Universidade do Minho
Escola de Ciências

Miguel Gil Afonso Santejo

Impact of cofilin-actin rods formation on Parkinson's Dementia

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Dissertação de Mestrado
Mestrado em Bioquímica Aplicada
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Trabalho efetuado sob a orientação da
Doutora Márcia Liz
e da
Doutora Sandra Paiva

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DIREITOS DE AUTOR E CONDIÇÕES DE UTILIZAÇÃO DO TRABALHO POR TERCEIROS

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STATEMENT OF INTEGRITY

I hereby declare having conducted this academic work with integrity. I confirm that I have not used plagiarism or any form of undue use of information or falsification of results along the process leading to its elaboration.

I further declare that I have fully acknowledged the Code of Ethical Conduct of the University of Minho.

ABSTRACT

The formation of inclusions of α-Synuclein (α-Syn), named Lewy bodies, is the major hallmark of Parkinson's disease, where they accumulate mainly in the *substantia nigra* of the brain triggering the characteristic motor symptoms of the disorder. Additionally, α-Syn accumulation in the hippocampus is associated with the cognitive impairment seen in PD patients which develop dementia. Structures that have been associated with synaptic dysfunction of the hippocampus in diseases presenting cognitive impairment are the cofilin-actin rods. Rods are formed by hyperactivation, by dephosphorylation, of cofilin leading to actin saturation. Preliminary data of our laboratory suggested that rods are formed in response to α-Syn in hippocampal neurons raising the hypothesis of whether these structures underlie cognitive impairment in PD.

In this master thesis we aimed to establish a cell-based system of cofilin-actin rod formation in hippocampal neurons in order to study the molecular mechanism underlying their formation. We successfully established the model by lentiviral-mediated overexpression of α-Syn, which recapitulates the high levels of the protein seen in PD cases with dementia. We confirmed cofilin-actin rod formation which occurred by α-Syn-mediated decrease in the levels of phospho-cofilin, a mechanism that probably uses Cellular Prion Protein (PrP^{c}) and NADPH oxidase (NOX). In this respect, increasing the levels of cofilin phosphorylation blocked α-Syn-induced rod formation. Aiming at addressing whether rods are specific to hippocampal neurons we used SH-SY5Y cells, a relevant model to study PD since they resemble dopaminergic neurons. In these cells rod formation was not observed in response either to α-Syn overexpression or to a general inducer of rod formation, what might support cell type specific rod formation. Also using the SH-SY5Y model we tested the neuroprotective effect of modified-analogues of the tripeptide GPE which was previously shown to have a beneficial effect on PD. We observed that the GPE analogues had an improved effect on blocking 6-OHDA-induced neurotoxicity in SH-SY5Y cell and might be tested in the future in the context of α-Syn overexpression and rod formation.

In summary, this thesis generated an *in vitro* system which will be crucial to test the consequences of α-Syn -induced rod formation to neuronal function. Moreover, raised the question of rods being specific to the hippocampus in the context of PD and disclosed new compounds that might used as neuroprotective agents for the disease.

Keywords: α-Synuclein; Parkinson Disease; Dementia; Hippocampus.

RESUMO

A formação de inclusões de α-sinucleína (α-Sin), denominados corpos de Lewy, são características da Doença de Parkinson (DP), onde se acumulam principalmente na *substantia nigra* do cérebro, desencadeando os sintomas motores característicos da doença. Além disso, a presença de α- Sin no hipocampo está associada à disfunção cognitiva observada em pacientes com DP que desenvolvem demência. Estruturas que têm sido associadas à disfunção sináptica do hipocampo em doenças que apresentam disfunção cognitiva são os rods de cofilina-actina. Os rods são formados por hiperativação, desfosforilação de cofilina levando à saturação de actina. Dados preliminares de nosso laboratório sugeriram que os rods são formados em resposta à α-Sin nos neurônios do hipocampo, levantando a hipótese de que essas estruturas estão subjacentes à disfunção cognitiva na DP.

Nesta dissertação de mestrado, objetivamos estabelecer um sistema *in vitro* de formação de rods de cofilina-actina em neurônios do hipocampo, a fim de estudar o mecanismo molecular subjacente à sua formação. Estabelecemos com sucesso o modelo de sobreexpressão de α-Sin mediada por lentivírus, que recapitula os altos níveis da proteína observada em casos de DP com demência. Confirmamos que a formação dos rods de cofilina-actina é induzida por α-Sin, levando a uma diminuição nos níveis de fosfo-cofilina, sendo este um mecanismo que provavelmente usa a proteína priónica celular (PrP^c) e NADPH oxidase (NOX). A este respeito, o aumento dos níveis de fosforilação de cofilina bloqueou a formação de rods induzida por α-Sin. Com o objetivo de abordar se os rods são específicos para os neurônios do hipocampo, usamos células SH-SY5Y, um modelo relevante para o estudo da DP, pois se assemelham a neurônios dopaminérgicos. Nessas células, a formação de rods não foi observada em resposta à sobreexpressão de α-Sin ou a um indutor geral de formação de rods, o que pode apoiar que a formação de rods é específica do tipo de célula. Também usando o modelo SH-SY5Y, testamos o efeito neuroprotetor de análogos do tripéptido GPE, que anteriormente demonstrou ter um efeito benéfico na DP. Observamos que os análogos do GPE tiveram um efeito significativo no bloqueio da neurotoxicidade induzida por 6-OHDA nas células SH-SY5Y e podem ser testados no futuro no contexto de sobreexpressão de α-Sin e formação de rods.

Em suma, esta tese gerou um sistema *in vitro* que será crucial para testar as consequências da formação de rods induzida por α-Sin na função neuronal. Além disso, levantou a questão de a formação de rods possa ser específica no hipocampo no contexto da DP e demonstrou novos compostos que poderiam ser usados como agentes neuroprotetores para a doença.

Palavras Chave: α-sinucleína; Doença de Parkinson; Demência; Hipocampo

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LIST OF ABBREVIATIONS

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

6-Hydroxydopamine - 6-OHDA

Actin-binding protein - ABP

Alzheimer's disease - AD

Amyloid precursor protein - APP

Amyloid β - Aβ

Brain-derived neurotrophic factor - BDNF

Cellular prion protein - PrP^{c}

Chronopin - CIN

Days in Vitro - DIV

Dementia with Lewy bodies - DLB

Filamentous actin - F-actin

Glial cytoplasmic inclusion - GCI

Globular actin - G-actin

Glycine-proline-glutamate - GPE

Green fluorescent protein - GFP

Huntington's disease - HD

Internal ribosome entry site - IRES

Lewy Bodies - LB

Lewy neurites – LN

LIM kinase - LIMK

Membrane-associated SNARE - t-SNARE

Multiple System Atrophy - MSA

N,N'-dimethyl-4-4'-bipiridinium - Paraquat

NADPH Oxidase - NOX

Non-Aβ amyloid component - NAC

Parkinson's disease - PD

Parkinson's disease dementia – PDD

Phosphate-buffered saline - PBS

Platelet-derived growth factor - PDGF

Pre-formed fibrils - PFF

Reactive oxygen species - ROS

Retinoic acid – RA

Room temperature – RT

Serine 3 - Ser3

Slingshot phosphatase - SSH

Soluble N-ethylmaleimide-sensitive fusion protein attachment protein receptors - SNARE

Transfection units - TU

Tyrosine hydroxylase - TH

Vesicle-associated SNARE - v-SNARE

Wild-type - WT

α-Synuclein - α-Syn

α-Synuclein phosphorylated in Ser9 - PS129- α-Syn

INTRODUCTION

Neurodegenerative diseases are characterized by the loss of neuronal systems, both anatomically and physiologically. This type of diseases involves the degeneration of neuronal cells due to molecular changes, that can cause loss of integrity of the different neuronal compartments (Conforti, Adalbert et al. 2007).

These diseases are usually classified as a movement disorders or dementias. Parkinson's disease (PD) and Huntington's disease (HD) are considered movement disorders, characterized mainly by the loss of motor control, akinesia, bradykinesia or ataxia. In the case of dementias, the most frequent symptoms are cognitive deficits which occur mainly in Alzheimer's disease (AD), and dementia with Lewy bodies (DLB). A common hallmark of these disorders is the aggregation and accumulation of misfolding proteins in neuronal cells, which might occur extracellularly or intracellularly, causing a neurotoxic effect (Weinreb, Zhen et al. 1996, Soto and Estrada 2008).

PD, the disorder mainly addressed in this master thesis, is characterized by the accumulation of aggregated α-Synuclein (α-Syn) in neurons causing neurotoxicity. The motor symptoms characteristic of PD are associated with the loss of dopaminergic neurons leading to the decrease in the levels of the neurotransmitter dopamine, which is responsible for different neurological processes including motor control. Additionally, several PD patients also present non-motor symptoms such as cognitive deficits and dementia (Fredericks, Norton et al. 2017).

Disruption of cognitive function has been associated with the formation of neurodegeneration-associated structures, named cofilin-actin rods. Cofilin-actin rods are formed by the dysregulation of cofilin-1, an actin-binding protein (ABP) responsible for the regulation of actin dynamics, through severing or stabilization of the actin filaments. This dysregulation can cause neurotoxicity, affecting neuronal function by disruption of axonal transport and synaptic activity (Minamide, Striegl et al. 2000, Cichon, Sun et al. 2012). Rod formation was mainly reported in AD and associated with the cognitive impairment characteristic of the disease.

The introductory section of this master thesis will address the pathophysiology of PD, focusing on the hippocampal pathology and cognitive dysfunction observed in the disease, and the impact of cofilin pathology on neurodegeneration.

1. Synuclein

α-Syn belongs to the family of presynaptic proteins called Synucleins, which also includes β-Synuclein and γ-Synuclein. Synucleins are involved in neurotransmitter release and vesicle turnover at the presynaptic terminals (Jakes, Spillantini et al. 1994). Although these 3 proteins have been studied in the context of several diseases, such as Creutzfeldt-Jakob disease and AD (Oeckl, Metzger et al. 2016), α-Syn emerged as a key player in PD, since it is the main constituent of neuronal inclusions named Lewy Bodies (LB), the major hallmark of the disorder.

1.1 Synuclein Biology

α-Syn is a small neuronal protein composed by 140 amino acids encoded by a single copy of a gene named *SNCA* gene, localized in the chromosome 4 of the human genome. The *SNCA* gene is composed by six exons (Venda, Cragg et al. 2010). The translation start codon ATG is encoded by exon 2 and the stop codon TAA is encoded by exon 6. The non-Aβ amyloid component (NAC) is encoded in exon 4. Two previously reported minor isoforms of α-Syn are alternatively spliced products from the splicing out of the exon 3 or exon 5. Exon 1 was found to have different splicing sites, producing different 5'-untranslated sequences in the cDNAs (Xia, Saitoh et al. 2001).

α-Syn sequence can be divided in three different regions: 1) the N-terminal domain, including residues 1-60, with amphipathic characteristics; this region contains six imperfect repeats of the consensus motif KTKEGV and forms amphipathic alpha-helices that are responsible for the binding to phospholipid membranes and vesicles; 2) the region including residues 61-95 contains a non-Aβ amyloid component (NAC) sequence, which is relatively hydrophobic and can promote aggregation of human α-Syn; 3) the C-terminal domain, from 96-140 residues, is an acidic and negatively charged region (rich in glutamate and aspartate residues) that can be responsible for multiple protein interactions like ion binding, polycation binding and polyamine binding (Figure 1) (Emamzadeh 2016).

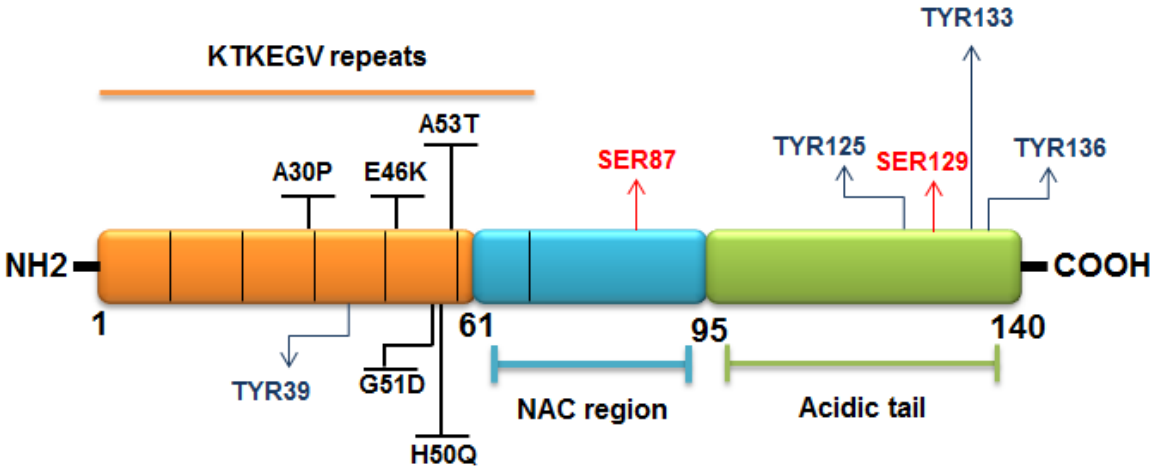

Figure 1 - Biochemical structure of α-Syn. A schematic representation of α-Syn structure highlighting: the N-terminal region (orange), the NAC region (light blue), the C-terminal domain (green), the genetic mutations (bold), and the serine (red) and tyrosine (dark blue) sites of phosphorylation. Adapted from Basso 2014.

α-Syn is ubiquitously expressed being present in tissues like muscle, kidney, liver, lung, heart, testis, blood vessels, cerebrospinal fluid, blood plasma, platelets, lymphocytes, and red blood cells. Additionally, this protein is highly expressed in the nervous system, particularly in some parts of the brain like the neocortex, hippocampus, striatum, thalamus, and cerebellum. In neurons, although present throughout the cell, α-Syn is enriched in the presynaptic terminals (Auluck, Caraveo et al. 2010). Many studies show that α-Syn localizes to and binds to mitochondria (Devi, Raghavendran et al. 2008, Nakamura, Nemani et al. 2008, Liu, Zhang et al. 2009). Overexpression of α-Syn in mouse midbrain neurons, causes mitochondrial dysfunction, promoting mitochondrial fission, that consequently leads to mitochondrial fragmentation (Nakamura, Nemani et al. 2011). In transgenic mice overexpressing A53T α-Syn there is mitochondrial degeneration, and the overexpression of A53T α-Syn in primary neurons increases mitophagy (Martin, Pan et al. 2006, Choubey, Safiulina et al. 2011). α-Syn nuclear localization is controversial, since the reports of its nuclear localization have not been consistent (Mori, Tanji et al. 2002, Yu, Li et al. 2007). However, α-Syn inhibits histone acetylation in the nucleus and histone deacetylase inhibitors were able to rescue neurotoxicity caused by α-Syn in SH-SY5Y cells and in transgenic *Drosophila* (Kontopoulos, Parvin et al. 2006). The association of α-Syn to the cytoskeleton is predicted by the *in vitro* observation of the α-Syn interaction with multiple cytoskeleton components such as tubulin (Zhou, Huang et al. 2010), kinesin light chain, dynein heavy chain, septin-4 (Woods, Boettcher et al. 2007) and the microtubule-associated Tau (Jensen, Hager et al. 1999).

It is proposed that α-Syn is degraded by the ubiquitin proteasome pathway (UPS) and autophagy–lysosome system (Burre, Sharma et al. 2018). The proteasome inhibition leads to α-Syn accumulation and it is suggested that the proteasome is involved in C-terminal proteolytic cleavage of α-Syn. The UPS usually degrades proteins with shorter half-life (<10 hours), and several research studies show that α-Syn half-life is around 16 hours (Shin, Dawson et al. 2009). The proteins with longer half-lives are usually degraded by autophagic pathways within lysosomes and α-Syn can also be degraded by this pathway. α-Syn degradation *via* autophagy–lysosome system occurs by the translocation of wild-type (WT) α-Syn to the lysosome, and mutant forms of α-Syn are resistant to this pathway (Xilouri, Vogiatzi et al. 2009).

1.2 Synuclein physiological function

α-Syn is widely expressed in the nervous system and in different types of neurons, what suggests a general role for the protein in neuronal function. In neurons, this protein is mainly located in the presynaptic terminals, being relatively absent in the cell body and in the dendrites/neurites (Burre, Sharma et al. 2018). However, α-Syn only localizes to the synapses in later stages of neuronal development, suggesting that the protein is not crucial for the formation of this structure, but may be involved in the maintenance and function of synaptic structures after they are formed (Bendor, Logan et al. 2013). α-Syn has affinity to membranes, being a natively unfolded structure when free on the cytosol and acquiring a α-helical structure when bound to membranes. Under physiologic conditions there is an equilibrium between these two structures. Moreover, it was also described that α-Syn can arrange itself in a soluble tetrameric form or oligomerize into multimers when bound to membranes (Marques and Outeiro 2012). Although α-Syn does not contain a typical transmembrane domain or a classical lipid anchor, its interaction with membranes can be explained by the N-terminal sequence. However, this interaction of α-Syn with membranes is weak. Despite this, α-Syn is enriched in neuronal termini bound to synaptic vesicles and it has high preference for highly curved membranes, such as synaptic vesicles (Varkey, Isas et al. 2010). In addition, α-Syn not only interacts with the synaptic vesicles but also with lipid rafts, showing that this protein can be important in neurotransmitter release and cell signaling (Fortin, Troyer et al. 2004).

The binding of α-Syn to phospholipids suggests a role in lipid transport (Sharon, Goldberg et al. 2001). α-Syn binds to fatty acids and it is suggested to act as a fatty acid transporter between the cytosol and membrane compartments (Lucke, Gantz et al. 2006). Furthermore, α-Syn has also an important role in the inhibition of phospholipases D1 and D2, suggesting its involvement in the cleavage of membrane lipids and membrane biogenesis (Burre, Sharma et al. 2018). α-Syn also plays an important role in dopamine synthesis and transport by the inhibition of the expression and activity of Tyrosine hydroxylase (TH) (Burre, Sharma et al. 2018). It is suggested that this inhibition results from the reduction of phosphorylation state of TH and by the stabilization of dephosphorylated inactive TH. Moreover, the increase of α-Syn expression in the *substancia nigra* negatively correlates with the expression of TH (Chu and Kordower 2007).

α-Syn is involved in vesicle trafficking by regulation of the interaction of Rab GTPases. Overexpression of the protein causes formation of cytoplasmic lipid droplets and vesicle formation by blocking trafficking from endoplasmic reticulum to Golgi. This is caused by the aggregation of several Rab GTPase proteins which is induced by α-Syn (Marques and Outeiro 2012). These proteins are involved in several mechanisms that regulate organelles trafficking such as formation, transport, tethering and fusion of transport vesicles (Hutagalung and Novick 2011). Additionally, α-Syn can also modulate SNARE-complex assembly at neuronal synapses. SNAREs in association with other proteins are involved in exocytosis and neurosecretion, playing an important role in vesicle docking, priming, fusion and synchronization of neurotransmitter release (Ramakrishnan, Drescher et al. 2012). α-Syn at the presynaptic terminal interacts with the vesicle-associated SNARE (v-SNARE) and membrane-associated SNARE (t-SNARE) proteins forming complexes involved in neurotransmitter release (Figure 2). Aggregation of α-Syn induces a destabilization in the formation of these protein complexes having an impact on processes such as neurotransmitter release and vesicle recycling (Lashuel, Overk et al. 2013). As α-Syn is involved in the regulation of Rab GTPases and SNARE proteins, it plays roles in both, neurotransmitter release and maintenance of synaptic vesicles.

In summary, the proper balance of α-Syn synthesis, aggregation and degradation is necessary for the normal function of this protein and a disruption of these mechanisms can lead to pathological effects and neurotoxicity.

