THAIANY QUEVEDO MELO

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Tese apresentada à Faculdade de Medicina da Universidade de São Paulo, para obtenção do título de Doutora em Ciências, Programa de Fisiopatologia Experimental.

Orientadora: Profa. Dra. Merari de F. R. Ferrari.

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Seu tempo me fez preparada,

Quando eu sabia o nada,

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Suas lições me fizeram saber,

Que o **Senhor** me guiaria,

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“We've all got both light and dark inside us. What matters is the part we choose to act on. That's who we really are.” (Sirius Black).

— J.K. Rowling, Harry Potter and the of the Phoenix

“Happiness can be found, even in the darkest of times, if one only remembers to turn on the light.” (Dumbledore).

— J.K. Rowling, Harry Potter and the Prisoner of Azkaban

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Sumário

Resumo

A doença de Parkinson é a doença motora neurodegenerativa mais comum do mundo. Agregados proteicos contendo principalmente alfa-sinucleína é a principal marca da doença. Recentemente, tem sido demonstrado que defeitos na dinâmica mitocondrial e da autofagia são causados pelo acúmulo da proteína. No nosso estudo, foram utilizados neurônios derivados de SH-SY5Y ou de hiPSC de pacientes com a doença de Parkinson hereditária, além de leveduras para analisar a dinâmica mitocondrial e da autofagia e o envolvimento de proteínas desses processos na toxicidade da alfa- sinucleína. Foi observado a diminuição do tráfego mitocondrial em neurônios derivados das células SH-SY5Y que expressavam alfa-sinucleína A53T. Além disso a proteína mutante ainda levou ao aumento de espécies reativas de oxigênio, perturbação da autofagia e aumento da sinalização apoptótica. Os neurônios então foram tratados com NAP, um peptídeo neuroprotetor, que preveniu os efeitos tóxicos da alfa-sinucleína mutante. Leveduras contendo deleções nos genes Gem (Miro), Ypt53 (Rab5) e Atg8 (LC3) e expressando alfa-sinucleína dos tipos A30P e A53T, demonstraram que a toxicidade da alfa-sinucleína é dependente das disfunções na mitocôndria e na autofagia. A agregação da alfa-sinucleína A53T foi prevenida na ausência de Gem. Além disso, a toxicidade da proteína envolvendo a disfunção mitocondrial e sinalização apoptótica causada pelo estresse do ER foi dependente dos genes Gem e Atg8, respectivamente. Neurônios derivados de hiPSC de pacientes contendo a triplicação do gene da alfa-sinucleína, mostraram diminuição do transporte de mitocôndrias e do potencial de membrana da mitocôndria. Análises sobre a quantidade de vesículas lisossomais desses neurônios, demonstraram acúmulos dessas vesículas, sugerindo que a autofagia está alterada. Em um ensaio sobre a sensibilidade dos neurônios dopaminérgicos, foi observado que os neurônios contendo a alpha-sinucleína mutante são mais susceptíveis à rotenona, quando comparado com os neurônios dopaminérgicos do controle. A exposição à rotenona também causou mudanças na distribuição de mitocôndrias, sugerindo que o tráfego retrógrado da organela está alterado.

