLS, M. A. et al. Large-scale meta-analysis of genome-wide association data identifies six new risk loci for Parkinson's disease. **Nature genetics**, v. 46, n. 9, p. 989–93, set. 2014.

Nature Outlook : Parkinson's disease. Disponível em: <http://www.nature.com/nature/outlook/parkinsons-disease/index.html>. Acesso em: 6 fev. 2017.

NAVARRO, A. et al. Human brain cortex: mitochondrial oxidative damage and adaptive response in Parkinson disease and in dementia with Lewy bodies. **Free radical biology & medicine**, v. 46, n. 12, p. 1574–80, 15 jun. 2009.

NISHIDA, E. et al. Cofilin is a component of intranuclear and cytoplasmic actin rods induced in cultured cells. **Proceedings of the National Academy of Sciences of the United States of America**, v. 84, n. 15, p. 5262–6, ago. 1987.

OHLOW, M. J. et al. Why Have Clinical Trials of Antioxidants to Prevent Neurodegeneration Failed? - A Cellular Investigation of Novel Phenothiazine-Type Antioxidants Reveals Competing Objectives for Pharmaceutical Neuroprotection. **Pharmaceutical Research**, v. 34, n. 2, p. 378–393, 28 fev. 2017.

OLANOW, C. W. Can we achieve neuroprotection with currently available anti-parkinsonian interventions? **Neurology**, v. 72, n. 7 Suppl, p. S59-64, fev. 2009.

OLANOW, C. W.; KIEBURTZ, K.; SCHAPIRA, A. H. V. Why have we failed to achieve neuroprotection in Parkinson's disease? **Annals of Neurology**, v. 64, n. S2, p. S101–S110, 6 jan. 2009.

OLANOW, C. W.; KIEBURTZ, K.; SCHAPIRA, A. H. V. Why have we failed to achieve neuroprotection in Parkinson's disease? **Annals of neurology**, v. 64 Suppl 2, p. S101-10, dez. 2008.

PÅHLMAN, S. et al. Retinoic acid-induced differentiation of cultured human neuroblastoma cells: a comparison with phorbolester-induced differentiation. **Cell**

differentiation, v. 14, n. 2, p. 135–44, jun. 1984.

PAN-MONTOJO, F. et al. Progression of Parkinson's Disease Pathology Is Reproduced by Intragastric Administration of Rotenone in Mice. **PLoS ONE**, v. 5, n. 1, p. e8762, 19 jan. 2010.

PARKER, W. D.; PARKS, J. K.; SWERDLOW, R. H. Complex I deficiency in Parkinson's disease frontal cortex. **Brain research**, v. 1189, p. 215–8, 16 jan. 2008.

PARKINSON, J. An essay on the shaking palsy. 1817. **The Journal of neuropsychiatry and clinical neurosciences**, v. 14, n. 2, p. 223–36; discussion 222, jan. 1817.

PARKINSON STUDY GROUP PRECEPT INVESTIGATORS. Mixed lineage kinase inhibitor CEP-1347 fails to delay disability in early Parkinson disease. **Neurology**, v. 69, n. 15, p. 1480–1490, 9 out. 2007.

PEELAERTS, W.; BAEKELANDT, V. α-Synuclein strains and the variable pathologies of synucleinopathies. **Journal of Neurochemistry**, v. 139, n. S1, p. 256–274, out. 2016.

PERIER, C. et al. Two molecular pathways initiate mitochondria-dependent dopaminergic neurodegeneration in experimental Parkinson's disease. **Proceedings of the National Academy of Sciences of the United States of America**, v. 104, n. 19, p. 8161–6, 8 maio 2007.

PETERSON, L. et al. Potential Benefits of Therapeutic Use of ***β* 2** -Adrenergic Receptor Agonists in Neuroprotection and Parkinson's Disease. **Journal of Immunology Research**, v. 2014, p. 1–8, 2014.

POLYMEROPOULOS, M. H. et al. Mutation in the alpha-synuclein gene identified in families with Parkinson's disease. **Science (New York, N.Y.)**, v. 276, n. 5321, p. 2045–7, 27 jun. 1997.