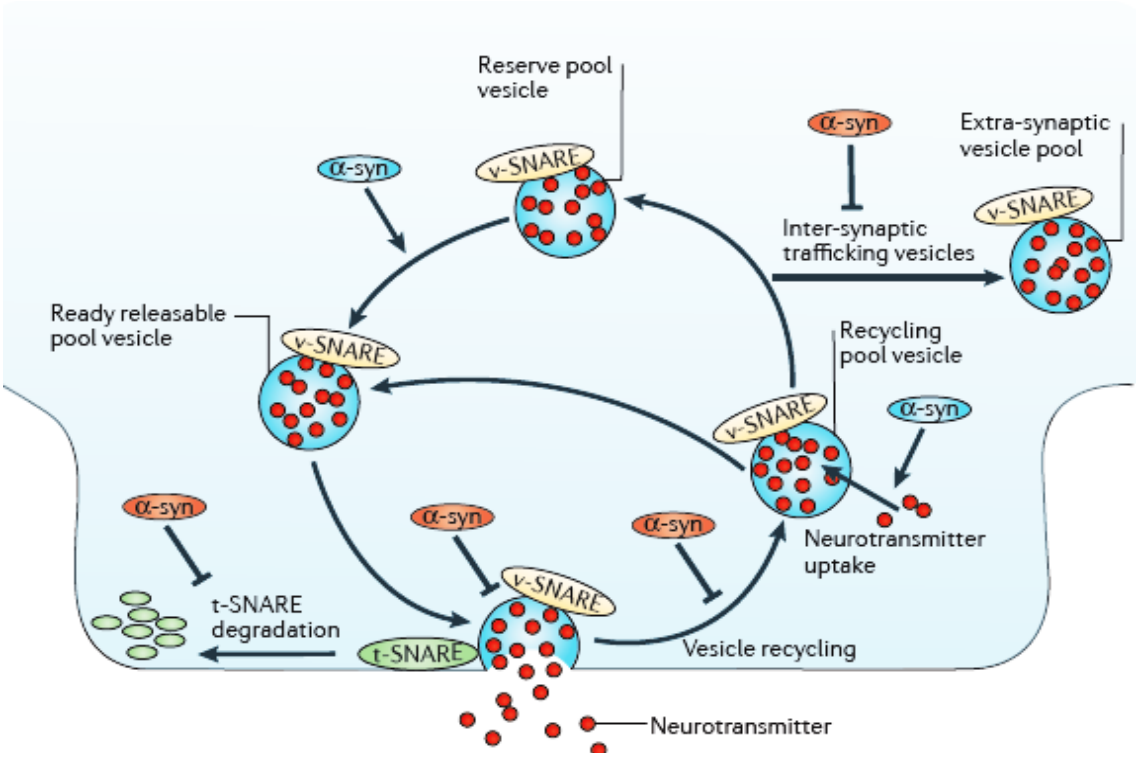

Figure 2 - Representation of the different pathways involved in the regulation of vesicle trafficking and vesicle refilling by the interaction between the v-SNARE and t-SNARE proteins and α-Syn. α-Syn in physiological conditions is represented in blue and α-Syn in pathological conditions in red. Adapted from (*Lashuel, Overk et al. 2013*)

1.3 Synuclein aggregation

The deposition of aggregated α-Syn intracellularly is well known to cause cell toxicity. As referred above, α-Syn is a highly dynamic protein, since it can be in a cytosolic soluble unfolded form or in a α-helical membrane-bound state acquiring a tetrameric form or oligomerize into

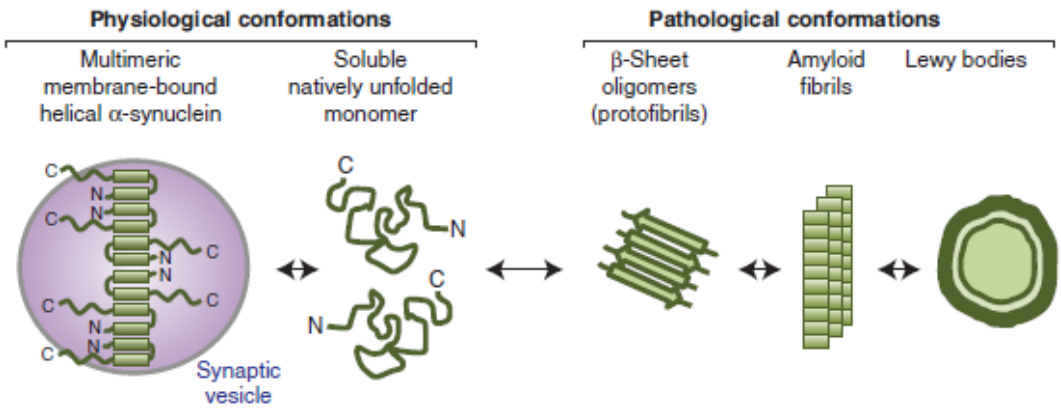

Figure 3 - Physiological and pathological conformations of α-Syn. Adapted from (*Burre, Sharma et al. 2018*)

multimers. In pathological conditions α-Syn might exist in intermediate states of fibrillization (Giasson, Uryu et al. 1999, Conway, Harper et al. 2000). The protein forms oligomeric species that gradually generate β-sheet-like oligomers (protofibrils), which aggregate leading to the formation of amyloid fibrils. The association of these amyloid fibrils culminate in the formation of insoluble aggregates named Lewy bodies which cause neurotoxicity (Figure3) (Lashuel, Overk et al. 2013). This aggregation process occurs by a nucleation-dependent mechanism, where first there is the formation of the oligomers structure (lag phase), and then a rapid growth phase with monomer addition, forming the fibrillar species (Figure 4) (Wood, Wypych et al. 1999).

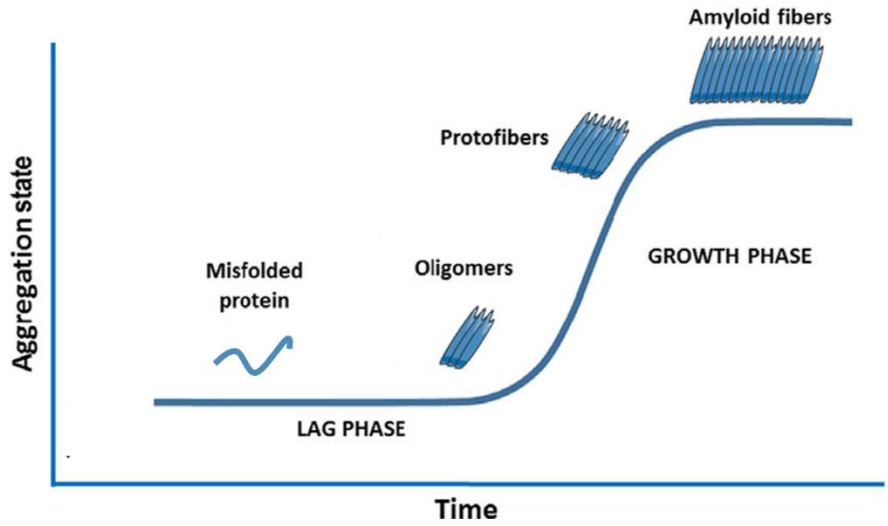

Figure 4 - Schematic representation of the kinetics of an amyloid fiber formation. This model involves the initial formation of oligomeric complexes that rapidly evolve into fibrils followed by amyloid formation. Adapted from (*Maiza, Chantepie et al. 2018*).

Additionally, it was shown that cells overexpressing human α-Syn lacking NAC domain (domain that is responsible for α-Syn aggregation) or overexpressing Heat shock proteins (HSPs), as protection mechanism for aggregation, exhibit decreased toxicity, highlighting the importance of the NAC domain and of protein aggregation neurotoxicity (Auluck, Chan et al. 2002, Qin, Hu et al. 2007). Furthermore, it was described that different fibrils can be formed from the same protein. So, these aggregates can present different structures, levels of toxicity and seeding and propagation characteristics (*in vitro* and *in vivo)* (Bousset, Pieri et al. 2013). This leads to a great difficulty in studying the impact of α-Syn aggregation in pathologic contexts, such as PD.

As referred above, recent studies have shown that α-Syn does not only exist intracellularly but is also present in biological fluids, such as cerebrospinal fluid and blood plasma. This opened a new field of study which suggests that α-Syn might behave as a prion-like protein causing the spread of the pathology throughout the brain, contributing to the progression of neurodegeneration (Marques and Outeiro 2012). It was already shown that aggregated forms of α-Syn can be transmitted from cell-to-cell and promote aggregate formation of the endogenous protein in the recipient cells, leading to the progression of the disease (Brundin, Melki et al. 2010).

1.4 Cellular models for studying α-Syn-mediated neurotoxicity

Cell-based models are widely used to study the mechanisms underlying neurodegeneration. In the case of PD, several cellular models were established to study α-Syn pathology, focused on understanding α-Syn aggregation and toxicity. In addition, these models provide exclusive opportunities to the identification of new therapeutic targets for PD (Lazaro, Pavlou et al. 2017).

The most common cell-based models in the study of PD and other synucleinopathies are the immortalized cell lines, which include: non-neuronal cells and cells which might be differentiated in neurons. The non-neuronal cell lines most commonly used are HEK-293 (human embryonic kidney 293) and H4 (human neuroglioma) (Delenclos, Burgess et al. 2019) and they are used to study α-Syn pathology, by the overexpression of WT α-Syn or by the overexpression of mutated forms of the protein, which are more prone to aggregate, such as A53T or A30P α-Syn (Lazaro, Rodrigues et al. 2014). Moreover, these cells are suitable to study the effects of toxins on α-Syn toxicity and for studies with co-expression with other proteins. Regarding cell lines with potential for neuronal differentiation, the most commonly used are PC12 (pheochromocytoma), LUHMES (mesencephalic) and SH-SY5Y (neuroblastoma) (Delenclos, Burgess et al. 2019). These cells can be differentiated into a particular neuronal subtype, which exhibit a dopaminergic phenotype, recapitulating the neuronal population more affected and studied in the context of PD. The most common differentiation agents used for PC12 and SH-SY5Y are retinoic acid (RA) and brain-derived neurotrophic factor (BDNF) (Xie, Hu et al. 2010). LUHMES cells are usually differentiated with dibutyryl cyclic adenosinemonophosphate and glial cell-derived neurotrophic factor (Scholz, Poltl et al. 2011). These cells are also used for the overexpression of WT and mutant α-Syn, and the consequences of α-Syn transmission for the neuronal function.

Besides immortalized cell lines, primary neurons from a specific brain-region prepared from embryonic or early post-natal mouse or rat pups are often used to study α-Syn pathology. These cell types are more physiologically significant and provide the opportunity to isolate neurons from a specific part of the brain, which can be more relevant to address specific questions (Delenclos, Burgess et al. 2019). The overexpression of α-Syn in primary mouse midbrain dopaminergic neurons is used as a model of PD to evaluate the levels of oxidative stress in this neurons which can be correlated with the loss of dopaminergic neurons that is characteristic of PD (Lieberman, Choi et al. 2017). Moreover, α-Syn aggregation and LBs formation were already described in primary hippocampal neurons, where are important for cognitive function (Henderson, Peng et al. 2018). Moreover, primary neuron cultures have been used for several studies including the process of α-Syn seeding with PFFs, the propagation of α-Syn between neurons, the consequences of WT and mutant α-Syn overexpression and the study of mutant α-Syn in neurons from transgenic animals (Volpicelli-Daley, Luk et al. 2014).

1.5 Synucleinopathies

Synucleinopathies are neurodegenerative diseases characterized by the accumulation of insoluble aggregates of α-Syn in neuronal or glial cells. The most common synucleinopathies include PD, Dementia with Lewy Bodies (DLB) and Multiple System Atrophy (MSA) (McCann, Stevens et al. 2014). These diseases are distinguished by the different localization and ultrastructure of the α-Syn aggregates. It is possible to observe three different types of α-Syn depositions: LBs, Lewy neurites (LNs) and glial cytoplasmic inclusions (GCIs). The first two are found specifically in PD and DLB while the GCIs are more present in MSA (McCann, Stevens et al. 2014).

LBs are well studied in the context of PD and DLB, composed by globular/spherical protein inclusions which range between 5-25μm in size (Spillantini, Crowther et al. 1998). The localization of LBs in neurons is confined to the cytoplasm and perinuclear compartment. LNs are similar to LBs, acquiring spindle-like structure in cellular processes (Spillantini, Crowther et al. 1998). The main constituent of LBs is fibrillar α-Syn, mostly phosphorylated in the S129 residue, however there are other molecules present, such as mitochondria-related proteins, and molecules implicated in the ubiquitin–proteasome system, in autophagy, and in aggresome formation. (Kuzuhara, Mori et al. 1988, Galvin, Lee et al. 1997, Engelender, Kaminsky et al. 1999, Fujiwara, Hasegawa et al. 2002). LBs are widely distributed in the central nervous system, including the olfactory bulb, hypothalamus,

posterior pituitary, nucleus basalis of Meynert, substantia nigra, locus coeruleus, dorsal raphe nucleus, dorsal vagal nucleus, cerebellum, hippocampus and spinal cord (Wakabayashi, Tanji et al. 2013, Adamowicz, Roy et al. 2017). LBs toxicity causes the loss of different neuronal types including dopaminergic, noradrenergic, serotonergic and cholinergic neurons (Marsden 1983, Gibb and Lees 1988).

From all the different types of synucleinopathies, PD is the most common and is the most diagnosed in clinical practice, being discussed in more detail in the following section.

2.Parkinson's disease

PD is a neurodegenerative disorder initially described in 1817 which although being recognized as affecting motor activity, also affects cognitive and behavioral functions. PD is the second most common neurodegenerative disease and affects about 10 million patients worldwide. Furthermore, it is expected that the number of affected individuals double by 2030 in the Western Europe's 5 most and the world's 10 most populous countries (Fredericks, Norton et al. 2017).

2.1 Parkinson´s disease: sporadic versus genetic forms

Although PD is caused by both multiple genetic and environmental factors, this disease is majorly sporadic and only approximately 10% of PD cases are derived from familial forms. Regarding the genetic causes, PD can develop from mutations or multiplications of different genes listed in the table 1.The *SNCA* gene encoding α-Syn and *LRRK2* gene (Leucine-rich repeat kinase) are two of the genes responsible for autosomal dominant inherited cases of PD. Autosomal recessive cases involving genes encoding for PRKN (Parkin), PINK-135 and DJ-1 were also reported (Spatola and Wider 2014). Almost all the genetic forms of PD present α-Syn pathology (Maiti, Manna et al. 2017).

Table 1 - Genes linked with familial forms of PD. (AD=autosomal dominant; AR= autosomal recessive; UR=unknown relevance). Adapted from Basso 2014.

Locus	Gene	Protein	Inheritance
PARK1/PARK4	SNCA	Alpha-synuclein	AD
PARK2	PARK2	Parkin	AR
PARK3	SPR(?)	(?)	AD (not validated)
PARK6	PINK1	Pten-induced kinase 1	AR
PARK7	PARK7	DJ1	AR
PARK8	LRRK2	Leucine-rich repeat kinase 2	AD
PARK9	ATP13A2	ATPase type 13A2	AR
PARK10	PARK10	(?)	UR
PARK11	GIGYF2 (?)	GRB10 interacting GYF protein 2	AR
PARK12	PARK12	(?)	UR
PARK13	Omi/HTRA2	HtrA serine peptidase 2	UR
PARK14	PLA2G6	Phospholipase A2, group VI	AR
PARK15	FBX07	F-box protein 7	AR
PARK16	PARK16	(?)	UR
PARK17	VPS35	Vacuolar protein sorting 35 Homolog	AD
PARK18	EIF4G1	Eukaryotic translation initiation factor 4 gamma, 1	AD (not validated)

The main cause of autosomal dominant inherited cases of PD are mutations or multiplications of the *SNCA* gene. The most common and well described α-Syn mutations that trigger autosomal dominant PD phenotype are A30P, E46K, H50Q, G51D and A53T and all these missense mutations are present at the N-terminal domain of the protein. These mutations affect not only the ability of this protein to bind lipids, but also its propensity to acquire a misfolded structure (George, Jin et al. 1995, Flagmeier, Meisl et al. 2016). Duplications and triplications of the *SNCA* gene are also responsible for PD and related with a severe phenotype of dementia aggravated during disease progression (Fuchs, Nilsson et al. 2007). These genetic causes are responsible for the α-Synuclein-induced pathology in PD and contribute for both motor and cognitive dysfunction in PD patients. (Scott, Tabarean et al. 2010).

Sporadic PD is caused by several factors, such as history of anxiety or depression, pesticide exposure, head injury, rural living, beta-blockers and farming occupation (Noyce, Bestwick et al. 2012). Although the exact influence of environmental factors in sporadic PD is not known, the influence of neuroinflammation, oxidative stress and α-Syn misfolding and aggregation have been identified as some elements of disease development.

Importantly, both genetic and sporadic PD are characterized by the deposition of α-Syn and formation of LBs. Considering this, the formation of LBs containing α-Syn are considered the hallmark of both forms of PD.

2.2 Parkinson's disease: typical motor symptomatology

PD major hallmarks are the formation of LBs, and the loss of dopaminergic neurons in the *substantia nigra*. This loss of dopaminergic neurons is responsible for the typical motor symptoms of the disease which constitute the most common approach to diagnose the disease (Parkkinen, O'Sullivan et al. 2011). Bradykinesia, that is responsible for slowness of initiation of voluntary movements, is one of the first symptoms observed. This causes a progressive reduction in speed and amplitude of repetitive actions (Hughes, Daniel et al. 1992). Beyond this, muscular rigidity, resting tremor or postural instability is also observed. With disease progression, more symptoms appear like speech disturbances and postural deformities (Hughes, Daniel et al. 1992). Dystonia, characterized by a sustained muscular contraction is frequently accompanied by abnormal movements and postures which may also appear later at the onset of the disease (Sigurlaug 2016).

2.3 Parkinson's disease: cognitive impairment and dementia

Besides motor symptoms, PD is also characterized by alterations in cognitive and behavioral functions. These non-motor symptoms include autonomic dysfunction, impaired sense of smell, gastrointestinal disturbances, and psychiatric symptoms such as sleep disturbances, depression, impulse control disorders, dementia, and psychosis (Fredericks, Norton et al. 2017).

Cognitive dysfunction and dementia are presently recognized as major complications in PD (Emre 2003). A fraction of patients display mild cognitive deficits in early PD, even before motor symptoms, and often those patients develop more severe cognitive problems and dementia later in the course of the disease (Janvin, Larsen et al. 2006). These patients are classified as having

Parkinson's disease with dementia (PDD). Recent studies following people with PD estimate that 50 to 80 percent may experience dementia at some stage of disease (Poewe, Gauthier et al. 2008)

Additionally, to PDD, DLB is also a synucleinopathy characterized by cognitive impairment and dementia. These two diseases have similar clinical features leading to difficulty in identifying and distinguishing between both. The most common method to distinguish is by the different temporal manifestation of the dementia (McKeith, Galasko et al. 1996). Patients are diagnosed with DLB if they develop dementia prior or during the first year of parkinsonism. If dementia appears at least one year after the onset of motor symptoms the patients are considered to have PDD (Meeus, Verstraeten et al. 2012). DLB has an estimated prevalence of 30% of all dementias and is the second most frequent neurodegenerative dementia after AD. In the case of PDD, as already referred, 50 to 80 percent of patients with PD develop dementia during the disease progression, representing 3.6% of all dementias (Poewe, Gauthier et al. 2008, Meeus, Verstraeten et al. 2012).

The most common clinical symptoms for both PDD and DLB are prominent abnormalities in attention, executive function, visuospatial function, language function, memory retrieval, and behavior (Watson and Leverenz 2010). However, there are some different clinical features which can help in the differentiation between PDD and DLB (Lippa, Duda et al. 2007). Regarding patients presenting DLB, these are more likely to make more conceptual and attentional errors, to have more hallucinations and psychoses and more adverse reactions to antipsychotic agents, when compared with patients presenting PDD. Even though the majority of DLB patients develop some features of parkinsonism, the PDD patients tend to have more prominent motor features (Lippa, Duda et al. 2007).

In PDD and DLB there is an early development of significant pathology in the hippocampus and surrounding cortical regions, which are involved in learning and memory (Squire 1992). As mentioned above, the formation of LBs is the pathological hallmark of synucleinopathies and in the case of DLB and PDD there is a substantial deposition of α-Syn and LBs formation in the hippocampus and cortex (Yang and Yu 2017).

2.3.1 α-Synuclein-induced hippocampal pathology

The hippocampus is a major component of the brain and is highly involved in the consolidation of new memories, emotional responses, navigation, and spatial orientation. Considering the important role of the hippocampus in cognition, the pathological effects of α-Syn in this region of the brain might have a significant impact in the cognitive deficits observed in synucleinopathies (Lavenex, Lavenex et al. 2007).

Several studies addressed the pathological effect of α-Syn in hippocampal neurons either by the α-Syn pathology caused by PFFs or by the overexpression of α-Syn. The addition of α-Syn PFFs (pre-formed fibrils that mediates the formation of α-Syn aggregates) to primary cultures of hippocampal neurons was shown to induce the formation of pathogenic inclusions containing α-Syn in neurons, leading to a reduction on synaptic activity and culminating in neuronal loss (Mahul-Mellier, Vercruysse et al. 2015). In addition, administration of PFFs to primary hippocampal neurons also leads to the propagation of pathological α-Syn from neuron-to-neuron (Wu, Takano et al. 2019). Besides PFFs, the overexpression of α-Syn is also involved in the hippocampal pathology, as already referred. In primary cultures of hippocampal neurons from a mouse model overexpressing human WT α-Syn under the promoter PDGF (Platelet-derived growth factor), it was observed a variation in the size of the vesicles, in particularly an enlargement, and decreased levels of several presynaptic proteins involved in exo- and endocytosis (Scott, Tabarean et al. 2010). Besides this, in the referred mouse model, it was described a decrease of several proteins involved in the synaptic vesicle machinery, inhibiting neurotransmitter release which eventually leads to synaptic dysfunction. The decrease in presynaptic proteins can be caused by the inhibition of the axonal transport, which affects the mobility of α-Syn and other co-transported cargoes to the synapse. Alternatively, the overexpression of α-Syn leads to aggregation of the protein at the synapse, hampering the target of other presynaptic proteins or altering the properties of proteins and vesicles located at the synapses (Scott, Tabarean et al. 2010). All these data suggest that α-Syn overexpression leads to dysfunction of neurotransmitter release and vesicle trafficking in hippocampal neurons, that can be related with the synaptic dysfunction and cognitive deficits in synucleinopathies (Scott, Tabarean et al. 2010). These pathological effects of α-Syn overexpression in primary hippocampal neurons resemble the pathological ones that are induced by Aβ in AD (independent of α-Syn), in which synaptic dysfunction precedes synapse loss (Froula, Henderson et al. 2018).

Another effect of the α-Syn-induced hippocampal neurodegeneration is the destabilization of actin cytoskeleton dynamics that might alter several cellular processes, including cell migration and exo-and endocytic trafficking. *In vitro* studies, using primary cultures of hippocampal neurons, showed that the overexpression of A30P mutant α-Syn leads to a dysregulation of the actin dynamics. This results in the depolymerization of actin filaments, which affects the cytoskeleton architecture impairing the neurite outgrowth and neuronal adhesion (Sousa, Bellani et al. 2009)

Regarding *in vivo* studies, several mouse models were generated in order to recapitulate the cognitive defects observed in PD and its correlation with the α-Syn-induced hippocampal pathology. In a mouse model overexpressing the A30P variant of α-Syn under the control of the murine Thy1 promoter it was demonstrated that the mice developed cognitive impairment correlated with the presence of α-Syn in the hippocampus (Freichel, Neumann et al. 2007). Another mouse model, overexpressing the WT α-Syn under the control of the Thy1 promoter, exhibited cognitive deficits at early age, similar to the non-motor symptoms characteristic of the preclinical stage of PD. In this mouse model, phosphorylated in Ser9 (PS129) α-Syn was increased and present in the cell soma and nucleus of the hippocampal and cortical neurons, but was nearly absent in nerve terminals, contrasting to what occurs in a WT mice (Schell, Hasegawa et al. 2009). This is usually associated with pathology, since PS129-α-Syn is highly present in LBs and its levels are significantly increased in PD. In a mouse line overexpressing WT α-Syn under CaMKII promoter, it was observed a reduction in the neurogenesis in the hippocampus resulting in cognitive defects (Nuber, Petrasch-Parwez et al. 2008). In mice overexpressing human WT α-Syn under the PDGF promoter it was also observed a reduction in hippocampal neurogenesis (Magen and Chesselet 2011).