Abstract

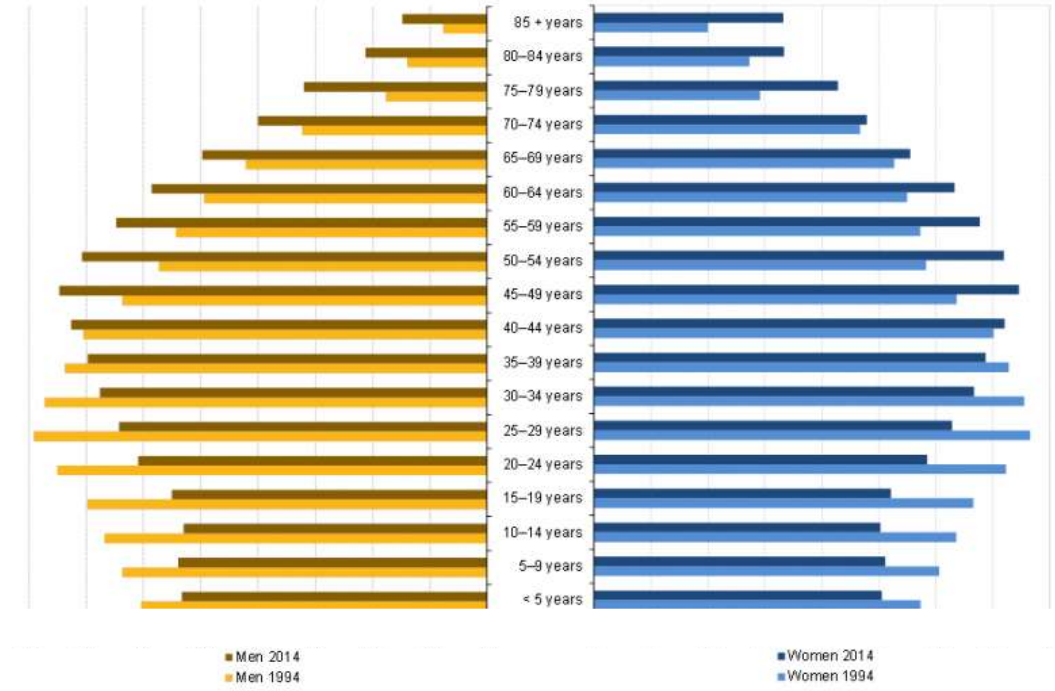
Parkinson's disease (PD) is the most common motor neurodegenerative disease in the world. Protein aggregates containing mainly alpha-synuclein are a hallmark of disease. Mitochondria and autophagy defects have been suggested to be caused by alpha-synuclein toxicity. In this study, we investigated mitochondria and autophagy dynamics related to alpha-synuclein toxicity in neurons derived form SH-SY5Y cells, hiPSC from patients with familial PD or yeast. We found that SH-SY5Y neuroblastoma cells expressing A53T α-synuclein showed significantly inhibited mitochondrial trafficking. A53T α- synuclein also caused the highest increase in ROS production in the dysmobilized mitochondria in comparison to wild-type or A30P α-synuclein. Treatment with NAP, the 8 amino acid peptide identified as the active component of activity-dependent neuroprotective protein (ADNP), completely annihilated the adverse effects of A53T on mitochondrial dynamics. During disruption of retrograde transport, we found disturbed autophagy and increased apoptosis signalization in neurons expressing A53T alpha-synuclein, suggest activation of the apoptosis pathway. Curiously, all groups expressing alpha-synuclein showed decreased levels of BCL-XL, revealing that mitochondria are susceptible to changes in the membrane potential in the presence of alpha-synuclein. Nevertheless, treatment with NAP was effective in blocking the apoptosis pathway and restore autophagy. We created a model to study A30P and A53T alpha-synuclein toxicity related to Gem (Miro), Ypt53 (Rab5) and Atg8 (LC3) genes in *Saccharomyces cerevisiae* in which these genes were knocked down. We found that A30P alpha-synuclein toxicity was dependent on mitochondrial and autophagy dysfunction. A53T alpha-synuclein was more toxic than A30P alpha-synuclein, and its aggregation was dependent on Gem expression. A53T alpha-synuclein toxicity involving damaged mitochondrial and apoptotic signaling caused by ER stress was dependent on Gem and Atg8 genes, respectively. In a study involving dopaminergic neurons derived from hiPSCs from patients containing triplicated alpha-synuclein gene ($SNCA^3$), we reported decreased mitochondrial trafficking and mitochondrial membrane potential, besides accumulation of lysosome vesicles. In a sensitivity assay, $SNCA^3$ neurons demonstrated more susceptibility to rotenone toxicity, which alters intracellular mitochondrial distribution, impairing retrograde transport of the organelle.