PONT-SUNYER, C. et al. The onset of nonmotor symptoms in Parkinson's disease (the ONSET PD study). **Movement disorders : official journal of the Movement Disorder Society**, v. 30, n. 2, p. 229–37, fev. 2015.

POON, G. Cinacalcet hydrochloride (Sensipar). **Proceedings (Baylor University. Medical Center)**, v. 18, n. 2, p. 181–4, abr. 2005.

POPE, B. J. et al. Solution structure of human cofilin: actin binding, pH sensitivity, and relationship to actin-depolymerizing factor. **The Journal of biological chemistry**, v. 279, n. 6, p. 4840–8, 6 fev. 2004.

POSTUMA, R. B. et al. Quantifying the risk of neurodegenerative disease in idiopathic REM sleep behavior disorder. **Neurology**, v. 72, n. 15, p. 1296–300, 14 abr. 2009.

POSTUMA, R. B. et al. Identifying prodromal Parkinson's disease: pre-motor disorders in Parkinson's disease. **Movement disorders : official journal of the**

Movement Disorder Society, v. 27, n. 5, p. 617–26, 15 abr. 2012.

POSTUMA, R. B. et al. MDS clinical diagnostic criteria for Parkinson's disease. **Movement Disorders**, v. 30, n. 12, p. 1591–1601, out. 2015.

PRESGRAVES, S. P. et al. Terminally differentiated SH-SY5Y cells provide a model system for studying neuroprotective effects of dopamine agonists. **Neurotoxicity research**, v. 5, n. 8, p. 579–98, jan. 2004.

PRINGSHEIM, T. et al. The prevalence of Parkinson's disease: a systematic review and meta-analysis. **Movement disorders : official journal of the Movement Disorder Society**, v. 29, n. 13, p. 1583–90, nov. 2014.

PU, J. et al. Redefining Parkinson's disease research using induced pluripotent stem cells. **Current neurology and neuroscience reports**, v. 12, n. 4, p. 392–8, ago. 2012.

QIAN, L. et al. β2-adrenergic receptor activation prevents rodent dopaminergic neurotoxicity by inhibiting microglia via a novel signaling pathway. **Journal of immunology (Baltimore, Md. : 1950)**, v. 186, n. 7, p. 4443–54, 1 abr. 2011.

RACZ, B.; WEINBERG, R. J. Spatial organization of cofilin in dendritic spines. **Neuroscience**, v. 138, n. 2, p. 447–456, 2006.

RADIO, N. M.; MUNDY, W. R. Developmental neurotoxicity testing in vitro: models for assessing chemical effects on neurite outgrowth. **Neurotoxicology**, v. 29, n. 3, p. 361–76, maio 2008.

RIZZO, G. et al. Accuracy of clinical diagnosis of Parkinson disease: A systematic review and meta-analysis. **Neurology**, v. 86, n. 6, p. 566–76, 13 jan. 2016.

ROSS, G. W. et al. Association of olfactory dysfunction with risk for future Parkinson's disease. **Annals of neurology**, v. 63, n. 2, p. 167–73, fev. 2008.

ROSS, H. J. The antiproliferative effect of trans-retinoic acid is associated with selective induction of interleukin-1 beta, a cytokine that directly inhibits growth of lung cancer cells. **Oncology research**, v. 8, n. 4, p. 171–8, jan. 1996.

SAITO, Y. et al. Molecular mechanisms of 6-hydroxydopamine-induced cytotoxicity in PC12 cells: involvement of hydrogen peroxide-dependent and -independent action. **Free radical biology & medicine**, v. 42, n. 5, p. 675–85, 1 mar. 2007.

SAKAMOTO, M. et al. Heterogeneity of nigral and cortical Lewy bodies differentiated by amplified triple-labeling for alpha-synuclein, ubiquitin, and thiazin red. **Experimental neurology**, v. 177, n. 1, p. 88–94, set. 2002.