Hippocampal pathology was reported in patients with synucleinopathies where manifestation of cognitive dysfunction correlated with hippocampal volume loss. As the hippocampal region is highly involved in cognition, the study of the mechanisms that are involved in this volume loss and its implications on the development of cognitive impairment and dementia are crucial for the better understanding of the disease progression (Yang and Yu 2017). Additionally, the presence of LBs in the hippocampus of patients with synucleinopathies was previously related with the cognitive impairment and hippocampal atrophy (Outeiro, Koss et al. 2019). In PD patients with long disease duration, the accumulation of α-Syn in cortical and limbic regions was shown to induce the development and severity of cognitive impairment. In addition, the observation of

parahippocampal LBs can help to distinguish demented and non-demented PD patients (Silbert and Kaye 2010). Furthermore, the observation of LBs in PD brains shows that α-Syn aggregates are located at the presynaptic sites and the dendritic spines are retracted, which can lead to neurotransmitter deprivation. This neurotransmitter deprivation can also be a consequence of the decreased number of synapses and spine densities in PD brains, which is correlated with the cognitive impairment, as already referred (Yang and Yu 2017).

In summary, the impairment of hippocampal function, induced by α-Syn aggregation, affects crucial mechanisms including spine morphogenesis and memory culminating in cognitive dysfunction and dementia (Aroniadou and Teyler 1991, Newcomer, Farber et al. 2000, Smith, Villalba et al. 2009, Mattison, Popovkina et al. 2014). All this suggests a major involvement of the hippocampal brain region in the progression of synucleinopathies and can explain the cognitive deficits observed in the different subtypes of the disease.

2.4 Neuroprotective agents as new therapies of PD

As already referred, the loss of dopaminergic neurons is a hallmark of PD, and there are several studies regarding the mechanisms underlying the loss of this specific type of neurons. However, there are limited treatment options for PD, and the most common neuroprotective agents target the oxidative stress, mitochondrial dysfunction, and inflammation (Seidl and Potashkin 2011). Caffeine and uric acid were already shown to be the neuroprotective agents and they were studied as a agents to decreases dopaminergic neuron toxicity in mice administrated with MPTP, paraquat and 6-OHDA (animal models of PD) (Seidl and Potashkin 2011). Levodopa is the most effective therapeutic approach for the motor dysfunction of PD. This drug is a dopamine precursor, and its action depends on its conversion to dopamine, with subsequent activation of dopamine receptors in dopaminergic neurons. With this, levodopa is able to act as a neuroprotector of dopaminergic neurons and to relief symptomatic motor dysfunction (Lewis, Huang et al. 2006). There are few neuroprotective agents used to target the cognitive defects of PD. Cyclosporin improved cognitive function in Thy1-α-Syn transgenic animals and administration of phenylbutyrate reduced deterioration in motor and cognitive functions in MPTP-treated animals. As such, this agents can be further tested to improve cognitive function in PD (Seidl and Potashkin 2011, Martinez and Peplow 2018).

The tripeptide glycine-proline-glutamate (GPE) and analogues are other promising neuroprotective agents and they were already shown to have potent neuroprotective effects in numerous animal models of neurodegenerative diseases such as PD, AD and Huntington's disease (Cacciatore, Cornacchia et al. 2012). GPE is a tripeptide naturally cleaved from the N-terminal tripeptide of insulin-like growth factor-1 in brain tissues by an acid protease and it has the ability to promote acetylcholine and dopamine release from neurons. GPE was already shown to act as a neuronal rescue agent in animal models of PD, preventing the loss of dopaminergic neurons in rats treated with 6-OHDA (Guan, Krishnamurthi et al. 2000). In addition, GPE analogues decreased neurotoxicity and acted as antioxidants against 6-OHDA-induced neurotoxicity in SH-SY5Y cells (Cacciatore, Baldassarre et al. 2012). However, GPE is easily degraded by enzymatic hydrolysis and has difficulty to cross cell membranes, such as the blood–brain barrier, limiting this way its therapeutic potential (Cacciatore, Cornacchia et al. 2012). Because of this unfavorable biochemical and pharmacokinetic properties new analogues were designed in order to increase its viability and its ability to cross cell membranes. Thus, the search for new analogues with better structure-activity relationship can be for the finding of new neuroprotective agents for PD.

3. Cofilin pathology in neurodegeneration

Structures that have been described as being involved in the synaptic dysfunction of hippocampal neurons are the cofilin-actin rods, which are composed by actin and cofilin. These structures were reported to occur upon different neurodegenerative stimuli, such as Aβ oligomers that are majorly involved in the progress of AD, a disorder characterized by cognitive impairment and dementia (Maloney and Bamburg 2007).

3.1. Actin

Actin is the major ubiquitous protein present in eukaryotic cells and is involved in several biological processes such as determination of the cell shape, cytokinesis, cell motility and cell-cell/cell-matrix interactions (Small, Rottner et al. 1999). Actin is present in the cells in two different forms: a monomeric form globular actin (G-actin) and a polymeric form designed filamentous actin (F-actin) (Reisler and Egelman 2007). F-actin is a dynamic polymer in which G-actin monomers are assembled and disassembled with different rates at barbed end (fast growing) and minus end (slow growing) (Atkinson, Hosford et al. 2004). F-actin and G-actin are present in equilibrium in the cell, and this dynamic state is controlled by different factors and mediators including the actin-binding proteins (ABPs). There are several types of ABPs with different isoforms and functions, and they are involved in different cellular functions: 1) provide actin monomers for the assembly of F-actin (profilin); 2) regulate the state of polymerization of filaments (ADF/cofilin, profilin) (figure 5); 3) bind to the growing ends of F-actin and block polymerization (gelsolin); 4) promote nucleation and formation of new actin polymer (gelsolin, Arp2/3, cofilin), 5) sever actin filaments (gelsolin,

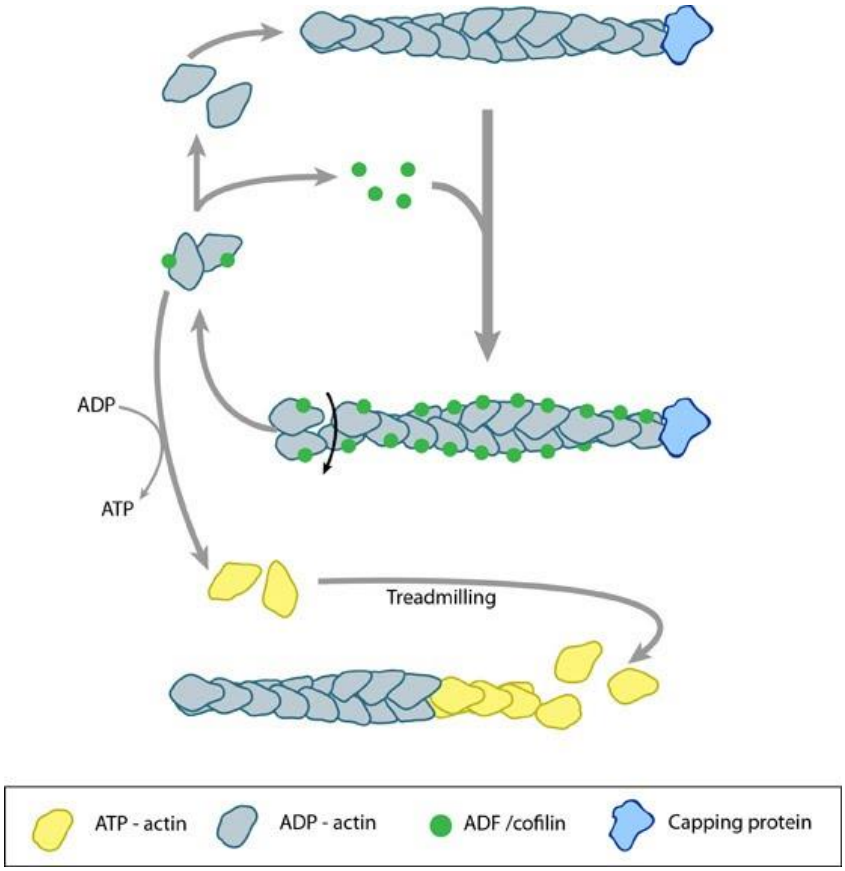

Figure 5 - Schematic representation of the filamentous actin nucleation and severing and the involvement of ADF/cofilin in this process. Adapted from MBInfo 2018.

ADF/cofilin), 6) bind to the sides of actin filaments (gelsolin, Arp2/3), and 7) cross-link actin filaments (Arp2/3) (dos Remedios, Chhabra et al. 2003).

3.2 Cofilin

ADF/Cofilin proteins belong to the actin depolymerizing family of proteins that are present in all eukaryotes (figure 5). In mammals there are three different types of these proteins: ADF, cofilin-1 and cofilin-2. Cofilin-1 is the more abundant form in non-muscle tissues and is highly expressed in the brain and cofilin-2 is the major form in differentiated muscle tissue. ADF is similar to cofilin in the regulation of actin dynamics activity, but they have different ratios of their binding to actin (Hotulainen, Paunola et al. 2005, Bamburg and Bernstein 2008). Although cofilin is widely distributed in the cytoplasm, its active form is more present in the regions where actin cytoskeleton is highly dynamic, such as the cleavage site of dividing cells and formation of neuronal growth cones (dos Remedios, Chhabra et al. 2003). Since cofilin-1 is the most abundant form in the brain, it may play an important role in the regulation of actin dynamics in neuronal function and as such will be the focus hereafter. The actin dynamics regulation is highly dependent on the concentrations of cofilin-1. When cofilin-1 is present at very low concentrations it acts as an actin stabilizer, at medium concentrations as a severing protein and at high concentrations as an actin nucleation protein (Andrianantoandro and Pollard 2006).

Cofilin-1 regulation is mediated by phosphorylation/dephosphorylation (Figure 6). Phosphorylation of the serine 3 (Ser3) residue leads to the inhibition of its binding to actin (Moriyama, Iida et al. 1996). There are four kinases that phosphorylate cofilin-1: LIM kinase -1 and LIM kinase -2 (LIMK1, LIMK2), TES kinase 1 and TES kinase 2 (TESK1, TESK2). LIM kinase is regulated by Rho kinase (ROCK) and p21-activated kinase (Pak1) proteins in a signaling cascade regulated upstream by Rho GTPases family (Edwards, Sanders et al. 1999).

The activation of cofilin-1 by dephosphorylation of its Ser3 residue is performed mainly by Slingshot phosphatase 1 (SSH1) and chronophin (CIN) (Niwa, Nagata-Ohashi et al. 2002). SSH1 is regulated by 14-3-3 proteins, that sequester phosphorylated SSH1 preventing its activation. Protein kinase D is responsible for the phosphorylation (inhibition) at Ser978 of SSH1 (Nagata-Ohashi, Ohta et al. 2004). The dephosphorylation (activation) of SSH1 is performed by calcineurin and it is calcium dependent (Wang, Shibasaki et al. 2005).

In addition, cofilin-1 can be regulated by other mechanisms that are independent of phosphorylation, which include the interaction of its actin binding domain to membrane phosphoinositides promoting inactivation of cofilin-1 (Yonezawa, Nishida et al. 1990). Oxidation is also important in cofilin-1 regulation, mainly induced by ROS formation. ROS can lead to dephosphorylation and consequently activation of cofilin-1, by oxidation of 14-3-3ζ preventing the sequestration of SSH1 (Kim, Huang et al. 2009).

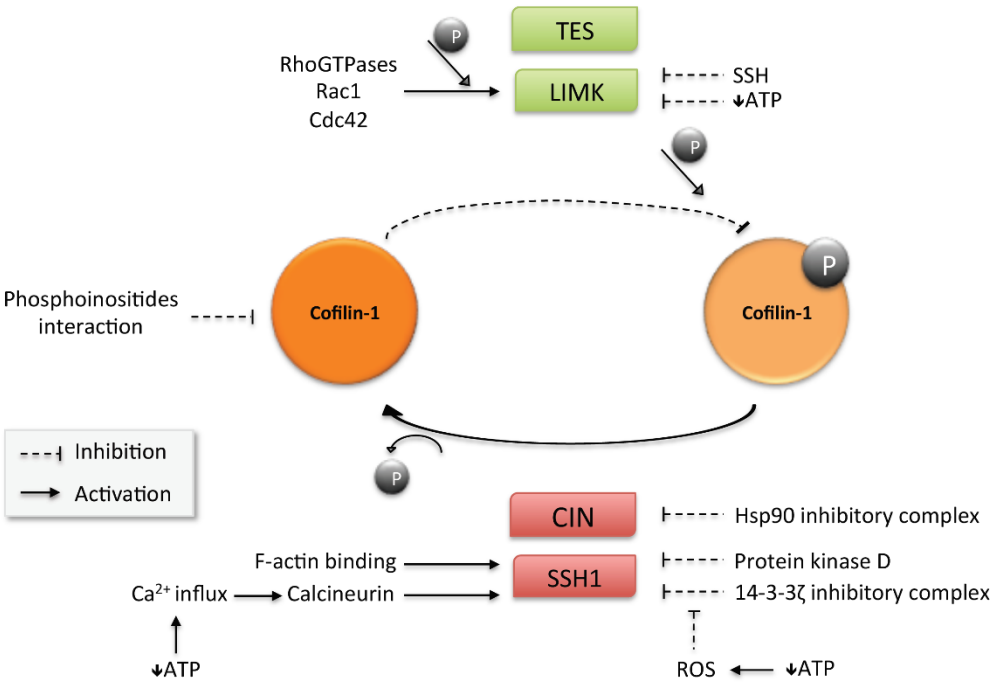

Figure 6 - Summary of the pathways involved in the regulation of cofilin activity discussed in the text. Adapted from Bamburg, Bernstein et al. 2010.

3.3 Cofilin-actin rods

When cofilin-1 activity is dysregulated, it can lead to pathological outcomes, such as the formation of cofilin-actin rods (Figure 7). These structures are formed by the hyperactivation of

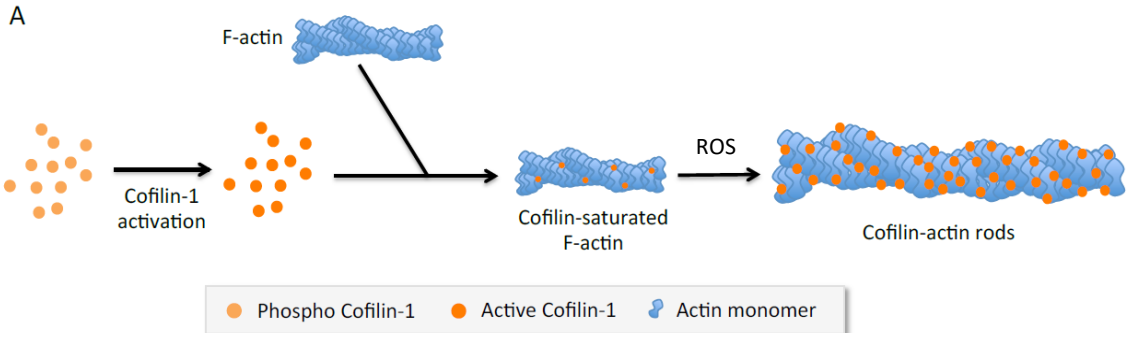

Figure 7 - Representation of cofilin-actin rods formation

cofilin-1 which leads to its saturated binding to actin filaments. Therefore, cofilin-actin rods are structures that are reorganized into rod shaped inclusions in neurons composed by cofilin and actin (Minamide, Striegl et al. 2000). The hyperactivation of cofilin-1 and the presence of ROS constitute the ideal conditions of rod formation (Figure 7) (Minamide, Striegl et al. 2000, Bernstein, Shaw et al. 2012). Even though rods are formed under stress conditions, their formation may have a temporarily neuroprotective effect, since its formation leads to a slower decline in ATP levels. This happens by the sequestration of cofilin-1 in the rods, decreasing this way the actin turnover which is a highly energetic process (Bernstein, Chen et al. 2006).

As cofilin-1 protein is widely expressed in the brain, the formation of these structures is particularly relevant in that region and can lead to neurodegeneration. Indeed, when these rods persist for long periods, they can cause neurotoxicity, affecting different functions, including disruption of microtubule integrity, synaptic deficits and block of intracellular trafficking, which leads to loss of dendritic spines (Cichon, Sun et al. 2012).

There are different pathways that can lead to rod formation, which include mitochondrial dysregulation (ATP-depletion), oxidative stress, high levels of glutamate, extracellular ATP and exposure to Aβ oligomers. This leads to an implication of the cofilin-actin rods in AD (Davis, Marsden et al. 2011).

The several signaling pathways that mediate rods formation are, in general, associated with the dephosphorylation of cofilin-1. One pathway is activated in response to excitotoxic levels of glutamate which leads to activation of AMPA receptors, resulting in ATP depletion and consequent Hsp90 inhibition. This pathway leads to CIN activation and consequently dephosphorylation and activation of cofilin-1 (Brennan, Suh et al. 2009, Bernstein, Shaw et al. 2012, Walsh, Minamide et al. 2014). Calcium influx is also involved in rod formation. In response to high levels of calcium, the calmodulin/calcineurin pathway is activated, leading to the dephosphorylation of SSH1, and consequent cofilin-1 activation. SSH1 is also activated is response to peroxide which induces oxidation of 14-3-3ζ. The oxidized 14-3-3ζ is released and SSH1 becomes available to activate cofilin-1 (Kim, Huang et al. 2009).

The other pathways are dependent of PrP^c and the activity of NADPH oxidase (NOX) which is activated by proinflammatory cytokines and Aβ. PrP^c is a glycoprotein that is present in cell membranes and its physiological function remains poorly understood (Walsh, Minamide et al. 2014).

The overexpression of PrP^C in hippocampal neurons, in the absence of other rod-inducing factors, leads to rod formation in 50% of neurons in a NOX-dependent pathway (Walsh, Minamide et al. 2014). RanBP9 is a scaffolding protein that is also involved in rods formation induced by Aβ, and it has an increased expression in AD brains and mouse models for AD (Lakshmana, Chung et al. 2010). This protein increases cofilin-1 dephosphorylation by the binding of β1-integrin and SSH1. In addition, Aβ oligomers bind to β1-integrins in a PrP^C-dependent manner, enhancing ROS production (Walsh, Minamide et al. 2014, Woo, Boggess et al. 2015). The cofilin-1 activation and ROS formation increase the formation of the cofilin-actin rods suggesting the involvement of Aβ and RanBP9 in the formation of these structures in AD (Figure 8). The regulation of Rho GTPases pathway is also involved in rods formation. Pak1 and LIMK are activated by Rho GTPases pathway leading to cofilin-1 phosphorylation (inactivation)(Mendoza-Naranjo, Contreras-Vallejos et al. 2012).

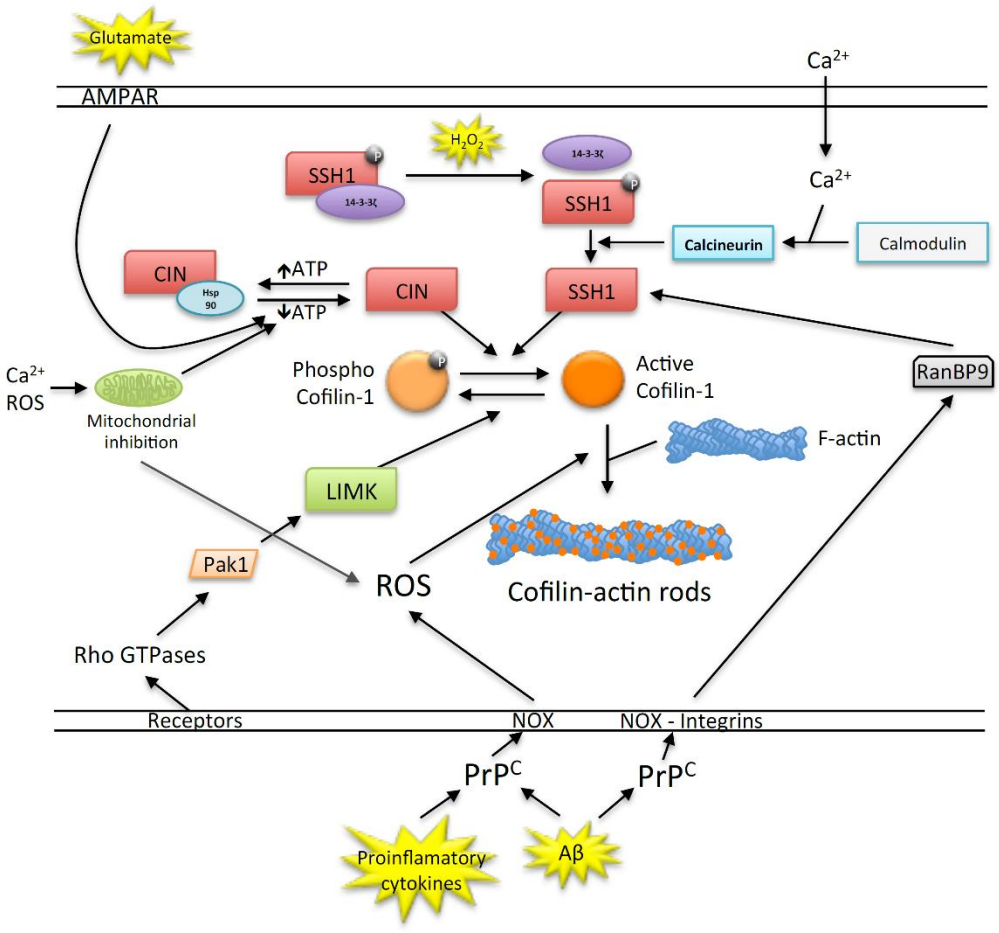

Figure 8 - Schematic of abbreviated signaling pathways contributing to rod formation adapted from Bamburg, Bernstein et al. 2010.