1. Introduction

1. Introduction

1.1. Demographic changes and neurodegenerative diseases

According to the World Health Organization (WHO) (2015), the world is aging as illustrated in the graph in Figure1. The aged population is larger in the high income Western countries than for instance in Mexico, Russia, South Africa and Brazil. However, Brazil also already experience changes in its demographic profile with a significant increase of aged population, as shown in Figure 2. Technological advances allow the development of new therapies, leading to an increased life expectancy. Consequently, the percentage of aged people older than 60 years is growing in the entire world. From 2015 to 2050, an increase of 22% in the aged world population is expected. That would lead to an increased burden of age-related health problems to the society, demanding it to adapt their health systems.



(*) Data refer to 1 January of each reference year. 1994: EU-27. 2014: provisional data given the non-availability of detailed data for Greece.
Source: Eurostat (online data code: demo_pjangroup)

Figure 1.: Illustration of European demographic changes from 1994 to 2014. Population of adults and aged men and women increased significantly from 1994 to 2014. Eurostat Statistic explained.

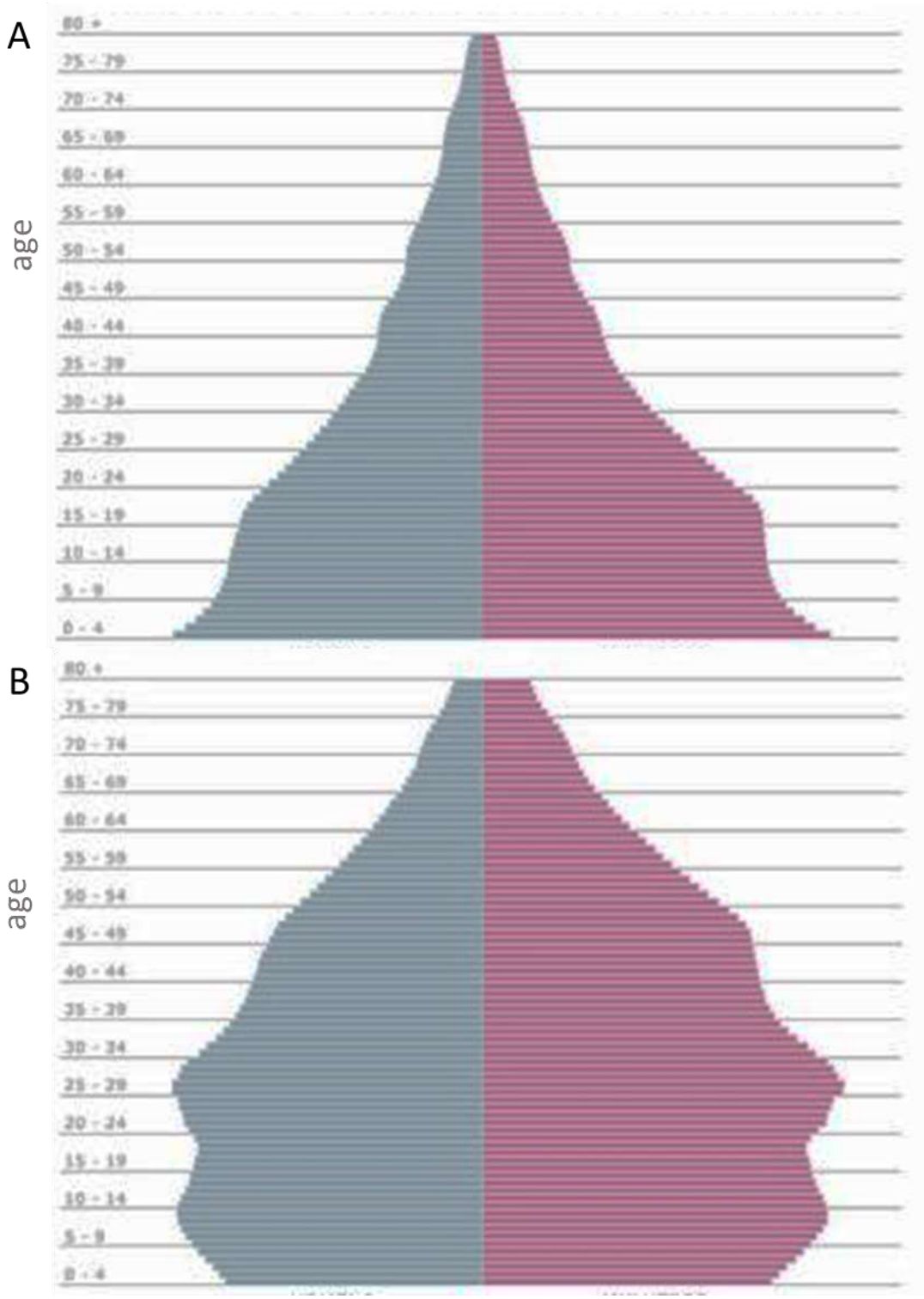


Figure 2.: Illustration of Brazilian demographic changes from 1980 (A) to 2010 (B). The population of men (blue) and women (pink) older than 60 years increased about 140 and 154% respectively. IBGE-Projeção da população.

A major age-related mental disorder is dementia, which is a chronic and progressive process that involves loss of memory and a decrease in social behavior and cognition abilities. Nowadays, 47.5 million people worldwide have dementia. It is expected that in 2050 there will be 135.5 million people living with dementia, mainly in low and middle-income countries such as Brazil and other countries in Latin America, where the prevalence of dementia is the highest compared to other regions in the world (Prince et al., 2013; Fagundes et al., 2011).

Dementia not only has an impact on people's life but also on their families and society, since it provides a major burden as far as care and costs are concerned. Until 2010, health systems around the world spent around US$ 604 billion per year on the treatment of dementia. The most common disease that leads to dementia is Alzheimer's disease (about 60-70% of cases of dementia is related to Alzheimer's disease), followed by diseases like vascular dementia, dementia with Lewy bodies and Parkinson's disease (PD). Yoritaka and collaborators (2016) have shown that the direct costs for PD outpatient clinics per month, at the University Hospital in Japan, are USD 485.74 per subject. Furthermore, 90.6% of the costs are related to drugs to treat PD (Table 1). They found that disease severity did not increase medical costs and they claimed that the costs in Japan are similar to Western countries.

Table 1. Monthly Cost of Out Clinic Parkinson's Disease per Subjects (US Dollars).