SAMPSON, T. R. et al. Gut Microbiota Regulate Motor Deficits and Neuroinflammation in a Model of Parkinson's Disease. **Cell**, v. 167, n. 6, p. 1469–1480.e12, 2016.

SÁNCHEZ-DANÉS, A. et al. Disease-specific phenotypes in dopamine neurons

from human iPS-based models of genetic and sporadic Parkinson's disease. **EMBO molecular medicine**, v. 4, n. 5, p. 380–95, maio 2012.

SANCHEZ, G. et al. Unaltered striatal dopamine release levels in young Parkin knockout, Pink1 knockout, DJ-1 knockout and LRRK2 R1441G transgenic mice. **PloS one**, v. 9, n. 4, p. e94826, 14 abr. 2014.

SAVICA, R. et al. Medical records documentation of constipation preceding Parkinson disease: A case-control study. **Neurology**, v. 73, n. 21, p. 1752–8, 24 nov. 2009.

SCHAPIRA, A. H. et al. Mitochondrial complex I deficiency in Parkinson's disease. **Lancet (London, England)**, v. 1, n. 8649, p. 1269, 3 jun. 1989.

SCHAPIRA, A. H. et al. Mitochondrial complex I deficiency in Parkinson's disease. **Journal of neurochemistry**, v. 54, n. 3, p. 823–7, mar. 1990a.

SCHAPIRA, A. H. et al. Anatomic and disease specificity of NADH CoQ1 reductase (complex I) deficiency in Parkinson's disease. **Journal of neurochemistry**, v. 55, n. 6, p. 2142–5, dez. 1990b.

SCHRAG, A. et al. Prediagnostic presentations of Parkinson's disease in primary care: a case-control study. **The Lancet Neurology**, v. 14, n. 1, p. 57–64, nov. 2014.

SCHÜLE, B.; PERA, R. A. R.; LANGSTON, J. W. Can cellular models revolutionize drug discovery in Parkinson's disease? **Biochimica et biophysica acta**, v. 1792, n. 11, p. 1043–51, nov. 2009.

SCHUSTER, S. et al. Antagonizing L-type Ca2+ channel reduces development of abnormal involuntary movement in the rat model of L-3,4-dihydroxyphenylalanine-induced dyskinesia. **Biological psychiatry**, v. 65, n. 6, p. 518–26, 15 mar. 2009.

SHI, M. et al. Significance and confounders of peripheral DJ-1 and alpha-synuclein in Parkinson's disease. **Neuroscience letters**, v. 480, n. 1, p. 78–82, 9 ago. 2010.

SHIBA, M. et al. Anxiety disorders and depressive disorders preceding Parkinson's disease: a case-control study. **Movement disorders : official journal of the Movement Disorder Society**, v. 15, n. 4, p. 669–77, jul. 2000.

SHIMURA, H. et al. Familial Parkinson disease gene product, parkin, is a ubiquitin-protein ligase. **Nature genetics**, v. 25, n. 3, p. 302–5, 1 jul. 2000.

SIAN, J. et al. Alterations in glutathione levels in Parkinson's disease and other neurodegenerative disorders affecting basal ganglia. **Annals of neurology**, v. 36, n. 3, p. 348–55, set. 1994a.

SIAN, J. et al. Alterations in glutathione levels in Parkinson's disease and other neurodegenerative disorders affecting basal ganglia. **Annals of neurology**, v. 36, n. 3, p. 348–55, set. 1994b.

SLONE, S. R. et al. Increased 14-3-3 phosphorylation observed in Parkinson's disease reduces neuroprotective potential of 14-3-3 proteins. **Neurobiology of disease**, v. 79, p. 1–13, jul. 2015.

SMITH, K. M.; DAHODWALA, N. Sex differences in Parkinson's disease and other movement disorders. **Experimental Neurology**, v. 259, p. 44–56, set. 2014.

SNOW, B. J. et al. A double-blind, placebo-controlled study to assess the mitochondria-targeted antioxidant MitoQ as a disease-modifying therapy in Parkinson's disease. **Movement disorders : official journal of the Movement Disorder Society**, v. 25, n. 11, p. 1670–4, 15 ago. 2010.