3.4 Cofilin pathology and neurodegeneration

Cofilin-1 pathology was mainly addressed in the context of AD. AD is a neurodegenerative disorder mainly acknowledged by cognitive impairment and dementia and is characterized by the deposition of Aβ aggregates and hyperphosphorylated Tau protein, which are present in extracellular senile plaques and in intracellular neurofibrillary tangles, respectively (Haroutunian, Perl et al. 1998, Haroutunian, Purohit et al. 1999). The cognitive defects are caused by the accumulation of these proteins in the hippocampus and cerebral cortex, regions that are highly involved in memory and cognition.

The formation of cofilin-actin rods has been reported in both familial and sporadic cases of AD. In familial AD rods are induced by the Aβ-mediated pathways involved in the activation of cofilin-1, due to the presence of high levels of Aβ. In sporadic AD, cofilin-actin rods are formed by Aβ or the presence of stress conditions, namely oxidative stress (Bamburg, Bernstein et al. 2010). The accumulation of cofilin-actin rods results in the formation of axonal swellings containing organelles, vesicles and microtubule-associated proteins including motor molecules (Lahiri, Ge et al. 2005). The inhibition of the intracellular trafficking is associated with the earliest defects observed in a transgenic mouse model for AD expressing human mutant amyloid precursor protein (APP). It is also suggested that the inhibition of retrograde transport of APP to the lysosomes can occur precluding the degradation of APP, promoting an increased accumulation and secretion of Aβ (Bamburg, Bernstein et al. 2010). Therefore, cofilin-actin rods formation can have an important role in the decline of neuronal function in AD and can also be responsible for the progression of the disease.

Cofilin-actin rods were also reported in the context of ischemic stroke (Shu, Chen et al. 2018). Ischemic stroke is a major cause of human death worldwide and is characterized by acute cerebral blood flow reduction induced by the blood vessel occlusion or cardiac arrest (Lo, Dalkara et al. 2003, Donnan, Fisher et al. 2008). This causes neuronal death in the infarct area and affects the structure and function of the surviving neurons in the surrounding peri-infarct area. These surviving neurons are mainly affected in their dendritic structure, causing spine loss (Kamal, Sheng et al. 2017). These structural effects on dendritic spines are related with the formation of cofilin-actin rods (Shu, Chen et al. 2018). In ischemic stroke, the formation of rods is related to the occurring energy depletion and excitotoxicity (Shu, Chen et al. 2018). Considering this, rod formation upon

ischemic stroke, may also have an important role for the symptoms of this disease and can be a target for new therapeutic approaches in ischemic stroke.

Besides AD and ischemic stroke, the dysregulation of cofilin-1 activity was also reported in PD (Lim, Kawamura et al. 2007) and it was previously shown an interaction of α-Syn with actin in cell-based models (Bras, Lopes et al. 2018). Since α-Syn pathology in the hippocampus is associated with dementia in PD, we hypothesized that α-Syn could be involved in the formation of cofilin-actin rods in hippocampal neurons, resulting in synaptic dysfunction and cognitive deficits observed in PD. Supporting this hypothesis, preliminary results from our group showed that α-syn oligomers induce the formation of cofilin-actin rods in primary cultures of hippocampal neurons (Figure 9). Considering those observations, the aim of this master thesis is to further investigate α-Syn-induced cofilin-actin rods formation in hippocampal neurons.

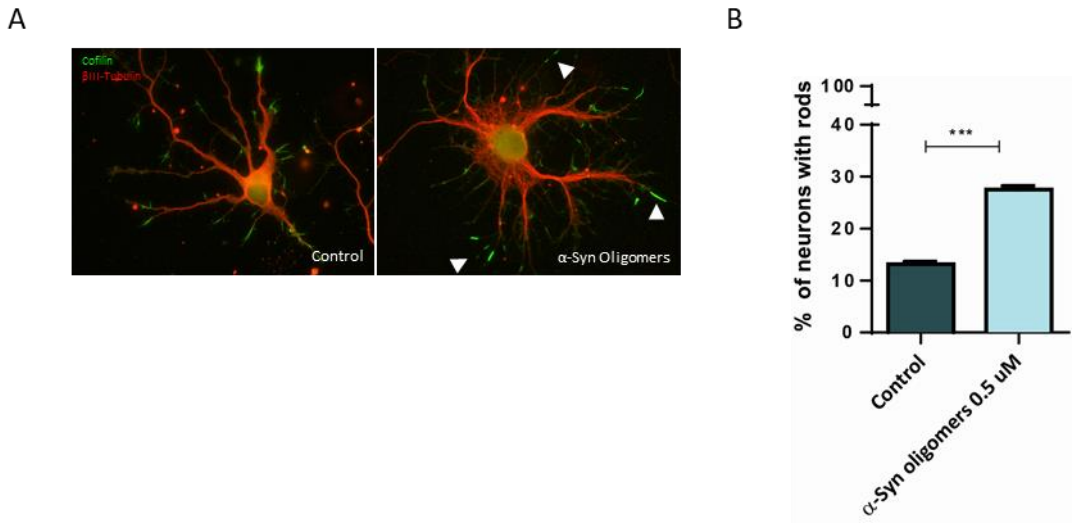

Figure 9 - **α-Syn oligomers induce rod formation**. A- Hippocampal neurons treated with α-Syn oligomers and immunostainned for cofilin. B- Quantification of the percentage of neurons with cofilin-actin rods.

2. OBJECTIVES

The primary goal of this study was to validate α-Syn-induced rod formation specifically in hippocampal neurons as well as the mechanisms involved in cofilin-actin rod formation, aiming at determining if cofilin-1 constitutes a therapeutic target for cognitive impairment in PD. To achieve the proposed aims, we established the following tasks:

- Set up the cell-based system to analyze α-Syn-induced rod formation in hippocampal neurons.

- Investigate the molecular pathways underlying α-Syn -induced rod formation.

- Assess the impact of cofilin-1 activity modulation in rod formation.

- Assess rod formation in other models relevant for PD

- Determine new possible therapies for PD

3. MATERIALS AND METHODS

3.1. Expression constructs and Lentiviral production

HBA-α-Syn-IRES-GFP and HBA-IRES-GFP plasmids were kindly provided by Tiago Outeiro (University Medical Center Göttingen, Göttingen). HBA-α-Syn-IRES-GFP is composed by a human beta actin promoter, followed by human α-Syn sequence, an Internal ribosome entry site (IRES) sequence and Enhanced Green Fluorescent Protein (GFP) sequence. The HBA-IRES-GFP is similar but it does not contain human α-Syn sequence (Figure 10 A and A`).

The CMV-α-Syn-IRES-GFP and CMV-Puro-IRES-GFP vector builder plasmids were constructed following our specifications (VectorBuilder Inc., Chicago, USA). CMV-α-Syn-IRES-GFP is composed by the cytomegalovirus promoter (CMV promoter), followed by human α-Syn sequence, an IRES sequence and Enhanced Green Fluorescent Protein (GFP) sequence. The CMV-Puro-IRES-GFP is similar but in the place for α-Syn sequence, it has the sequence for puromycin resistance in order to the distance between the promoter and the sequence of GFP be similar in both plasmids (Figure 10 B and B`). For the expression of the capsid and package proteins for the construction of lentiviruses VSVG and pPAX plasmids were used.

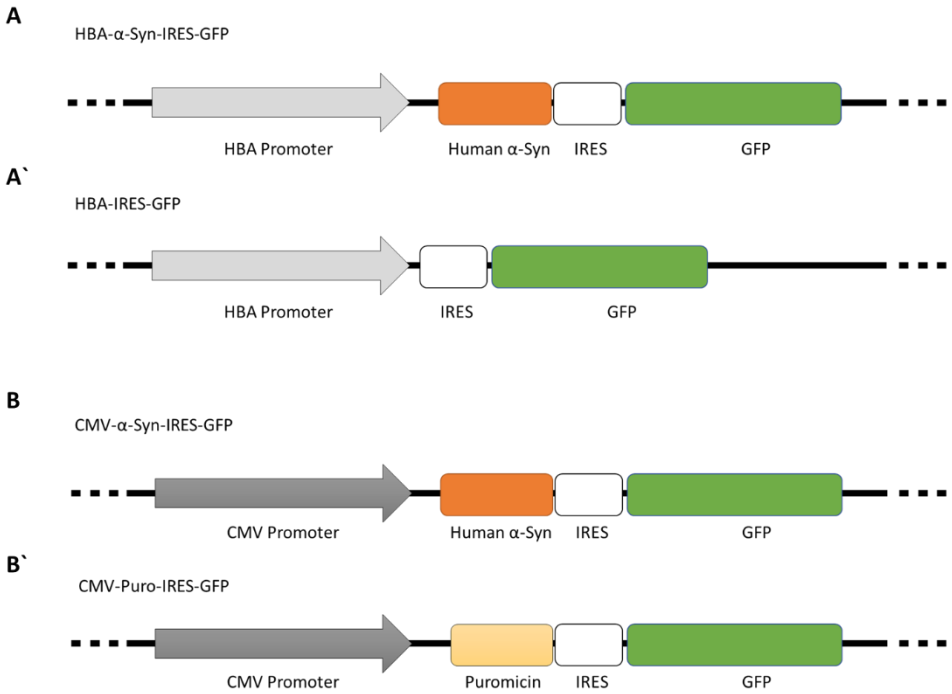

Figure 10 - Map of the plasmids used for lentivirus production. In A and A` are represented the plasmids HBA-α-Syn-IRES-GFP and HBA -IRES-GFP, respectively. A is composed by HBA promoter, followed by α-Syn sequence, an IRES sequence and GFP sequence. A` is similar but it doesn't have α-Syn sequence. In B and B` are represented by CMV-Syn-IRES-GFP and CMV-α-Puro-IRES-GFP respectively. B is composed by a CMV promoter, followed by α-Syn sequence, an IRES sequence and GFP sequence. B` is similar but in the place for α-Syn sequence, it has the sequence for Puro resistance.

For lentivirus production, Human Embryonic Kidney 293T cells (HEK 293T) were used. Initially HEK 293T cells were plated at a density of $4x10^6$ cells/dish in a petri dish coated with 0.1% gelatin from porcine skin (Sigma-Aldrich), and cultured in Dulbecco's Modified Eagle Medium (DMEM, VWR) with 10% Fetal Bovine Serum (FBS) and 1% Penicillin-Streptomycin (P/S, ThermoFisher) overnight. For transfection, performed with lipofectamine2000 (Invitrogen), cells were incubated in DMEM with 1% P/S. DNA complexes were formed by incubating 6ug of the plasmid of interest, 3ug of pPAX and 3ug of VSVG in 250ul of Optimem with 48ul of Lipofectamine2000 in 250ul Optimem, for 30 min at room temperature (RT). Then, complexes were added to the cells, cultured in DMEM with 1% P/S, dropwise and incubated for 5h at 37ºC. After the incubation, medium was replaced to DMEM, 10% FBS and 1% P/S. After 48h, the supernatants were recovered, centrifuged for 10 min at 500g and filtered using a 0.45um filter (Enzifarma). The filtered supernatants were concentrated using a centricon (GE Healthcare Life Sciences) and kept at -80 ºC.

For virus titration, HEKS 293T were plated at a density of 50.000 cells/well in a 6-well plate, in DMEM medium with 10% FBS and 1% P/S. Lentivirus were added to the wells in the following dilutions: 1:400, 1:800, 1:2000, 1:4000, 1:10000, 1:100000. Cells were cultured for 3 days and then resuspended with Trypsin 0,05% Ethylenediamine tetraacetic acid (EDTA, Sigma-Aldrich) for 3 min at 37ºC. Cell suspensions were centrifugated at 1200 rpm for 5 min. The supernatants were discarded, and cells were resuspended in phosphate buffered saline (PBS). The total number of transduced cells was analyzed by Flow Cytometry using FACS Accuri (BD Biosciences). The transfection units (TU) per ul were determined by the following equation:

$$TU/uL = \frac{number\ of\ cells * \%\ of\ infected\ cells\ (GFP\ positive)}{volume\ of\ viral\ particles\ added\ (uL)}$$

3.2. Hippocampal neuronal cultures

3.2.1 Primary hippocampal neuron cultures

Primary cultures of rat hippocampal neurons were prepared from embryonic day 18 Wistar rat embryos. Collected hippocampus were digested in 0.06% trypsin (Sigma-Aldrich) in Hanks' Balanced Salt Solution (HBSS, Sigma-Aldrich) at 37ºC for 15 minutes. The digestion was stopped

with 10%FBS (Invitrogen) in HBSS. Cells were mechanically dissociated and resuspended in neurobasal medium (Invitrogen) with 10%FBS, 2% N21 (R&D - Citomed), 0.25% L-Glutamine (Invitrogen) and 1% P/S. Cells were counted and plated in coverslips coated with 0.02mg/mL of poly-D-lysine (Sigma-Aldrich). After 2h, the medium was replaced by neurobasal, 2% N21, 0.25% L-Glutamine and 1% P/S and cells were maintained at 37ºC with 5% CO_2 until further use.

3.2.2 Rat hippocampal neurons viral transduction

Rat hippocampal neurons were cultured in a 24-well plate at a density of 15.000 cells/well. Three days after platting cells were treated with 1uM of (+)-MK 801 maleate (Sigma-Aldrich) for 30min at 37ºC to inhibit spontaneous rod formation. Infection was performed at DIV 4 with either HBA-α-Syn-IRES-GFP and HBA-IRES-GFP lentiviruses or CMV-α-Syn-IRES-GFP and CMV-Puro-IRES-GFP lentiviruses (15000TU). Cell culture supernatants were collected (for dot blot analysis) at DIV 7 or DIV 14, and cells were fixed with 4% of paraformaldehyde (PFA) in cytoskeleton preservation buffer (10 mM 2-(N-morpholino) ethanesulfonic acid (MES, Sigma-Aldrich) pH 6.1; 3 mM $MgCl_2$; 138 mM KCl; 2mM ethylene glycol-bis(β-aminoethyl ether)-N,N,N′,N′-tetraacetic acid (EGTA, Sigma-Aldrich); 0.32 M sucrose (VWR)), for 30 min at RT. Cells were then analyzed by immunocytochemistry.

3.2.3 Rat hippocampal neurons transfection

RFP-N1-Cofilin-S3E or RedTrack-CMV-NOX-DN plasmids were kindly provided by James Bamburg (Colorado State University, Colorado). Rat hippocampal neurons were cultured at a density of 15.000 cells/well in a 24 well plate. At DIV 3 cells were treated with 1uM of (+)-MK 801 maleate for 30min at 37ºC and at DIV5 were transfected with calcium phosphate co-precipitation with the constructs: HBA-α-Syn-IRES-GFP, HBA-IRES-GFP, RFP-N1-Cofilin-S3E, or RedTrack-CMV-NOX-DN. For the calcium phosphate co-precipitation, cell culture medium was recovered and replaced by neurobasal medium. DNA complexes were formed by incubating 2ug of the plasmids of interest in Tris-EDTA buffer (TE) pH 7.3 (10mM Tris-HCl pH 7.5; 0.5mM EDTA pH 8). Then 2mM of $CaCl_2$ in 10mM of 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid (HEPES, Invitrogen) was added dropwise and this mixture was added to HEBES buffer (0.3 M NaCl; 1mM KCl; 100mM Dextrose (Sigma-Aldrich); 40mM HEPES; 1.4 mM Na_2-HPO_4). The mixture was incubated for 30

min at RT to form the complexes. Then, the complexes were added to the cells and incubated for 45 min at 37°C. The medium was replaced by neurobasal medium acidified with 10% CO2 for 20min at 37°C. This acidic medium was then replaced with the culture medium recovered in the first step. The cell culture supernatant was collected (for dot blot analysis) at DIV 7 and cells fixed with 4% of PFA in Cytoskeleton preservation buffer for 30 min at RT. Cells were then analyzed by immunocytochemistry.

3.2.4 Rat hippocampal neurons treatments with N-Oxocarbamate (CN03) and ATP depletion

Primary cultures of hippocampal neurons were cultured at a density of 15.000 cells/well in a 24 well plate. At DIV 13 the culture medium were removed and replaced with medium containing 0.25ug/ml of CN03. At DIV 14 the culture medium was removed and replaced for ATP depletion solution (Sodium azide 10 mM (Sigma-Aldrich), 2-Deoxy-D-glucose 10 mM (Sigma-Aldrich) in PBS) and incubated for 30min at 37°C. Cells were then fixed with 4% of PFA in Cytoskeleton preservation buffer for 30 min at RT and analyzed by immunocytochemistry.

3.2.5 MTT test for cell viability in rat hippocampal neurons

The MTT test is based on the conversion of MTT to blue formazan crystals by viable cells. The MTT 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide) powder was dissolved in 0.1% of DMSO (Sigma) to a final concentration of 5 mg/ml.

Rat hippocampal neurons were plated at a density of 4000cells/well in a 96 well-plate. 24h after plating, 6-OHDA was added to the medium at a final concentration of 15uM. One day after, the culture medium was removed and 0.5mg/ml MTT in PBS was added per well and incubated for 3h at 37°C. Then, cells were centrifugated at 800g for 15 min and supernatant was removed. MTT crystals were solubilized with DMSO for 30 min at 37°C and absorbance was measured at 540nm using a BioTek™ Synergy™ Mx Monochromator-Based MultiMode Reader (BioTek).

3.3. SH-SY5Y Cell cultures

3.3.1 SH-SY5Y cells differentiation

SH-SY5Y cells were cultured in DMEM/F12 (Sigma-Aldrich), 10% FBS and 1% P/S. In the next day, the medium was replaced with DMEM/F12, 2% B27 (Invitrogen), 1% P/S, 10 μM all-trans-retinoic acid (Fisher Scientific). After 5 days the medium was replaced with DMEM/F12, 2% B27, 1% P/S, 50ng/ml BDNF (Peprotech EC).

3.3.2 SH-SY5Y cells viral transduction

SH-SY5Y cells were differentiated at a density of 6500 cells/well, in a 24 well plate with coverslips coated with 10 μg/ml poly-D-lysine (Sigma-Aldrich) in PBS for 1h and with 5 μg/ml Laminin (Sigma-Aldrich) in PBS for 1h. One day after differentiation, cells were infected with either α-Syn-IRES-GFP or IRES-GFP lentiviruses (10000TU). Three days after infection cells were fixed with 4% of PFA in Cytoskeleton preservation buffer for 30 min at RT. Cells were then analyzed by immunocytochemistry.

3.3.3 SH-SY5Y cells transfection

SH-SY5Y cells were differentiated in a 6 well plate at a density of 20000 cells/well. 24h after differentiation cells were transfected with the constructs HBA-α-Syn-IRES-GFP, HBA-IRES-GFP and RedTrack-CMV-PrP^{c}. For transfection, performed with lipofectamine2000, cells were incubated in DMEM/F12 with 1% P/S. DNA complexes were formed by incubating 3ug of the plasmid of interest with 15ul of Lipofectamine2000 in 250ul Optimem, for 20 min at RT. Then, complexes were added to the cells, dropwise and incubated for 5h at 37°C. After incubation, medium was replaced DMEM/F12, 2% B27, 1% P/S, 50ng/ml BDNF. Two days after transfection cells were re-plated at a density of 15000 cells/well in a 24-well in DMEM/F12, 2% B27, 1% P/S, 50ng/ml BDNF. After 24h, cells were fixed with 4% of PFA in Cytoskeleton preservation buffer for 30 min at RT. Cells were then analyzed by immunocytochemistry

3.3.4 Studies of cell viability and neuroprotection

GPE and analogues were kindly provided by Ivo Dias (Faculdade de Ciências da Universidade do Porto, Porto). GPD, APD, APE are the analogues that were synthetized from GPE with different peptide sequence, in order to improve its stability and viability.

3.3.4.1 MTT test for cell viability in undifferentiated SH-SY5Y cells

SH-SY5Y cells were plated at a density of 4000cells/well in a 96 well-plate in DMEM/F12 (Sigma-Aldrich), 10% FBS and 1% P/S. 24h after plating, GPE and analogues were added at the final concentration of 100uM. After 1h, 6-OHDA was added to the medium containing GPE and analogues, at a final concentration of 15uM. In the next day, MTT assay was performed as described above.

3.3.4.2 MTT test for cell viability in differentiated SH-SY5Y cells

SH-SY5Y cells were plated at a density of 4000cells/well in a 96 well-plate in DMEM/F12 (Sigma-Aldrich), 10% FBS and 1% P/S. 24h after plating, the medium was removed and replaced with DMEM/F12, 2% B27, 1% P/S, 10 μM all-trans-retinoic acid. After 3 days the medium was replaced with DMEM/F12, 2% B27, 1% P/S, 80nM phorbol 12-myristate 13-acetate (PMA). 4 days after, GPE and analogues were added at the final concentration of 100uM. After 1h, 6-OHDA was added to the medium at a final concentration of 15uM. In the next day, MTT assay was performed as described above.

3.4. Immunocytochemistry

3.4.1 Cofilin and β3-tubulin immunofluorescence

Cells were permeabilized with methanol at -20ºC for 3min and blocked with 2.5% of normal donkey serum in 1% BSA/PBS for 1h at RT. Incubation with primary antibodies was performed overnight at 4ºC in 1% BSA/PBS at a final dilution of 1:2000 (rabbit anti-T-Cofilin (Bamburg lab), mouse anti-β3-tubulin (Promega)), followed by incubation with secondary antibodies for 1h at RT (Alexa Fluor donkey anti-mouse IgG-568 Life Technologies and Alexa Fluor donkey anti-rabbit IgG-

Alexa568, Life Technologies). The coverslips were mounted with fluoromount (SouthernBiotech) and let set overnight before analysis with Fluorescence microscopy.