Factor	Category	n	cost		Univariate model			Multivariate inital model			Multivariate final model (Backward elimination p<0.05)		
			mean	SD	Coefficient Estimate	95% Confidence Interval	p value [for factor] for category	Coefficient Estimate	95% Confidence Interval	p value [for factor] for category	Coefficient Estimate	95% Confidence Interval	p value [for factor] for category
total		715	485.7	376.3		-	-		-	-		-	-
age	< 65 years	234	565.4	412.0	Reference	-	-	Reference		-	Reference	-	-
	≥ 65 years	481	447.0	351.7	-118.4	(-176.5, -60.2)	<0.001 ***	-65.6	(-132.3, 1.2)	0.054	-90.1	(-148.6, -31.5)	0.003 **
modified Hoen & Yahr stage	0	19	583.9	279.8	Reference	-	[0.410]	Reference	-	[0.367]			
	1	22	597.8	404.5	13.9	(-217.4, 245.3)	0.906	-6.4	(-304.3, 232.1)	0.955			
	2	172	508.7	386.6	-75.2	(-325.3, 103.4)	0.409	-82.3	(-257.4, 92.7)	0.356			
	3	222	449.0	355.3	-135.2	(-311.8, 41.4)	0.133	-135.2	(-310.3, 39.9)	0.130			
	4	183	484.3	369.9	-99.6	(-277.6, 78.4)	0.272	-86.7	(-265.8, 92.4)	0.342			
	5	70	494.7	472.3	-89.2	(-280.3, 101.9)	0.360	-52.4	(-245.2, 140.5)	0.594			
	Unknown	27	470.2	251.6	-113.7	(-334.9, 107.5)	0.313	-121.9	(-340.0, 95.7)	0.272			
disease duration	< ten years	277	431.3	327.0	Reference	-	-	Reference	-	-	Reference	-	-
	≥ ten years	438	520.2	550.0	88.9	(32.5, 145.2)	0.002 **	73.4	(13.8, 133.0)	0.016 *	67.5	(9.5, 125.6)	0.023 *
working	-	459	456.9	368.4	Reference	-	-	Reference	-	-			
	+	241	540.8	386.6	84.0	(25.4, 142.5)	0.005 **	41.0	(-26.0, 108.0)	0.230			
	Unknown	15	485.2	376.5	28.4	(164.7, 221.4)	0.773	9.1	(-181.2, 199.4)	0.925			
wearing off	-	255	389.6	303.8	Reference	-	-	Reference	-	-	Reference	-	-
	+	460	539.0	401.5	149.4	(92.7, 206.0)	<0.001 ***	126.0	(66.5, 185.4)	<0.001 ***	122.8	(63.9, 181.6)	<0.001 ***
hallucination	-	432	508.9	375.3	Reference	-	-	Reference	-	-	Reference	-	-
	+	283	450.4	375.7	-58.6	(-114.9, -2.2)	0.042 *	-62.8	(-121.7, -3.9)	0.037 *	-65.0	(-121.6, 8.5)	0.024 *
pain	-	484	480.9	368.9	Reference	-	-	Reference	-	-			
	+	231	496.0	392.1	15.0	(-44.1, 74.1)	0.619	20.1	(-38.3, 78.5)	0.500			

Table 1: Yoritaka and colleagues (2016) collected data about direct costs of 411 women and 313 men (724 patients in total) with PD who were participating in the "Specified Disease Treatment Research Program" at the University Hospital in Japan, during the period between June and December 2008.

1.2. Parkinson's disease development

PD was clinically described by James Parkinson in 1817 (Parkinson 1817; Goetz 2011), and nowadays, it is considered to be the most common neurodegenerative movement disorder (Tanner 1992). It is estimated that PD affects 2% of the population older than 60 years old. According to the Parkinson's Disease Foundation (2016), men are 1.5 times more affected by PD than women, and there are approximately 10 million people affected by PD around the world. Clinical symptoms of PD involve motor dysfunction as muscle rigidity, bradykinesia, balance disturbances, resting tremor, and non-motor symptoms such as cognitive decline, depression and deficit in olfactory and gustatory systems in the early stages of the disease, and mood alterations, sleep disturbances and dementia especially in the late stages of the disease (Paillusson et al., 2016; Saito et al., 2016; Chao et al., 2015; Cecchini et al., 2014; Weintraub et al., 2008).

Aging seems to be the main risk to develop PD. Degeneration of the substantia nigra (SN) is the main hallmark of PD and, therefore, it has been extensively investigated. The degeneration of the dopaminergic neurons located in the compact part of the SN has been suggested to start in the distal axon retrogradely disturbing and inhibiting fast axonal transport. The dopaminergic phenotype slowly disappears as evidenced by the decreased levels of dopaminergic markers such as tyrosine hydroxylase (TH). As a compensatory reaction, the expression levels of dopamine receptors such as D1 and D2 have been found to increase. Interestingly, these changes have also been observed during normal healthy aging (Rangel et al., 2015; Thanos et al., 2016; Keeler et al., 2016).

The first studies on PD pathology and aging showed a depletion of neurons as well as a decrease of pigmentation in the SN in both PD patients and healthy elderly subjects. The SN appears as a black structure in post-mortem brain tissue due to the cellular presence of neuromelanin, which is a pigment that accumulates throughout life at this region (Cabello et al., 2002; Rudow et al., 2008; Zucca et al., 2015).