SOTO-OTERO, R. et al. Autoxidation and neurotoxicity of 6-hydroxydopamine in the presence of some antioxidants: potential implication in relation to the pathogenesis of Parkinson's disease. **Journal of neurochemistry**, v. 74, n. 4, p. 1605–12, abr. 2000.

SPILLANTINI, M. G. et al. [alpha]-Synuclein in Lewy bodies. v. 388, n. 6645, p. 839–840, 28 ago. 1997.

SPILLANTINI, M. G.; DIVANE, A.; GOEDERT, M. Assignment of Human α-Synuclein (SNCA) and β-Synuclein (SNCB) Genes to Chromosomes 4q21 and 5q35. **Genomics**, v. 27, n. 2, p. 379–381, maio 1995.

STOESSL, A. J. Positron emission tomography in premotor Parkinson's disease. **Parkinsonism & related disorders**, v. 13 Suppl 3, p. S421-4, jan. 2007.

STUCHBURY, G.; MÜNCH, G. Optimizing the generation of stable neuronal cell lines via pre-transfection restriction enzyme digestion of plasmid DNA. **Cytotechnology**, v. 62, n. 3, p. 189–94, jul. 2010.

SULTANA, R.; PERLUIGI, M.; ALLAN BUTTERFIELD, D. Lipid peroxidation triggers neurodegeneration: a redox proteomics view into the Alzheimer disease brain. **Free radical biology & medicine**, v. 62, p. 157–69, set. 2013.

TANG, Y. et al. Cerebral Metabolic Differences Associated with Cognitive Impairment in Parkinson's Disease. **PloS one**, v. 11, n. 4, p. e0152716, 2016.

TEPPOLA, H. et al. Morphological Differentiation Towards Neuronal Phenotype of SH-SY5Y Neuroblastoma Cells by Estradiol, Retinoic Acid and Cholesterol. **Neurochemical research**, v. 41, n. 4, p. 731–47, abr. 2016.

TRETIAKOFF, K. Contribution à l'étude de l'anatomie pathologique du locus niger de Soemmering avec quelques déductions relatives à la pathogénie des troubles du tonus musculaire et de la maladie de Parkinson. **Thesis, University of Paris**, 1919.

UBL, A. et al. 14-3-3 protein is a component of Lewy bodies in Parkinson's disease-mutation analysis and association studies of 14-3-3 eta. **Brain research. Molecular brain research**, v. 108, n. 1–2, p. 33–9, dez. 2002.

UHL, G. R.; HEDREEN, J. C.; PRICE, D. L. Parkinson's disease: loss of neurons from the ventral tegmental area contralateral to therapeutic surgical lesions. **Neurology**, v. 35, n. 8, p. 1215–8, ago. 1985.

VALENTE, E. M. et al. Hereditary early-onset Parkinson's disease caused by mutations in PINK1. **Science (New York, N.Y.)**, v. 304, n. 5674, p. 1158–60, 21 maio 2004.

WALSH, D. M. et al. Naturally secreted oligomers of amyloid β protein potently inhibit hippocampal long-term potentiation in vivo. **Nature**, v. 416, n. 6880, p. 535–539, 4 abr. 2002.

WANG, T.; HAY, J. C. Alpha-synuclein Toxicity in the Early Secretory Pathway: How It Drives Neurodegeneration in Parkinsons Disease. **Frontiers in neuroscience**, v. 9, p. 433, jan. 2015.

WEINERT, M. et al. Isolation, culture and long-term maintenance of primary mesencephalic dopaminergic neurons from embryonic rodent brains. **Journal of visualized experiments : JoVE**, n. 96, p. e52475, 19 jan. 2015.

WERNER, C. J. et al. Proteome analysis of human substantia nigra in Parkinson's disease. **Proteome Science**, v. 6, n. 1, p. 8, 2008.