3.4.2 α-Synuclein and PS129- α-Synuclein staining

Cells were permeabilized with 2.5% triton X-100 at RT for 20 min and blocked with 5% of normal donkey serum in 1% BSA/PBS for 1h at RT. Subsequently, cells were incubated with primary antibodies in 1% BSA/PBS, overnight at 4°C (mouse anti-α-Synuclein (BD Biosciences) at 1:1000, rabbit anti-PS129- α-Synuclein (Abcam) at 1:500), followed by incubation with secondary antibodies for 1h at RT (Alexa Fluor donkey anti-mouse IgG-568 Life Technologies and Alexa Four donkey anti-rabbit IgG-Alexa568, Life Technologies). The coverslides were mounted with fluoromount and let set overnight before analysis with fluorescence microscopy.

3.5. Imaging and quantification of rod formation

Transduced rat hippocampal neurons (cultured for 7 days) and transfected or transduced SH-SY5Y cells were quantified for the presence of rods in an upright epifluorescence microscope (Zeiss Axio Imager Z1, Carl Zeiss) at 40x magnification. The percentage of neurons with cofilin-actin rods were quantified.

For analysis of Cofilin-actin rod formation in transduced rat hippocampal neurons (cultured for 14 days) and transfected rat hippocampal neurons, images were captured in an automated fluorescence widefield high-content screening microscope (IN Cell Analyzer 2000, GE Healthcare) at 40x magnification. Images were analyzed using Fiji software and the measurement of cofilin-actin rods formation was performed by the ratio between the total number of rods and the number of neurons (nuclei).

3.6. Immunoblot

3.6.1 Western blot

Rat hippocampal neurons were cultured in a 6 well plate at a density of 200.000 cells/well. At DIV4 cells were infected with either HBA-α-Syn-IRES-GFP or HBA-IRES-GFP lentiviruses. At DIV 14 cells were harvested and lysed in 0.3%Triton X-100 (Sigma-Aldrich), 1x protein inhibitors

Cocktail (100x, Sigma-Aldrich), 1mM Sodium orthovanadate (Sigma-Aldrich). Cell lysate was sonicated (2x during 10 cycles, Output Power 50 Watts, Branson sonifier 250) and cleared by centrifugation at 15000rpm for 5min at 4◦C (VWR CT15E). Protein quantification was performed by the Lowry method. Samples of 25ug or 5ug of protein extracts were run in 12% SDS-PAGE gels and then transferred to a nitrocellulose membrane (0.45μm GE HealthCare) for 2 hours, using a semi-dry transfer system (CBS scientific EBU-4000) in transfer buffer (1xTGS (Bio Rad) with 20% methanol (Sigma-Aldrich)).

Membranes were incubated with blocking solution 5% milk (Sigma-Aldrich) in TBS-T or 5%BSA (NZYTech) in TBS-T depending on the primary antibodies used (listed in Table 2) for 1 hour at RT. Incubation with primary antibodies (Table 2) was performed in either 5% milk in TBS-T or 5%BSA in TBS-T overnight at 4◦C followed by incubation with secondary antibodies conjugated with Horseradish-Peroxidase, diluted in 5% milk in TBS-T for 1 hour at RT. Immunodetection was performed by chemiluminescence using ECL (Bio-Rad) reagent.

3.6.2 Dot blot

Supernatants collected from either transfected or transduced cultured rat hippocampal neurons were centrifuged at 4000g for 5 min at 4◦C to remove cell debris and were applied to a nitrocellulose membrane using Fisherbrand™ Dot Blot Hybridisation Manifold System according to the manufacturer's recommended protocol. Membranes were blocked for 1h at RT with 5%milk in TBS-T and incubated with the primary antibody anti-mouse α-Syn at 1:1000 dilution in 5%milk in TBS-T, overnight at 4◦C. Incubation with secondary antibodies conjugated with Horseradish-Peroxidase, diluted in 5% milk in TBS-T was performed 1h at RT. Immunodetection was performed by chemiluminescence using ECL reagent

Table 2 - List of the Antibodies used for Western Blot, with the respective dilution and blocking solution used.

Antibody	Dilution	Blocking	Company
Anti-mouse α-Synuclein	1:1000 in 5%MILK/TBS-T	5%MILK/TBS-T	BD Biosciences
Anti-rabbit P129-α-Syn	1:500 in 5%MILK/TBS-T	5%MILK/TBS-T	Abcam
Anti-mouse Cofilin	1:500 in 5%BSA/TBS-T	5%BSA/TBS-T	Abcam
Anti-rabbit P-cofilin	1:1000 in 1%BSA/TBS-T	5%MILK/TBS-T	Cell signaling
Anti-rabbit LIMK	1:500 in 5%BSA/TBS-T	5%MILK/TBS-T	ECM Biosciences
Anti-rabbit Vinculin	3:10000 in 5%BSA/TBS-T	5%MILK/TBS-T	Thermo Fisher Scientific

3.7 Statistical analysis

All the data was analyzed using Student's t-test and expressed as mean±SEM. All statistical analyses were performed using GraphPad Prism 6 software: *p<0.05; **p<0.01; ***p<0.001; ****p<0.0001.

4. RESULTS

4.1 Impact of α-Syn overexpression on cofilin-actin rods formation in hippocampal neurons

Previous results from our group showed that cofilin-actin rods are induced by the extracellular addition of α-Syn oligomers to primary cultures of hippocampal neurons (Figure 9). Cognitive dysfunction in PD patients is correlated with *SNCA* gene duplications and triplications and high levels of α-Syn pathology in the hippocampus (Ferese, Modugno et al. 2015). Additionally, in an animal model of PD, the overexpression of α-Syn under the Thy1 promoter (Thy1-α-Syn mice) is associated with the presence of α-Syn in the hippocampus and cognitive impairment (Tsai, Chiu et al. 2009). These facts suggest that the overexpression of α-Syn in the hippocampus might be relevant to study cognitive impartment in PD. As such, we aimed to establish a cell model of α-Syn overexpression in hippocampal neurons to study rod formation. This cell-based system will be the basis for the future validation of rods in Thy1-α-Syn mice and correlation with cognitive defects *in vivo*.

4.1.1 α-Syn overexpression in hippocampal neurons induces cofilin-actin rods formation

In order to set up a model of α-Syn overexpression to analyze rod formation in hippocampal neurons, we used a lentiviral expression system. We initially constructed plasmids containing a CMV (cytomegalovirus) promoter, followed by α-Syn or puromycin sequence, an Internal ribosome entry site (IRES) sequence and GFP (Green Fluorescent Protein) sequence. The CMV promoter is widely used for transient and stable transgene expression (Barrow, Perez-Campo et al. 2006), and was previously used to overexpress α-Syn in primary neuron cultures (Prasad, Kumar et al. 2004). IRES is a RNA sequence that allows the initiation of translation from any position within a mRNA immediately downstream from where the IRES is located (Mokrejs, Vopalensky et al. 2006). Using this system, the expression of α-Syn and GFP is independent, thus α-Syn will not be tagged to GFP not affecting its structure and function. In the control vector the α-Syn sequence was replaced by a puromycin resistance sequence, to assure that the distance between the promoter and the GFP sequence was similar in both plasmids. The lentiviruses containing the sequence for α-Syn

overexpression is represented as CMV-α-Syn-IRES-GFP and the control vector is represented as CMV-Puro-IRES-GFP.

To investigate whether cofilin-actin rods are formed upon α-Syn overexpression, we infected rat hippocampal neurons at DIV4 with lentiviruses for either CMV-Puro-IRES-GFP or CMV-α-Syn-IRES-GFP. At DIV7 we quantified rod formation by cofilin immunostaining and found that the

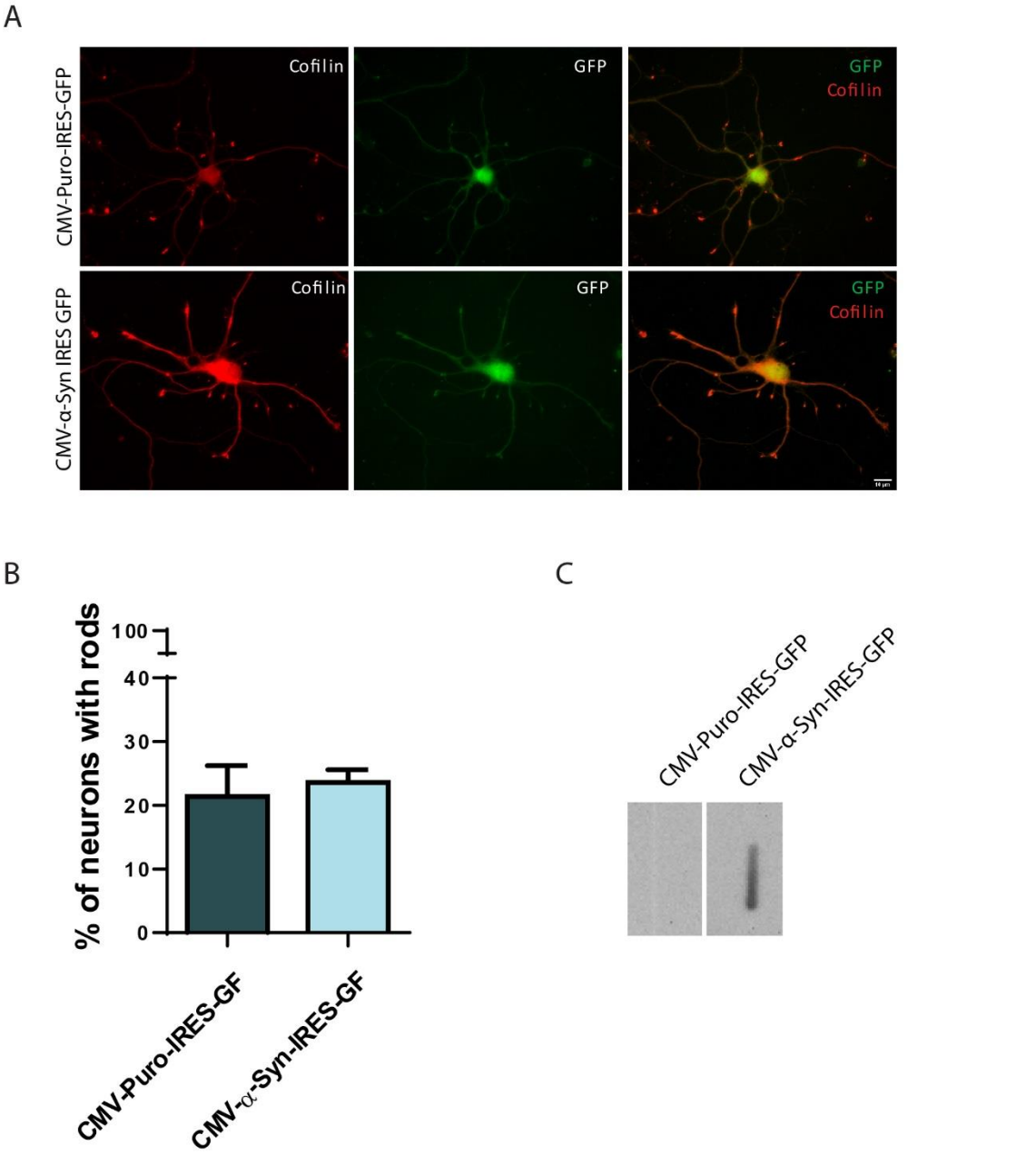

Figure 11 – **Overexpression of α-Syn under the CMV promoter does not induce rod formation.** A- Hippocampal neurons treated with lentivirus expressing CMV-Puro-IRES-GFP (control) or CMV-α-Syn-IRES-GFP at DIV4 and immunostainned for cofilin at DIV7 (red). B- Quantification of the percentage of neurons with cofilin-actin rods. C - Dot blot for α-Syn of the supernatants recovered from neurons treated with the respective lentiviruses. Number of neurons quantified: ≥ 100 in each of the three different experiments.

overexpression of α-Syn did not induce differences in the percentage of neurons with rods when compared to control transduced neurons (Figure 11A, B). α-Syn was previously shown to be secreted from cells supporting the idea of a spreading pathology. As such, we analyzed the presence of α-Syn in the supernatant of the transduced hippocampal neuronal cultures by dot blot, what confirmed secretion of α-Syn in the CMV-α-Syn-IRES-GFP transduced neurons (Figure 11C). These results suggest that rod formation seen in the tested conditions is not related with the presence of α-Syn as there were no differences between CMV-Puro-IRES-GFP or CMV-α-Syn-IRES-GFP transduced neurons. The 20% value for the number of cells with rods could be related with toxicity of the expressing constructs.

α-Syn is expressed *in vitro* after DIV6 and at DIV14 is predominantly localized to the presynaptic terminals, its localization in physiological conditions (Murphy, Rueter et al. 2000). Taking this in consideration, we decided to access rod formation in mature neurons where α-Syn has a more relevant role. For this, we infected rat hippocampal neurons at DIV4 with the lentiviruses for CMV-Puro-IRES-GFP or CMV-α-Syn-IRES-GFP and analyzed rod formation at DIV14 (Figure 12A). Due to cell complexity we quantified the rod index (ratio between the total number of rods and the number of neuronal nuclei). Similarly to what was seen at DIV7, the overexpression of α-Syn did not induce rod formation when compared to control infected cells (Figure 12B). We also analyzed the presence of α-Syn in the supernatant of the transduced hippocampal neurons and no differences were observed between the two conditions what might explain the absence of α-Syn-induced rods (figure 12C). One can appreciate the presence of α-Syn in control conditions, further validating that α-Syn is expressed later in developing hippocampal neurons.

The results obtained with the CMV-Puro-IRES-GFP or CMV-α-Syn-IRES-GFP constructs suggest that the α-Syn expression levels might not be sufficient, and that the constructs might induce some toxicity.

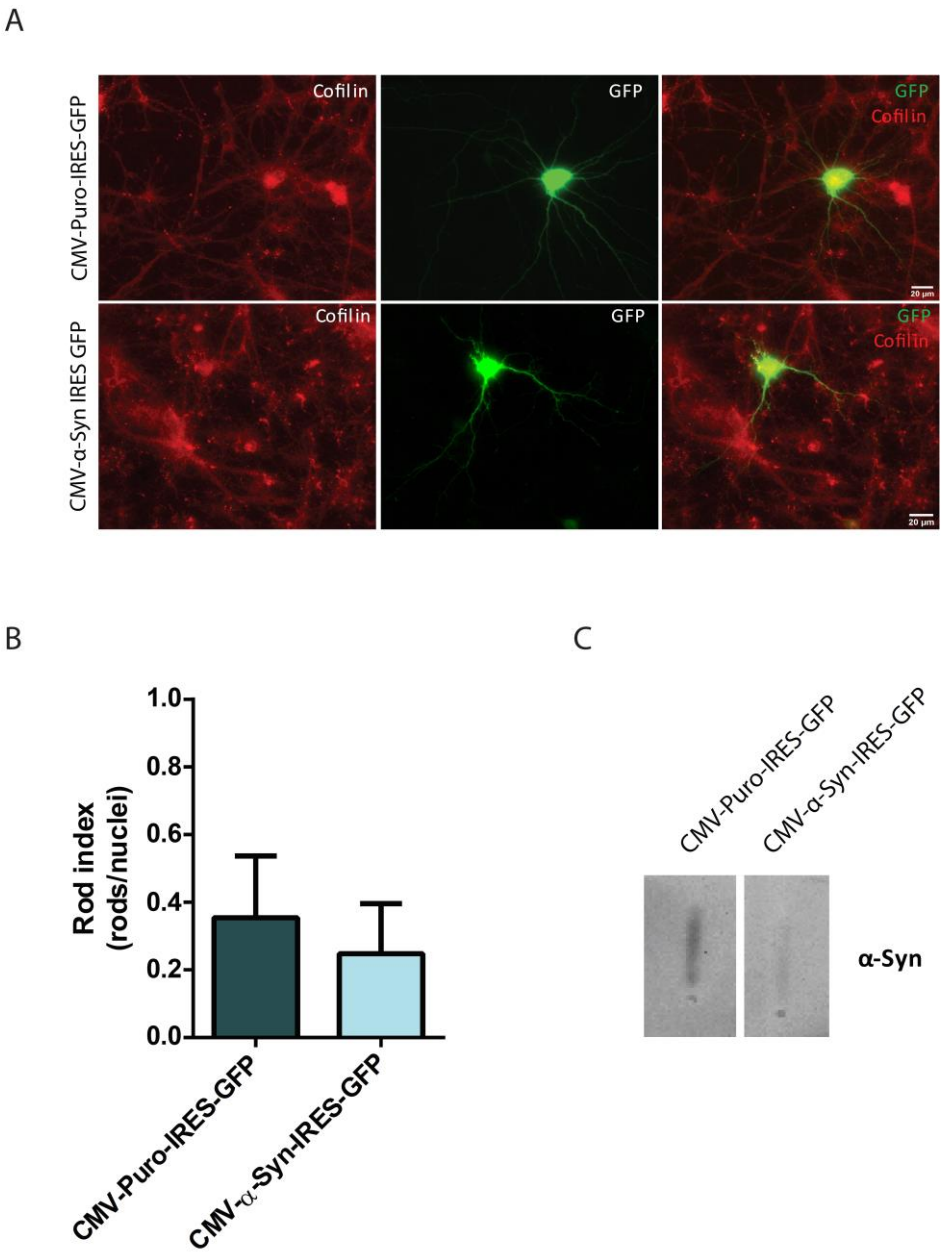

Figure 12 - **Overexpression of α-Syn under the CMV promoter does not induce rod formation in mature neurons**. A- Hippocampal neurons treated with lentivirus expressing CMV-Puro-IRES-GFP (control) or CMV-α-Syn-IRES-GFP at DIV4 and immunostainned for cofilin at DIV14

Previous studies showed that α-Syn overexpression using lentivirus leads to an increase in α-Syn levels by 4.5-fold and impairs neuronal differentiation in LUHMES cells (Oliveira, Falomir-Lockhart et al. 2015). The constructs used in the referred study overexpressed α-Syn under the HBA promoter which was already shown to drive high levels of transgene expression in other *in vitro*

models, such as the PC12 cells (Muller, Sullivan et al. 1990). In this respect, we aimed to use these constructs, which were previously validated, in order to overcome the problems we found with the lentivirus that we designed. These constructs are composed by the HBA promoter, followed by α-Syn sequence, an IRES sequence and a GFP sequence. The control vector does not present α-Syn sequence and the promoter is followed by the IRES sequence. The lentiviruses containing the sequence for α-Syn overexpression are represented as HBA-α-Syn-IRES-GFP and the control ones are represented as HBA-IRES-GFP.

In order to demonstrate that the overexpressing system was working we analyzed α-Syn overexpression by immunostaining and western blot in primary cultures of hippocampal infected at DIV4 and analyzed at DIV7. Using both techniques we were able to confirm that α-Syn was being expressed in neurons infected with HBA-α-Syn-IRES-GFP lentiviruses (Figure 13A, B). The absence of α-Syn in control neurons at DIV7 corroborates the later physiological expression of the protein

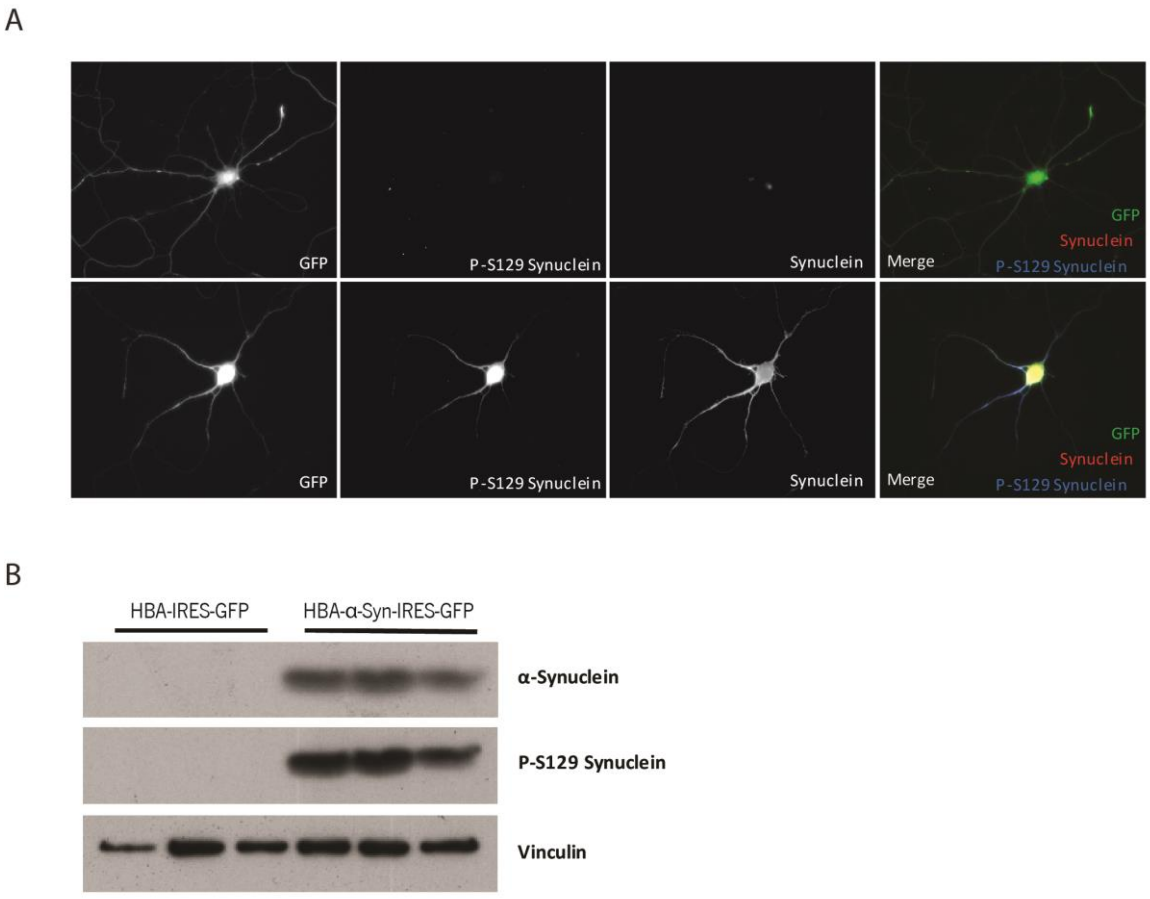

Figure 13 - **Overexpression of α-Syn under the HBA promoter using HBA-α-Syn-IRES-GFP and HBA-IRES-GFP lentivirus**. A-Hippocampal neurons treated with lentivirus expressing HBA-IRES-GFP (control) or HBA-α-Syn-IRES-GFP at DIV4 and immunostainned for synuclein (red) and PS129-α-Syn (blue) at DIV7. B- Western blot analysis for α-Syn and PS129-α-Syn in transduced hippocampal neurons. Vinculin was used as loading control.

in neurons. One of the pathological hallmarks of PD is the presence of PS129-α-Syn in the LBs, which is the predominant form of α-Syn included in these structures. Therefore, we analyzed the presence of PS129-α-Syn in our setup and observed high levels of the phosphorylated protein upon α-Syn overexpression, as demonstrated by immunostaining and western blot analysis (Figure 13A, B).

After confirming α-Syn overexpression, we assessed rod formation in hippocampal neurons infected at DIV4 and immunostainned for cofilin at DIV7. We observed that the overexpression of α-Syn is significantly inducing rod formation comparing with the control condition (Figure 14A, B). Additionally, we observed that α-Syn was present in the supernatants of the α-Syn-overexpressing cultured neurons (Figure 14C).

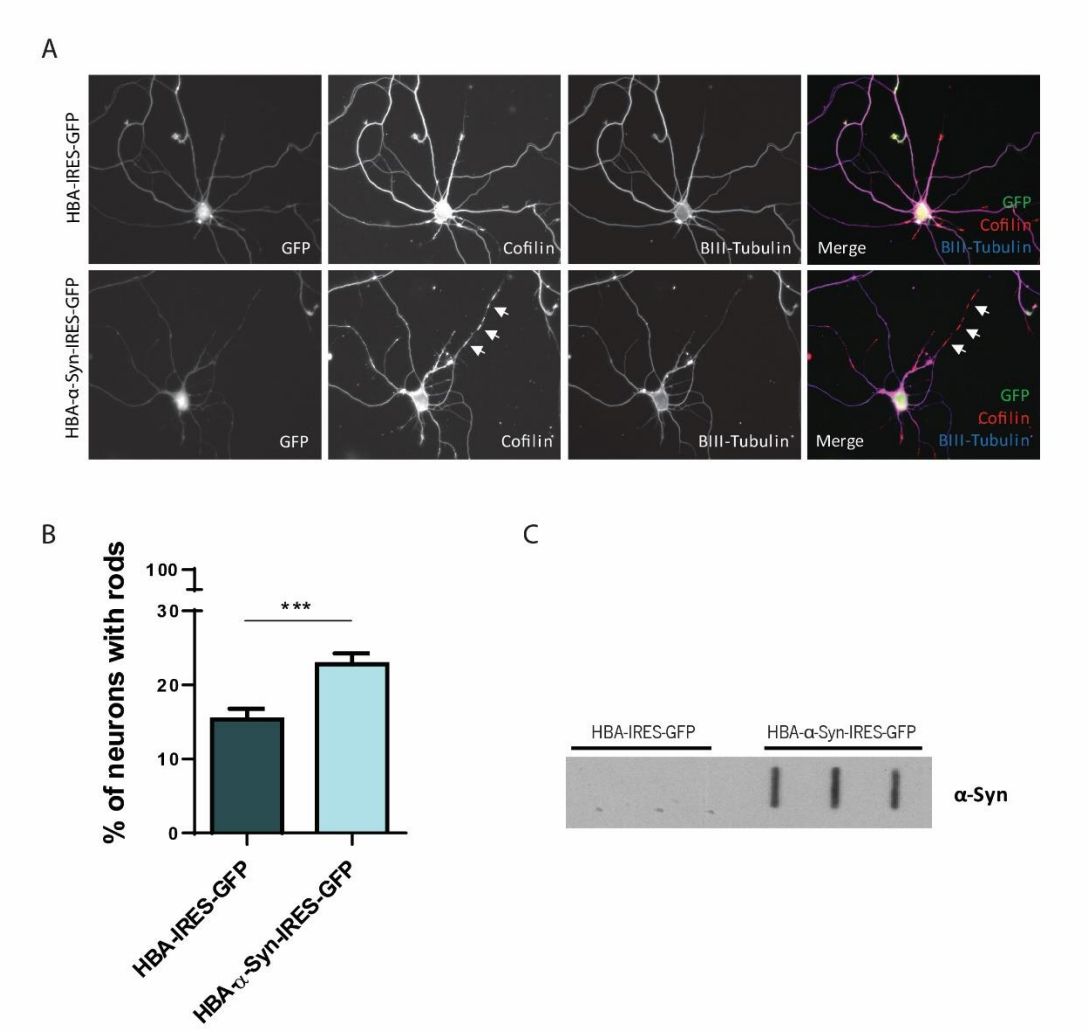

Figure 14 - **Overexpression of α-Syn under the HBA promoter induces rod formation.** A- Hippocampal neurons treated with lentivirus expressing HBA-IRES-GFP (control) or HBA-α-Syn-IRES-GFP at DIV4 and immunostainned for cofilin at DIV7 (red). B- Quantification of the percentage of neurons with cofilin-actin rods. C – Dot blot for α-Syn of the culture medium recovered from neurons treated with the respective lentiviruses. Number of

neurons quantified: ≥ 100 in each of the three different experiments. Statistical significance determined by t-test: ***P<0.001.

We also validated our results in long-term cultured hippocampal neurons, since it is more relevant physiologically, as explained before. For this, we infected hippocampal neurons at DIV4 and analyzed at DIV14. Similarly to what we observed at DIV7, we confirmed α-Syn overexpression in neurons by immunocytochemistry and western blot (Figure 15A and B). Interestingly, high levels of phosphorylated α-Syn (PS129-α-Syn) were seen in neurons overexpressing α-Syn, suggesting that this can contribute to the pathology.

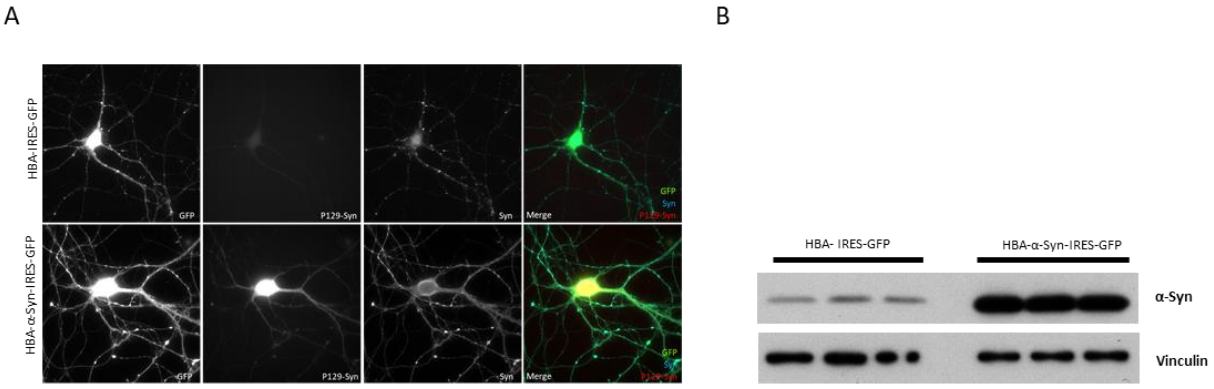

Figure 15 - **Overexpression of α-Syn under the HBA promoter using HBA-α-Syn-IRES-GFP and HBA-IRES-GFP lentivirus**. A-Hippocampal neurons treated with the lentivirus expressing HBA-IRES-GFP (control) or HBA-α-Syn-IRES-GFP at DIV4 and immunostainned for synuclein (red) and PS129-α-Syn (blue) at DIV14. B- Western blot analysis for α-Syn in transduced hippocampal neurons. Vinculin was used as loading control.

Next, we analyzed rod formation in the long-term cultures. Similarly to what was seen at DIV7, DIV14 α-Syn transduced neurons present increased rod formation when compared to control neurons, as determined by quantification of the rod index (Figure 16A, B). Moreover, we also detected α-Syn in the supernatants from the α-Syn overexpressing neurons confirming secretion of the protein (Figure 16C).

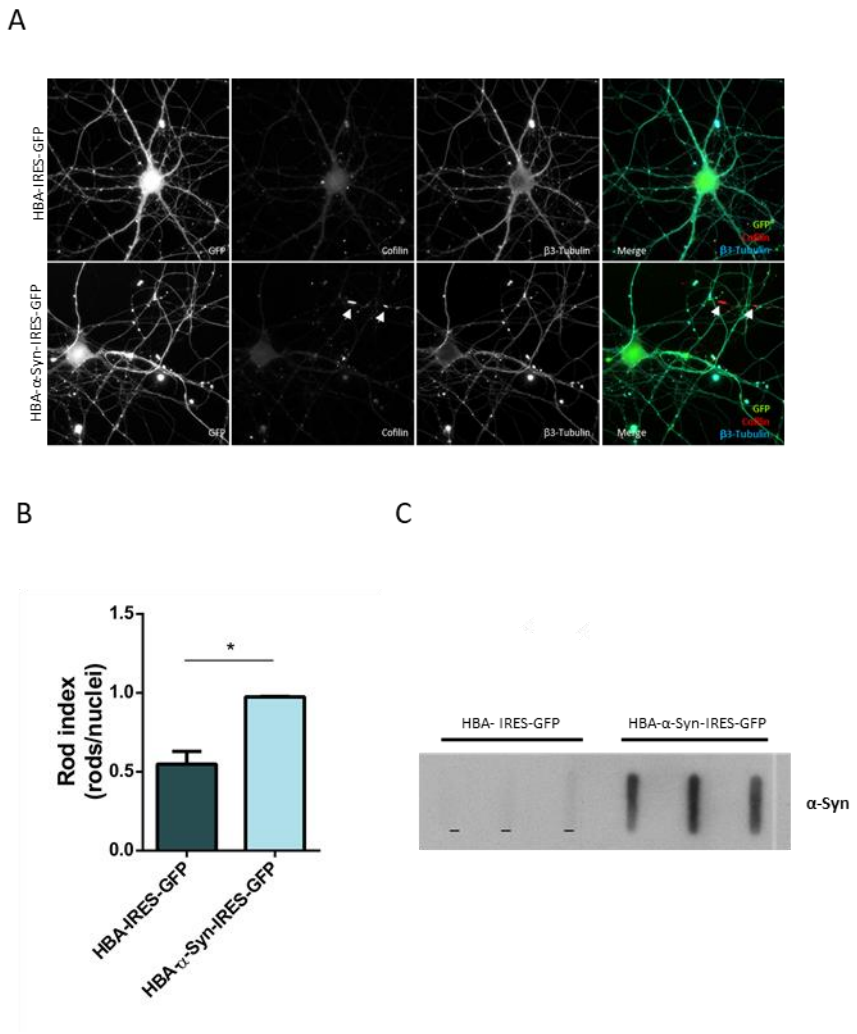

Figure 16 - **Overexpression of α-Syn under the HBA promoter induces rod formation.** A- Hippocampal neurons treated with the lentivirus expressing HBA-IRES-GFP (control) or HBA-α-Syn-IRES-GFP at DIV4 and immunostainned for cofilin at DIV14 (red). Arrows indicate cofilin-actin rod structures. B- Rod Index quantification. C – Dot blot for α-Syn of the culture medium recovered from neurons treated with the respective lentiviruses. Number of neurons quantified: ≥ 60 in each of the two different experiments. Statistical significance determined by t-test: *P<0.05.

We observed that lentiviral-mediated overexpression of α-Syn induces cofilin-actin rods formation in hippocampal neurons. Concerning the fact that we planned to validate some molecular players mediating α-Syn induced rod formation, by performing their genetic manipulation using transfection, we tested rod formation in neurons overexpressing α-Syn by transfection of the lentiviral constructs. For that, we transfected hippocampal neurons at DIV4 with HBA-α-Syn-IRES-GFP or HBA-IRES-GFP plasmids and performed analysis at DIV14. We observed that the overexpression of α-Syn is this model was also inducing cofilin-actin rods formation (Figure 17A, B). With this, we could confirm that, independently of the method, the α-Syn overexpression results in rod formation enabling us the use of the most adequate method for each experimental question.

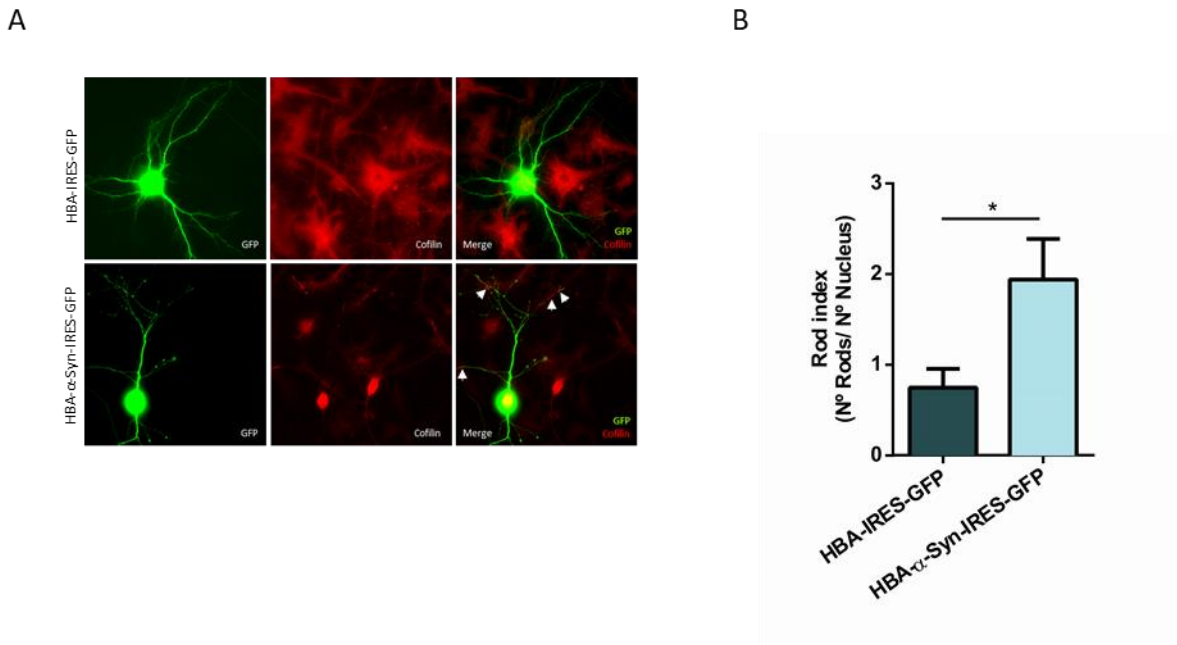

Figure 17 - **Overexpression of α-Syn under the HBA promoter induces rod formation** A- Hippocampal neurons transfected with the plasmids HBA-IRES-GFP (control) or HBA-α-Syn-IRES-GFP and at DIV5 and immunostainned for cofilin at DIV7 (red). Arrows indicate cofilin-actin rod structures. B- Rod index quantification. Number of neurons quantified: ≥ 30 in each of the three different experiments. Statistical significance determined by t-test: *P<0.05.

4.1.2 α-Syn-containing supernatants from transduced hippocampal neurons induce rod formation

As already mentioned, α-Syn spreading is thought to contribute to the propagation of the disease between neurons and ultimately between different regions of the brain. As we observed that in our settings α-Syn is being secreted to the culture medium, we tested whether secreted α-Syn is able to induce rod formation in WT neurons. For that, we recovered the supernatants from DIV14 transduced hippocampal neurons and added them to DIV5 WT hippocampal neurons. Two days later we quantified the percentage of neurons with rods and observed that α-Syn-containing supernatants promote cofilin-actin rods formation, comparing with control condition (Figure 18). This suggests that extracellular α-Syn has an effect on neurons and that is capable of triggering rods formation supporting the hypothesis of α-Syn spreading pathology.

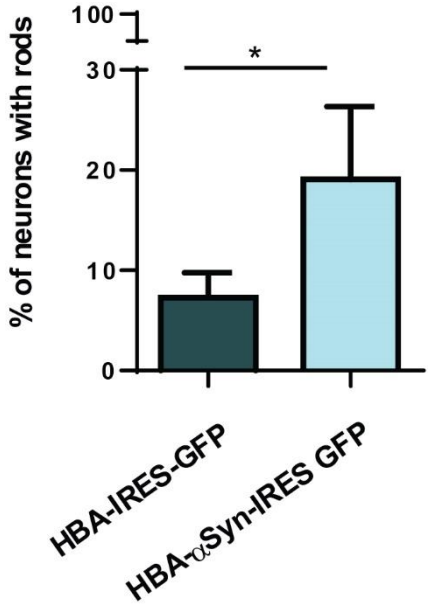

Figure 18 - **Supernatants containing α-Syn induce rods formation**. Supernatants from transduced hippocampal neurons were incubated in WT rat hippocampal neurons at DIV5 for 2 days. Quantification of the cofilin-actin rods represented by the percentage of neurons with rods. Number of neurons quantified: ≥ 100 in each of the three different experiments Statistical significance determined by t-test: *P<0.05.

4.1.3 Molecular mechanism underlying α-Syn-induced rod formation

In AD, cofilin-actin rods are induced by Aβ, activating the PrP^c pathway which is NOX-dependent (Walsh, Minamide et al. 2014). More importantly, PrP^c via NOX activation is able to induce rods formation either by the increase in the activation of cofilin-1 or by an increase in ROS formation. In addition, previous results from our group showed that rod formation induced by α-Syn is decreased in a PrP^c KO mice comparing with the WT mice, showing that PrP^c is involved in α-Syn-induced rod response (Figure 19). We hypothesized that rod formation induced by Aβ and α-Syn might be activated by a similar pathway. Therefore, we wanted to search if α-Syn-induced rods involve mechanisms downstream of PrP^c including NOX1 and cofilin-1 activation.

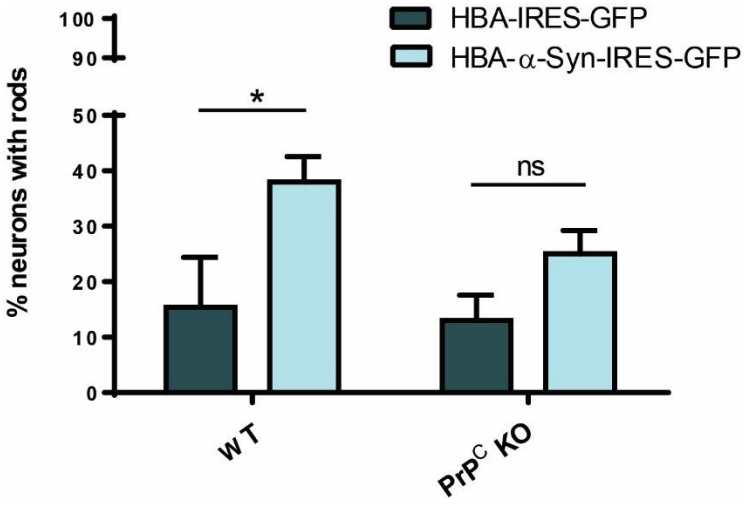

Figure 19 - **PrP^{c} is involved in rod response induced by α-Syn**. Rod formation in either HBA-IRES-GFP or HBA-α-Syn-IRES-GFP transduced hippocampal neurons from WT and PrP^{c} KO mice. Statistical significance determined by t-test: *P<0.05.

4.1.3.1 Effect of NOX 1 modulation on rod formation induced by α-Syn

The formation of ROS by NOX is activated by the PrP^{c} pathway, and this pathway is required for the formation of rods induced by α-Syn. As such, we evaluated the consequences of NOX modulation for rod formation. For that, we used a dominant negative (DN) mutant of gp22^{PHOX} subunit of NOX. Gp22^{PHOX} is one of the two transmembrane proteins that assemble the catalytic core of NOX (Nguyen, Green et al. 2017). The dominant negative mutant of gp22^{PHOX} abolishes the function of this subunit (Sheppard 1994) and consequently inhibits the function and activity of NOX. Moreover, the decrease in ROS formation by NOX inactivation was previously demonstrated in neurons (Pircalabioru, Aviello et al. 2016). To test whether modulation of NOX activity has an impact in α-Syn-induced rod formation we co-expressed either the HBA-α-Syn-IRES-GFP or the control plasmid HBA-IRES-GFP with the RedTrack-CMV-NOX-DN (dominant negative mutant of gp22^{PHOX}) plasmid in primary cultures of hippocampal neurons (Figure 20). Although in this experiment α-Syn overexpression (HBA-α-Syn-IRES-GFP) did not lead to a statically significant increase in rod formation, we observed that co-expression with the dominant negative NOX (HBA-α-Syn-IRES-GFP + RedTrack-CMV-NOX-DN) reverted that tendency, suggesting that NOX might be involved in α-Syn-induced rod formation. Surprisingly, co-transfection of RedTrack-CMV-NOX-DN with the HBA-IRES-GFP control plasmid increased rod formation, when compared to control cells (single transfection with HBA-IRES-GFP. Although these results warrant further investigation, new experiments must be

performed to confirm the impact of NOX activity on α-Syn-induced rod formation. These experiments should include a control empty vector for the RedTrack-CMV-NOX-DN one.