WHO | Neurological Disorders: Public Health Challenges. **WHO**, 2012.

WILLEMS, E. et al. Porcine carotid vascular effects of eletriptan (UK-116,044): a new 5-HT1B/1D receptor agonist with anti-migraine activity. **Naunyn-Schmiedeberg's archives of pharmacology**, v. 358, n. 2, p. 212–9, ago. 1998.

WIRDEFELDT, K. et al. Epidemiology and etiology of Parkinson's disease: a review of the evidence. **European journal of epidemiology**, v. 26 Suppl 1, p. S1-58, jun. 2011.

WOO, J. A. et al. RanBP9 at the intersection between cofilin and Abeta pathologies: rescue of neurodegenerative changes by RanBP9 reduction. **Cell death & disease**, v. 6, p. 1676, mar. 2015a.

WOO, J. A. et al. Slingshot-Cofilin activation mediates mitochondrial and synaptic dysfunction via Abeta ligation to beta1-integrin conformers. **Cell death and differentiation**, v. 22, n. 6, p. 921–934, jun. 2015b.

WU, S. S.; FRUCHT, S. J. Treatment of Parkinson's disease : what's on the horizon? **CNS drugs**, v. 19, n. 9, p. 723–43, 2005.

www.atcc.org/Products/All/CRL-2266.aspx. Disponível em: <http://www.atcc.org/Products/All/CRL-2266.aspx>. Acesso em: 19 maio. 2016.

XICOY, H.; WIERINGA, B.; MARTENS, G. J. M. The SH-SY5Y cell line in Parkinson's disease research: a systematic review. **Molecular neurodegeneration**, v. 12, n. 1, p. 10, 24 jan. 2017a.

XICOY, H.; WIERINGA, B.; MARTENS, G. J. M. The SH-SY5Y cell line in

Parkinson's disease research: a systematic review. **Molecular Neurodegeneration**, v. 12, n. 1, p. 10, 24 dez. 2017b.

XIE, H.; HU, L.; LI, G. SH-SY5Y human neuroblastoma cell line: in vitro cell model of dopaminergic neurons in Parkinson's disease. **Chinese medical journal**, v. 123, n. 8, p. 1086–92, 20 abr. 2010.

YACOUBIAN, T. A. et al. Differential neuroprotective effects of 14-3-3 proteins in models of Parkinson's disease. **Cell Death and Disease**, v. 1, n. 1, p. e2, jan. 2010.

YE, X. et al. MTERF4 regulates the mitochondrial dysfunction induced by MPP(+) in SH-SY5Y cells. **Biochemical and biophysical research communications**, v. 464, n. 1, p. 214–20, 14 ago. 2015.

YORITAKA, A. et al. Immunohistochemical detection of 4-hydroxynonenal protein adducts in Parkinson disease. **Proceedings of the National Academy of Sciences of the United States of America**, v. 93, n. 7, p. 2696–701, 2 abr. 1996.

ZECCA, L. et al. Substantia nigra neuromelanin: structure, synthesis, and molecular behaviour. **Molecular pathology : MP**, v. 54, n. 6, p. 414–8, dez. 2001.

ZHAO, Y. et al. Altered activation in visual cortex: Unusual functional magnetic resonance imaging finding in early Parkinson's disease. **Journal of International Medical Research**, v. 42, n. 2, p. 503–515, 1 abr. 2014.

ZIBETTI, M. et al. Motor and nonmotor symptom follow-up in parkinsonian patients after deep brain stimulation of the subthalamic nucleus. **European neurology**, v. 58, n. 4, p. 218–23, jan. 2007.

ZIMPRICH, A. et al. Mutations in LRRK2 Cause Autosomal-Dominant Parkinsonism with Pleomorphic Pathology. **Neuron**, v. 44, n. 4, p. 601–607, 18 nov. 2004.

ZIMPRICH, A. et al. A mutation in VPS35, encoding a subunit of the retromer complex, causes late-onset Parkinson disease. **American journal of human genetics**, v. 89, n. 1, p. 168–75, 15 jul. 2011.