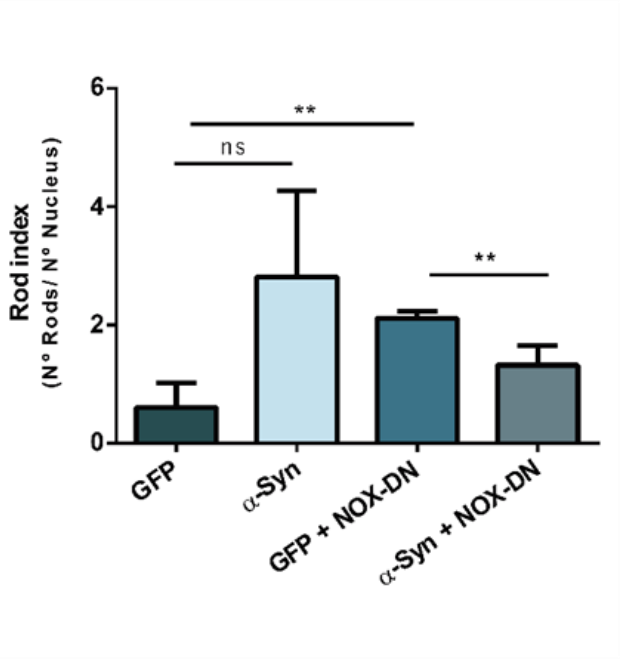

Figure 20 - **Overexpression of NOX-DN decreases rod formation induced by α-Syn**. Rod index quantification of hippocampal neurons transfected with the plasmids HBA-IRES-GFP (GFP) or HBA-α-Syn-IRES-GFP (α-Syn) or co-transfected with the referred plasmids plus RedTrack-CMV-NOX-DN (GFP+NOX-DN) or (α-Syn+NOX-DN). Number of neurons quantified: ≥ 15 in each of the three different experiments. Statistical significance determined by t-test: **P<0.01.

4.1.3.2 Cofilin-1 activation mediates cofilin-actin rods formation induced by α-Syn

Cofilin-1 activation is an essential step for cofilin-actin rods formation. As such, we analyzed the phosphorylation levels of Cofilin-1 when α-Syn is overexpressed. The phosphorylation of Cofilin-1 in its Ser3 residue leads to its inactivation, so we measured the levels of phosphorylated cofilin (P-Cofilin) and total cofilin (T-Cofilin) by western blot (Figure 21). Our results showed that P-Cofilin levels were decreased when α-Syn was overexpressed without changes in the total levels of the protein. This result confirms a decrease in the phosphorylated inactive form of Cofilin-1 and consequently an activation of the protein when α-Syn is overexpressed.

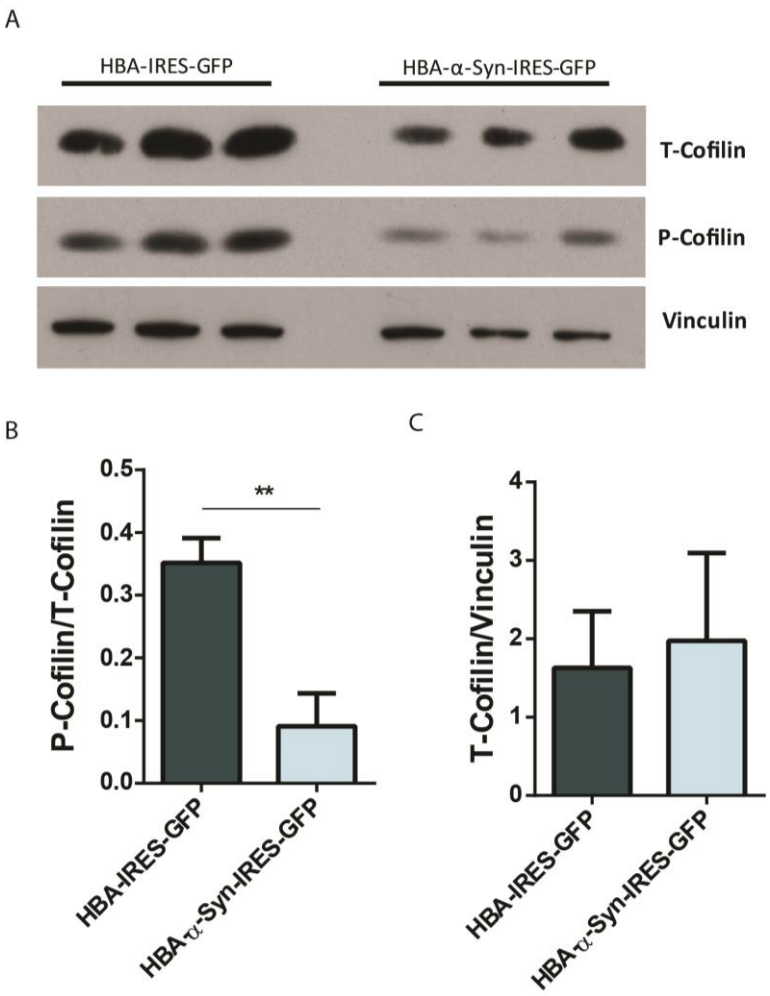

Figure 21 - Expression of α-Syn increases the levels of activated Cofilin-1. A - Western blot analysis of total Cofilin (T-Cofilin) and phosphorylated Cofilin (P-Cofilin) in hippocampal neurons treated with the lentivirus expressing HBA-IRES-GFP (control) or HBA-α-Syn-IRES-GFP. B – Quantification of the ratio P-Cofilin/T-Cofilin. C – Quantification of the ratio T-Cofilin/Vinculin. Statistical significance determined by t-test: **P<0.01

Following confirmation that α-Syn overexpression induces Cofilin-1 activation, we evaluated whether the elevation of Cofilin-1 phosphorylation levels could suppress α-Syn-induced rod formation. To analyze that, we took advantage of a Cofilin-S3E mutant which mimics the phosphorylated form of Cofilin-1 (inactive) to test whether this could modulate rod formation induced by α-Syn (Vitriol, Wise et al. 2013). We co-transfected HBA-α-Syn-IRES-GFP or the control plasmid HBA-IRES-GFP with RFP-N1-Cofilin-S3E in primary cultures of hippocampal neurons and quantified rod formation. We observed that the overexpression of Cofilin-S3E is sufficient to rescue cofilin-actin rods formation induced by α-Syn (Figure 22). This is a very relevant finding since it allows its posterior application to study the specific effects of α-Syn-induced rod formation on neuronal function.

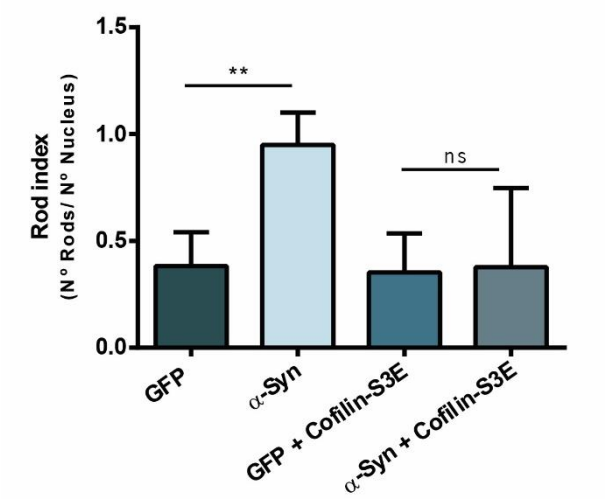

Figure 22 - **Modulation of Cofilin-1 activity decreases rod formation induced by α-Syn**. A- Hippocampal neurons transfected with the plasmids HBA-α-Syn-IRES-GFP or HBA-IRES-GFP (control) and RFP-N1-Cofilin-S3E at DIV5 and immunostainned for cofilin at DIV7. B - Rod Index quantification. Number of neurons quantified: ≥ 15 in each of the three different replicates. Statistical significance determined by t-test: **P<0.01.

4.1.3.3 Effect of chemical activation of RhoA, and consequent Cofilin-1 phosphorylation, on rod formation.

We demonstrated that increasing the levels of phosphorylated Cofilin-1 decreases rod formation induced by α-Syn. As such, we intended to test in our settings the effect of a Rho Activator (CN03), which was already used to modulate cofilin-actin rods formation in the context of ischemic stroke (Shu, Chen et al. 2018). CN03 is a compound that increases LIMK activity by RhoA activation (Shu, Chen et al. 2018). An increase in LIMK activity leads to an increase in Cofilin-1 phosphorylation, thus inactivation of the protein. ATP depletion is a general inducer of rod formation in neurons and is useful to study ischemic stroke since this disease is characterized by energy depletion. In this context, CN03 was shown to reduce cofilin-actin rods formation induced by ATP depletion in an ischemic stroke cell model (Shu, Chen et al. 2018). As such, we decided to address whether we could use CN03 to decrease rod formation induced by α-Syn. First, we validated whether we could recapitulate the effect of CN03 on inhibiting rod formation induced by ATP depletion. For that, CN03 was added to DIV6 hippocampal neurons and at DIV7 ATP depletion was performed and neurons were fixed for analysis of rod formation. As expected, we observed that ATP depletion significantly increased rod formation comparing with untreated neurons (Figure 23). However, the treatment with CN03 was not able to inhibit rod formation induced by ATP depletion. This could

have happened due to the different culture times of the neurons used comparing to what was previously described (Shu, Chen et al. 2018). Thus, new experiments must be done to optimize the effect of CN03 in rods formation and to further test the effect of CN03 on our cell-based system with α-Syn overexpression as inducer of rods.

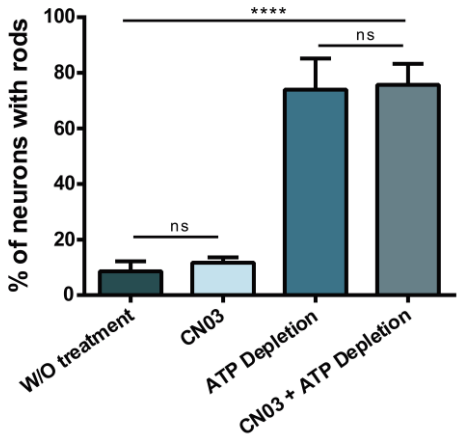

Figure 23 - **CN03 cannot rescue rod formation induced by ATP depletion**. Neurons were incubated with CN03 at DIV6 and at DIV7 ATP depletion was performed during 30min. Quantification of the cofilin-actin rods represented by the percentage of neurons with rods. Number of neurons quantified: ≥ 100 in each of the three different experiments. Statistical significance determined by t-test: ****P<0.0001.

4.2. Cofilin-actin rods formation in a cell model of dopaminergic neurons

We observed previously that the overexpression of α-Syn induces cofilin-actin rods formation in hippocampal neurons. We wanted to validate whether this occurs specifically in hippocampal cells or also has an impact in other neurons affected in PD, namely dopaminergic neurons. Since SH-SY5Y cell line can be differentiated in a relevant dopaminergic neuron-like cell model used to mimic a PD-like phenotype *in vitro*, we hypothesized whether α-Syn could also induce rod formation in this cell type.

4.2.1 α-Syn does not induce cofilin-actin rods in SH-SY5Y-derived cell model of PD

In order to analyze cofilin-actin rods in SH-SY5Y cells, we differentiated these cells for 7 days and then we transduced them with either HBA-IRES-GFP or HBA-α-Syn-IRES-GFP. Three days

after, we quantified rod formation by cofilin immunostaining (Figure 24) and found that the overexpression of α-Syn did not induce differences in the percentage of neurons with rods when compared to control transduced neurons. Although we observed rod formation in both conditions, the number of rods were very low to perform a quantification (1-2 rods in approximately 300 cells). This suggests that α-Syn might not be expressed at sufficient levels in these cells or that the pathways required for rod formation are not being activated by α-Syn.

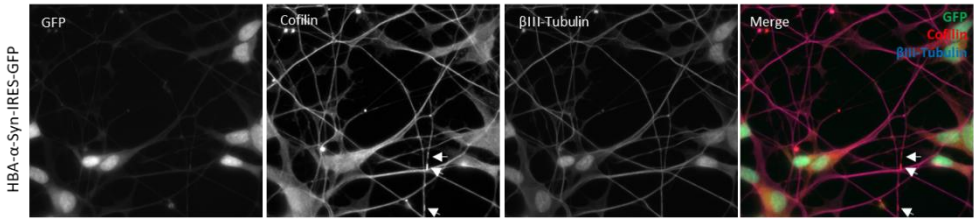

Figure 24 - **Overexpression of α-Syn in SH-SY5Y does not induce rod formation.** A-differentiated SH-SY5Y cells treated with lentivirus expressing HBA-α-Syn-IRES-GFP at day 7 and immunostainned for cofilin (red) at day 10.

4.2.2 Overexpression of α-Syn and PrP^c does not induce cofilin-actin rods in the SH-SY5Y cell model

As referred above, the activation of PrP^c pathway leads to the formation of cofilin-actin rods (Walsh, Minamide et al. 2014) and previous data from our laboratory demonstrated that PrP^c is involved in rod formation induced by α-Syn. As such, we decided to analyze whether the co-overexpression of α-Syn and PrP^c could potentiate rod formation in differentiated SH-SY5Y cells. For that, we differentiated cells for 5 days and we co-transfected either with HBA-IRES-GFP or HBA-α-Syn-IRES-GFP and RedTrack-CMV-PrP^c palsmids. The transfected cells were then re-plated, in order to observe individualized cells. We quantified rod formation (Figure 25) and observed that the overexpression of either PrP^c alone or PrP^c plus α-Syn did not induce rod formation. This further reinforces that in our settings α-Syn is not inducing rod formation in SH-SY5Y cells.

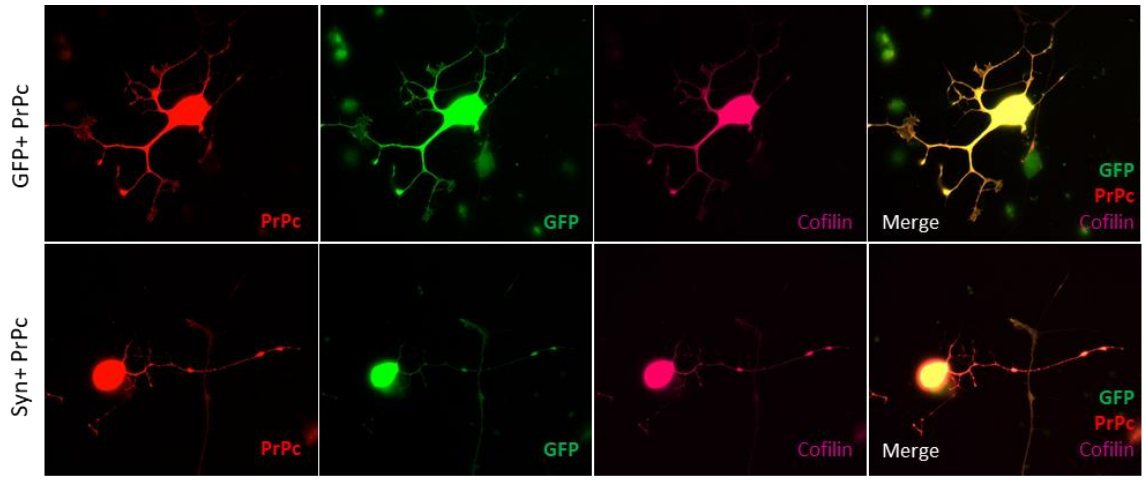

Figure 25 - **Overexpression of α-Syn and PrP^c in SH-SY5Y does not induce rod formation.** A- Differentiated SH-SY5Y cells transfected with the plasmid HBA-IRES-GFP (control) or HBA-α-Syn-IRES-GFP and RedTrack-CMV-PrP^c at day 5 and immunostainned for cofilin at day 8.

4.2.3 ATP depletion does not induce cofilin-actin rods in the SH-SY5Y cell model

As we did not observe rod formation in SH-SY5Y cells using the overexpression of α-Syn and/or the overexpression of PrP^c, we questioned whether these cells were able to form rods upon a more general stimulus. As demonstrated above, ATP depletion is a general inducer of rod formation in cells and is not PrP^c-dependent. As such, we tested if ATP depletion is able to induce rod formation in SH-SY5Y cells. To test that, we differentiated cells for 7 days and we re-plated them in order to obtain individualized cells. Cells recovered for 24h and ATP depletion was performed (Figure 26). We observed only three or four rods being formed in the ATP depletion condition with the quantification of approximately 300 cells, suggesting that ATP depletion has not a major effect on these cells. Nevertheless, as we saw few rod structures only in the ATP-depletion condition, we think these cells might be more resistant to the insults and new experiments should be performed with longer times of ATP depletion, for example.

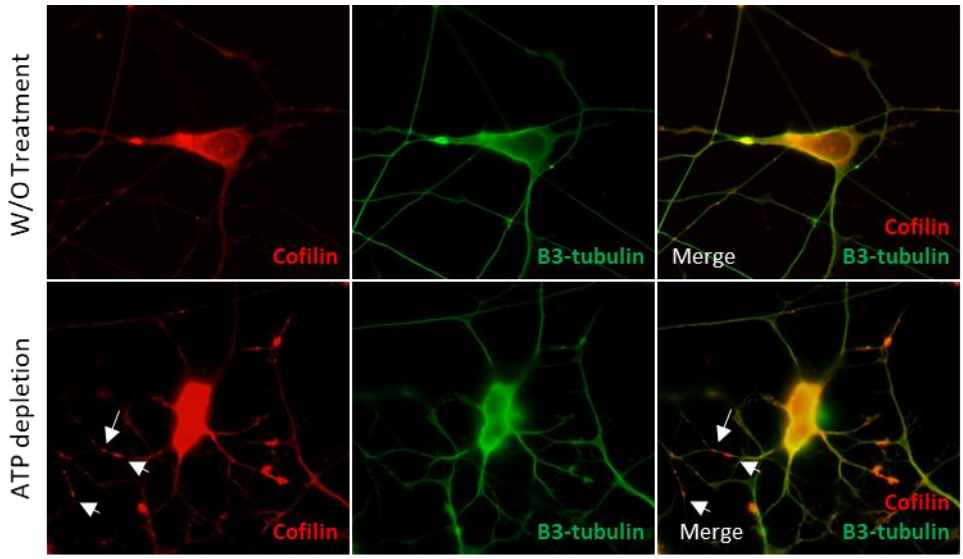

Figure 26 - **ATP depletion in SH-SY5Y does not induce rod formation.** A- Differentiated SH-SY5Y cells were cultured for 10 days and ATP depletion were done for 30 minutes. Cells were immunostainned for cofilin.

4.3 New therapies for PD

As referred before, there are several cell models for the study of the mechanisms underlying neuronal loss in PD. These studies are usually directed to study oxidative stress, mitochondrial dysfunction, and inflammation, which are responsible for the degeneration of these neurons. However, there are limited treatment options for these mechanisms of degeneration. One of the promising neuroprotective agents that was previously used in the search for new therapies for PD is GPE, which was shown to reduce toxicity in the PD *in vitro* model of SH-SY5Y treated with 6-OHDA (Cacciatore, Baldassarre et al. 2012). The 6-OHDA is capable of inducing degeneration of dopaminergic neurons in animal models of PD, recapitulating the damage of the nigrostriatal pathway which is responsible for the motor functions of PD. Since α-Syn pathology is not restricted to the dopaminergic neurons and the nigrostriatal pathway, this makes it a more important target for the study of the neuroprotective agents such as the GPE and analogues.

Aiming at testing strategies to prevent neurotoxicity induced by α-Syn overexpression, we tested the neuroprotective effect of GPE and new analogues. The analogues used were GPD, APD, APE and they are synthetized from GPE in order to improve its stability and viability.

4.3.1 Effect of GPE-derived analogues on SH-SY5Y cell viability

GPE is a compound already used as a neuroprotective agent in PD models. We started by validating the results previously described for GPE neuroprotection in the 6-OHDA-induced PD model using SH-SY5Y and also test the neuroprotective effects of GPE analogues (Cacciatore, Baldassarre et al. 2012). To address these questions we performed the MTT assay, which is a colorimetric assay for assessing cell metabolic activity. We cultured undifferentiated SH-SY5Y and incubated the neuroprotective compounds for 1h. Then the 6-OHDA was added to induce the PD phenotype in the SH-SY5Y cells. We observed in the MTT test that GPE (syntethized) and GPE ctrl (comercial GPE) did not protect cells from 6-OHDA induced toxicity. However, GPD, APD and APE (analogues of GPE) significantly increase the viablity of SH-SY5Y upon treated 6-OHDA (Figure 27A). This suggests that an increase in the stability and viability of the compounds by the alteration of the petide sequence is important for the neuroprotective effects of the tripeptide. However, we were not able to validate the reported neuroprotective effect of GPE in our settings. More experiments shoud be done to validate if these contitions are the ideal to test these neuroprotective agents.

Since the differentiation increases the dopaminergic phenotype of SH-SY5Y cells, we also tested the neuroprotective effects of GPE and derived analogues in differentiated cells. For that we added the compounds for 1h to differentiated SH-SY5Y and induced the PD phenotype with 6-OHDA. We observed, by the measurement of cell viability, that none of the compounds were able to increase SH-SY5Y viablity upon treatment with 6-OHDA (Figure 27B). This migth be related with the decreased susceptibility to oxidative stress of differentiated SH-SY5Y (Cacciatore, Baldassarre et al. 2012) or with the concentration of GPE that may not be sufficient to achieve a neuroprotective effect. It is also described that GPE and analogues can only have a neuroprotective effect upon 6-OHDA toxicity in SH-SY5Y when they are combined with another neuroprotective compound (Cacciatore, Baldassarre et al. 2012). So the combination of more than one neuroprotective agent should be necessary to decrease 6-OHDA toxicity or the synthetization of new analogues to increase neuroprotective properties in differentiated SH-SY5Y.

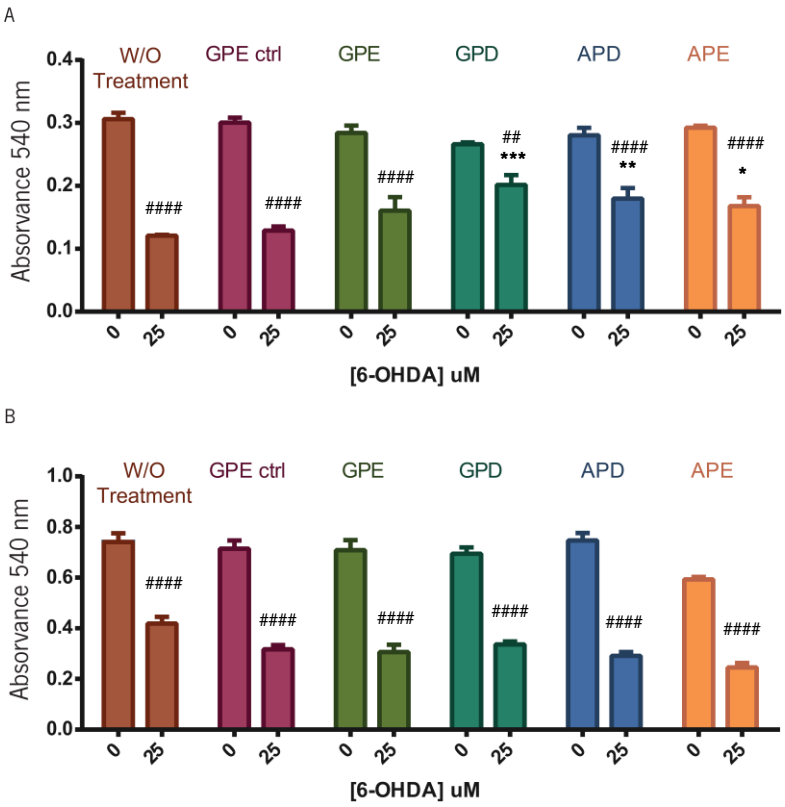

Figure 27 - **GPE analogues are neuroprotective in undifferentiated SH-SY5Y but not in differentiated SH-SY5Y cell.** The undifferentiated and differentiated cells were incubated for 1 h with the compounds, and were then treated with 25uM of 6-OHDA for a further 24 h. A- MTT test in undifferentiated SH-SY5Y. B- MTT test in differentiated SH-SY5Y. Statistical significance represented by * and #. # represents the comparison of the different conditions with and without 25uM 6-OHDA: ##P<0.01, ####P<0.0001. * represents the comparison of all neuroprotective agents with the control condition (w/o treatment) when cells were treated with 25uM 6-OHDA: *P<0.05, **P<0.01, ***P<0.001.

4.3.2 Effect of α-Syn overexpression on SH-SY5Y cell viability

Our major aim was to study the effect of neuroprotective agents on toxicity induced by α-Syn with the goal to test in the future their effect on rod formation. As such, after testing GPE and analogues in the reported conditions we aimed to test them in a scenario of overexpression of α-Syn. We started by analyzing the effect of α-Syn overexpression in cell viability of undifferentiated SH-SY5Y using either HBA-α-Syn-IRES-GFP or HBA-IRES-GFP (control) lentiviruses. We also used 6-OHDA insult as a control for decreased cell viability. As expected, 6-OHDA decreased cell viability of SH-SY5Y cells (Figure 28). Unexpectedly, we observed that α-Syn overexpression did not induce cytotoxicity (Figure 28). This suggests that α-Syn expression levels might not be sufficient to increase SH-SY5Y toxicity, similar what was suggested with the results of rod formation. As such,

the analysis of the levels of α-Syn overexpression in SH-SY5Y cells is a critical point in these experiments.

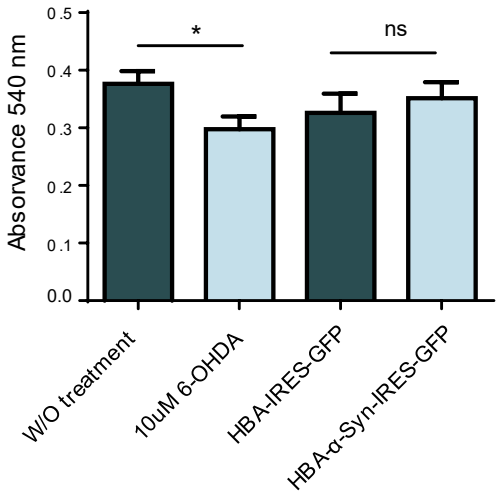

Figure 28 -**6-OHDA induce cell death in SH-SY5Y cell model while α-Syn does affect cell viability.** MTT test was performed in undifferentiated SH-SY5Y incubated with 10uM of 6-OHDA for 24h or infected with/HBA-IRES-GFP/HBA-α-Syn-IRES-GFP lentivirus for 48h or. Statistical significance determined by t-test: *P<0.05

5. DISCUSSION

PD is a neurodegenerative disease usually characterized as a movement disorder, since the loss of dopaminergic neurons in *substancia nigra* is responsible for the impairment of motor functions. However, cognitive defects in PD are currently considered a significant object of study and a hallmark of the disease progression. Although there are many studies addressing the cognitive deficits in PD, the molecular mechanisms underlying these symptoms are still unknown. The degeneration of hippocampal neurons is related with problems in memory, thus, is an important region of the brain for the study of cognitive defects. The formation of cofilin-actin rods in the context of AD was related with cognitive defects of the disorder and, in PD, preliminary data to this thesis showed cofilin-actin rods formation in hippocampal neurons, induced by α-Syn, suggesting the involvement of these structures in the disruption of cognitive function in PD.

The presence of α-Syn in the hippocampus of the PD mouse model (Thy1-α-Syn mice) and in PD patients with *SNCA* gene duplications and triplications is correlated with cognitive disfunction, thus, α-Syn pathology in this brain region might be relevant to study cognitive impartment in PD. As such, we hypothesized that cofilin-actin rods formation is related with α-Syn pathology in the hippocampus, and this might be relevant for the cognitive impairment in PD. Therefore, the major goal of this master thesis was to set up a cell-based system consisting on the overexpression of α-Syn in primary cultures of hippocampal neurons to assess cofilin-actin rods formation.

We decided to mediate the overexpression of α-Syn through the use of lentiviruses since this technique is widely use to overexpress protein in non-dividing cells, with less vulnerability to insertional mutagenesis and they present high levels of infectivity (Merten, Hebben et al. 2016). The first lentivirus that we produced overexpressed α-Syn under CMV promoter and they did not induce the formation of cofilin-actin rods in rat hippocampal neurons, cultured either for short or long time periods. However, it was possible to observe the secretion of α-Syn to the culture medium in short-term culture neurons, confirming that the protein was being expressed and secreted. Taking this in consideration, our findings suggest that the overexpression of α-Syn under the CMV promoter might not be at sufficient levels to induce rods formation. Indeed, although the CMV promoter is widely used for transient and stable transgene expression (Barrow, Perez-Campo et al. 2006), its strength can vary considerably from cell type to cell type (Qin, Zhang et al. 2010). In addition, this promoter expression weakens over time and it can be silenced in some cells at some point (Qin, Zhang et al. 2010) and can be suppressed in neurons co-cultured with glial cells, as in our cultures

primary hippocampal cultures, a process mediated by glial secretion of cytokines (Kugler, Meyn et al. 2001). It is important to refer that we never measured the expression levels of α-Syn in this model and this would be important to support the presented speculations. To overcome the problems with our model we used lentivirus-mediated expression under the HBA promoter which was already successfully used to overexpress α-Syn in *in vitro* models of LUHMES and PC12 (Muller, Sullivan et al. 1990, Ferese, Modugno et al. 2015, Oliveira, Falomir-Lockhart et al. 2015). Beta-actin promoters are ubiquitous, with high neural expression. In addition, these promoters only show lower expression in motor neurons with high expression levels of GFP in rat hippocampal neurons (Gray, Foti et al. 2011). We observed that the constructs with the HBA promoter induced α-Syn overexpression, and that the protein was present in both, inside the neurons and in the culture medium. Importantly, using these lentiviral constructs we observed that α-Syn is inducing the formation of cofilin-actin rods in primary cultures of rat hippocampal neurons. The deposition of α-Syn in LBs inside the neurons is considered a hallmark in PD and inside these inclusions it is known that α-Syn is post translationally modified (Zhang, Li et al. 2019). The most studied post translational modification is the phosphorylation on Ser-129 and 90% of α-Syn in LBs is PS129-α-Syn, in contrast with the physiological conditions, in which only 5% of α-Syn is phosphorylated (Oueslati 2016). Considering this, PS129-α-Syn is considered a pathological form of the protein. Although we never searched for the inclusions of α-Syn in our model, we observed the presence of PS19-α-Syn in α-Syn-transduced hippocampal neurons what could have an important role in the formation of rods or at least in α-Syn neurotoxicity. We validated α-Syn-induced rod formation both in short-term and in long-term cultured hippocampal neurons, since in the long-term cultures α-Syn is predominantly localized to the presynaptic terminals where has a more relevant role. Moreover, these long-term cultures allow the study of the effect of α-Syn on mature dendrites and synapses, where the protein has a more relevant synaptic function. This will allow us to study the pathological impact of α-Syn in the function and morphology of these structures, thus giving us clues to the direct effects on synaptic activity.

There is a new line of study in neurodegenerative diseases which focuses on the "Prion-like" spreading of misfolded aggregated proteins from neuron to neuron, as a way of spreading of pathology in these diseases (Bras, Lopes et al. 2018). The progression of PD was already associated with this mechanism, and it is already shown that α-Syn is secreted by neurons, and more importantly, α-Syn is transmitted between neurons. In addition, the administration to of aSyn PFFs

in hippocampal neurons also leads to the propagation of pathological α-Syn from neuron-to-neuron (Froula, Henderson et al. 2018). Using our model, we showed that α-Syn was secreted to culture medium, and we observed that applying/incubating this culture medium to WT hippocampal neurons resulted in the formation of cofilin-actin rods. This further shows the importance of the propagation of α-Syn for the progression of the disease. However, we did not assess whether α-Syn exerts its effect inside or outside of the cells and further experiments should be considered to test whether α-Syn internalization is necessary for the induction of rods formation. We could also have seen the effect of α-Syn spreading directly on α-Syn-transfected neuron cultures. In this way, we could compare the induction of rods in transfected versus non-transfected cells, since the transfection efficiency is approximately 30%. If we would observe an induction of cofilin-actin rods in untransfected cells it would be caused by α-Syn secretion by the transfected cells.

After we set up our model, we searched for the mechanisms underlying aSyn-induced rod formation. In AD, the formation of cofilin-actin rods is induced by Aβ through the activation of the PrP^c pathway (Walsh, Minamide et al. 2014). This pathway is NOX-dependent and induce cofilin-actin rods formation by the increase in the activation of cofilin-1 and by an increase in ROS formation (Minamide, Striegl et al. 2000). Moreover, previous studies from our group show that PrP^c is involved in rod response induced by α-Syn. The inactivation of this pathway using a PrP^c KO mice, significantly decreased rods formation induced by α-Syn. So, we searched for mechanisms downstream of PrP^c (Walsh, Minamide et al. 2014). Our results showed that NOX inactivation might be decreasing rods formation induced by α-Syn. However, our results are not conclusive, as the construct of used to overexpress NOX-DN might be increasing rods formation, in contrary to what was expected. New experiments should be done with the same settings and with the control empty vector for the RedTrack-CMV-NOX-DN. The evaluation of ROS formation might also be important to evaluate the activity of NOX induced by α-Syn. Therefore, if there is an increase in ROS, it is due to an increase in α-Syn-induced NOX activity, giving further evidence of the activation of this pathway by α-Syn. The activation of cofilin-1 is dependent of its dephosphorylation and our results showed that the overexpression of α-Syn decreases the levels of phosphorylated cofilin, increasing this way the activation of cofilin. This suggests that α-Syn is mediating rods formation by the increase in the activation of cofilin. With these results, we showed the activation of one of the pathways involved in rods formation induced by α-Syn, which consists on Cofilin-1 activation. Our results are consistent with previous literature which showed that there are two essential conditions

for rod formation, hyperactivation of cofilin-1 and oxidative stress (Minamide, Striegl et al. 2000, Bernstein, Shaw et al. 2012). Modulation of Cofilin-1 activation, using a phosphomimetic form of cofilin (cofilin S3E or S3D) was already used as a therapeutic mechanism in animal models of AD, to improve cognitive and behavioral deficits (Shaw and Bamburg 2017). Considering this, we assessed whether the modulation of cofilin-1 activation can impact on α-Syn-induced rods formation. We observed that by increasing the levels of phosphorylated cofilin, by using a cofilin S3E phosphomimetic expressing construct, the formation of α-Syn-induced rods significantly decreased. This further confirms that cofilin-1 activation is indeed related with the cofilin-actin rods formation, as already described. Importantly, the modulation of cofilin-1 might be an important target to investigate the specific consequences of cofilin-actin rods formation in neuronal function (alteration of morphology and function of spines and dendrites) and to assess the impact of the modulation of cofilin -1activity in the reversion the neuronal pathology caused by α-Syn-induced rod formation. Cofilin-1 modulation can also be a promising target for new therapies aiming at reducing the symptoms associated with α-Syn-induced rod formation. Moreover, since the current therapies for PD usually target motor symptoms, our study could open new windows for the treatment of cognitive defects of this disease.

As we observed a beneficial impact of increasing cofilin-1 phosphorylation (inactivation) we considered to use a pharmacological approach with the same purpose. For that we used the CN03. CN03 is a chemical compound which increases LIMK 1 activity by the activation of its upstream regulator Rho, increasing this way the phosphorylation of Cofilin-1. It was already described, in the context of ischemic stroke, that CN03 decreases the formation of cofilin-actin rods (Shu, Chen et al. 2018). Moreover, it is suggested that in ischemic stroke rods are induced by energy depletion and oxidative stress. We would like to validate these results and then use the same settings in our cell-based model. With this we could check whether α-Syn-induced rod formation can be used as a target for CN03 and thus decrease rod formation. Our results showed that CN03 was not able to decrease rods formation induced by ATP depletion, in contrast with the results obtained previously (Shu, Chen et al. 2018). This could be explained by the different stages of neuronal development in vitro, since we used less developed neurons than the ones in the described study. Moreover, long-term cultured neurons show more susceptibility to excitotoxicity than short-term cultured neurons (Calvo, Sanz-Blasco et al. 2015) and indeed, in our setup we found approximately 70% of neurons with rods in ATP depletion condition in contrast with the 96% of neurons with rods described (Shu, Chen et al. 2018). In addition, in the reffered study, the treatment with CN03 rescued rod formation

after ATP depletion in only 26% of neurons which results in a percentage of neurons with rods very simillar with the one we obtained without the treatment. This could explain our results and suggests that the concentration of CN03 was not sufficient to reduce rods formation in our setup or that CN03 can only prevent rod formation in neurons more susceptible to ATP-depletion, such as the long-term cultured neurons. Moreover, in the reported work it was performed the quantification of number of rods per nuclei and this quantification can be more sensitive since it is possible to observe if CN03 is impacting in the number of rods per neuron, even with the same percentage of neurons with rods. Using this approach, we would be able to see if in our neurons CN03 is reducing the number of rods per nuclei, although maintaining the same percentage of neurons with rods.

The loss of dopaminergic neurons is a hallmark of PD, so using a PD model that mimics this phenotype is important to study rod formation. SH-SY5Y cell line is used as model of PD, since after differentiation these cells present a dopaminergic phenotype. Considering this, we wanted to search whether the overexpression of α-Syn induces cofilin-actin rods formation in SH-SY5Y cell line, as we demonstrated in primary cultures of hippocampal neurons. This can give us evidence if in dopaminergic cells there is formation of rods, allowing to understand if rods formation is specific to neurons responsible for cognitive function. We observed that in SH-SY5Y overexpression of α-Syn did not induce rod formation. We suggest that α-Syn expression is not sufficient to induce rods formation. The beta-actin promoter, similar to CMV, transgene expression can vary considerably from cell type to cell type and as already referred, this promoter show lower expression in motor neurons (Gray, Foti et al. 2011). Although we never checked for the levels of α-Syn expression in SH-SY5Y cells, it might be the case that the CMV promoter could not be the appropriate to this cell model. In order to enhance the formation of rods, we overexpressed α-Syn and PrP^{c} since both proteins induce the formation of these structures. Our results demonstrated that the formation of cofilin-actin rods was not induced by the overexpression of α-Syn and PrP^{c}. This suggests that rods formation in these cells might be induced by a pathway that is not being activated by α-Syn or PrP^{c}. As such, we decided to use a more general rod inducer stimulus namely ATP depletion, which is not PrP^{c}-dependent. In differentiated SH-SY5Y cells ATP depletion did not induce significant rod formation. This could be explained by the relevant metabolic differences between rodents (rat primary hippocampal neurons) and humans (human derived cell line SH-SY5Y) cells, which could affect its susceptibility to cytotoxicity (Constantinescu, Constantinescu et al. 2007). In addition, the differentiation in SH-SY5Y cells decreases its susceptibility to cytotoxic agents (Cheung, Lau et al. 2009). Considering these differences, the time or the concentration of

ATP depletion may not have been enough to increase rods formation. Moreover, the metabolic differences, could lead to different pathways for the induction of rods formation. It is described that under stress conditions there is an early accumulation of cofilin-1 in mitochondrial fractions in SH-SY5Y cells. Importantly, only dephosphorylated cofilin-1 was translocated to the mitochondria in these cells (Chua, Volbracht et al. 2003). This suggests that, in our conditions, activated cofilin-1 (dephosphorylated) could be translocated to mitochondria, and so, might not be available to bind to actin and form the cofilin-actin rods. As cofilin-1 activation is a crucial step for rods formation, this suggest that in SH-SY5Y rods could be formed transiently, which can explain the reduced number that we observe when we overexpress α-Syn or in the ATP depletion in SH-SY5Y cells. So, in further experiments we should address if cofilin-1 is in fact being activated and if it is translocated to mitochondria when we activate pathways of rod formation, such as, PrP^c overexpression or ATP depletion. Moreover, we could also overexpress a constitutively active mutated form of cofilin (S3A) in SH-SY5Y cells and observe if this form is translocated to mitochondria or is present in the cytosol (where is necessary for rods formation).

The search for new therapies for PD is a topic of high interest and one of the promising neuroprotective agents is the GPE. It was already shown GPE was able to reduce the the toxicity induced by 6-OHDA in SH-SY5Y cells. The use of 6-OHDA in SH-SY5Y mimics the degeneration of the dopaminergic neurons, being this considered a hallmark of PD. Although these neuroprotective agents were used in dopaminergic models of PD induced by 6-OHDA, we hypothesized if our model of α-Syn overexpression could be a target for the neuroprotective compounds. Moreover, this would allow to observe if these agents could be used to target the cognitive defects observed in PD. Aiming at testing strategies to prevent neurotoxicity induced by α-Syn overexpression, we first validated the neuroprotective effect of GPE and new analogues. The analogues used, are new compounds synthesized in order to improve the stability and viability, by the alteration of the peptide sequence of the tripeptide GPE. We observed that GPE was not able to block 6-OHDA induced toxicity, but GPE analogues significantly reduced toxicity. Although GPE was not able to reproduce previous results (Cacciatore, Baldassarre et al. 2012), GPE analogues should be considered for further study. We observed that GPE and analogues were not able decrease 6-OHDA induced toxicity in differentiated SH-SY5Y. In differentiated cells, the effect of the oxidant is less pronounced than undifferentiated cells, since the differentiation decreases SH-SY5Y susceptibility to oxidative stress (Cacciatore, Baldassarre et al. 2012). Considering this, the effect of the neuroprotective agents could not be sufficient to decrease toxicity, as this differentiated cells are

already less susceptible to the toxicity of 6-OHDA. New GPE analogues should be designed to decrease toxicity in differentiated SH-SY5Y. The use of an combination of GPE and (R)-α-lipoic acid already was shown to induce a neuroprotective effect in differentiated SH-SY5Y (Cacciatore, Baldassarre et al. 2012), so the combination of the analogues or GPE with other neuroprotective agents, such as caffeine or uric acid (Cacciatore, Baldassarre et al. 2012), could be also used to improve cell viability and consequently decrease cell toxicity.

Although the neuroprotective agents were not able to decrease 6-OHDA toxicity in differentiated cells they are still promising, since they have an effect in non-differentiated Sh-SY5Y cells. Therefore, we intended to study the neuroprotective effects of GPE and analogues in a model of α-Syn overexpression SH-SY5Y cells. Previous studies demonstrated that α-Syn overexpression in SH-SY5Y decreases cell viability, and using chondroitin sulfate as a neuroprotective agent it was possible to reduce α-Syn-induced toxicity (Ju, Gao et al. 2017). The effect of GPE and analogues could be further validated in our cell model of α-Syn overexpression in hippocampal neurons and more importantly we could assess whether these compounds are able to reduce cofilin-actin rods formation induced by α-Syn. As cofilin is ubiquitously expressed in the body (Tsai, Chiu et al. 2009), the modulation of this protein is not specific to neurons and it can lead to unwanted effects in the other cells. So, using this neuroprotective agents, is possible to target only neurons to decrease rods formation, without affecting the function of cofilin in other cell types. However, our results showed that α-Syn did not induce cell death in SH-SY5Y cells. This suggests as referred above, that α-Syn is probably not being expressed at sufficient levels in SH-SY5Y cells. In future experiments, we can test the effect of α-Syn overexpression in the viability of hippocampal neurons, since we have already optimized this model for α-Syn pathology. Furthermore we can evaluate the effects of GPE and analogues in rods formation induced by α-Syn in the referred model.

6. CONCLUSIONS AND FUTURE PERSPECTIVES

In summary, in this thesis we successfully generated an in vitro system which recapitulates the high levels of the α-Syn seen in PD cases with dementia, by lentiviral-mediated overexpression of the protein. We observed that the formation of cofilin-actin rods is meditated by α-Syn, by an increase in the phosphorylation of cofilin, through a mechanism that probably involves the PrP^C and NOX activation. Our result also showed that the suppression of cofilin-1 rod formation by an increase in the levels of cofilin-1 phosphorylation might be target to reduce rods formation induced by α-Syn.

We observed that α-Syn overexpression or a general inducer of rod formation did not induce rods formation in SH-SY5Y cells, what might support that rod formation is cell type specific. Moreover, our outcomes showed that GPE analogues resulted in significant neuroprotective effects against 6-OHDA-induced neurotoxicity in SH-SY5Y cells and can be considered a suitable candidate for further studies in the context of α-Syn-induced rod formation.

For future work, we expect to determine the impact of cofilin-actin rods formation in synaptic function and to assess the impact of cofilin-1 activity modulation in the neuronal function. Beside this, we expect to observe the formation of cofilin-actin rods in hippocampus of the animal model of PD that overexpress α-Syn under the Thy1 promoter. The different mechanisms involved in rods formation will be further validated in this animal model, to understand if they can be used to reduce the symptoms of dementia or restore the normal neuronal function.

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