As a consequence of the loss of the nigrostriatal dopaminergic projections, the levels of dopamine gradually decrease in the striatum during PD progress. Curiously, striatal dopamine levels also seem to decrease in aged healthy brains in a range about 10-13% per decade of life, and denervation of striatum is also found in the aged healthy brain (Carlsson and Winblad, 1976; Riederer and Wuketich, 1976; Kish et al., 1992; Kish et al., 1988; Hornykiewicz, 1989; Haycock et al., 2003). Some researchers suggested that an increased dopamine turnover might be a compensatory mechanism in the degeneration of dopaminergic neurons in PD. Interestingly, a similar compensatory mechanism was observed by other researchers investigating aging of the SN (Barrio et al., 1990; Sossi et al., 2002; Greenwood et al., 1991).

Disturbed dopamine metabolism may increase oxidative stress. Although dopamine levels are decreased during aging and PD, oxidative stress is present in both situations. This changed redox state in dopaminergic neurons is thought to be caused by mitochondria dysfunction. It is known that oxidative stress can lead to progressive accumulation of oxidative damage, which is known to accelerate aging and PD development (Jang and Remmen, 2009; Zucca et al., 2015; Kuter et al., 2016).

It is important to point out that a major factor in the process of human and animal aging involves heritability of longevity, suggesting that the aging process is not only modulated by life-style, but also has an important genetic component. Studies focusing on genes associated with the vulnerability of dopaminergic neurons have shown that genes involved in dopaminergic degeneration are also associated with normal aging. The most studied genes associated with PD appeared to be involved in the quality control of mitochondria and the modulation of oxidative stress; the same genes are associated with aging acceleration. Alpha-synuclein is an important pre-synaptic protein that plays a role in all neurons in the recycling of vesicles in synapses. Overexpression or anomalous conformation of this protein due to the presence of point mutations as well as a high oxidative environment lead to the oligomerization of α-synuclein and the formation

of amyloidogenic filaments. This will lead to the formation of aggregates and Lewy bodies, which are found in both PD and healthy normal brains (Passarino et al., 2016; Prinzinger, 2005; Yang et al., 2016; Devi et al., 2008; Weihofen et al., 2009; Giasson et al., 2000; Li et al., 2004; Polito et al., 2016).

Various researchers are investigating the differences between aging and PD in the brain. PD differs from aging mainly with respect to the higher level of cell loss (Rodriguez et al., 2015). It seems that PD is a consequence of aging restricted to a specific cell population in the brain, whereas aging itself affects all cells in the body. Moreover, only 4-5% of aged people develop PD. With so many similarities between aging and PD development, it is hard to conclude what changes lead to the disease. PD is thought to be the consequence of a complicated interaction between a wide variety of potentially toxic external stimuli and variable genetic susceptibility explaining the high clinical diversity among PD patients.

Therefore, together these changes observed in aged brain could be similar in the PD brains, leaving unclear the threshold between healthy aging and neurodegeneration.

1.3. Risk factors for Parkinson's disease

The etiology of PD is still largely unknown. Among the PD cases, 95% are considered sporadic and only 5% has a known genetic cause. Manifestation of PD has been thought to involve the chronic exposure to a set of environmental factors including pesticides, like rotenone, and herbicides. Familial PD implicates genetic susceptibility caused by one of several (point) mutations in the genes encoding for LRRK2, (leucine-rich repeat kinase 2), DJ-1, PINK1, parkin, GBA (glucocerebrosidase gene, Gaucher's disease), UCH-L1, PODXL (podocalyxin-like), SYNJ1 (PARK20), ATP13A2, SNCA (alpha-synuclein) and others. Autosomal dominant mutations in the

alpha-synuclein gene (SNCA) can lead to duplication or triplication of the gene, generating several copies of alpha-synuclein. A30P (G88C) and A53T are examples of PD linked point mutations in the α-synuclein gene involving the replacement of alanine by proline or threonine, respectively, at the indicated sites (Vilageliu and Grinberg, 2016; Sudhaman et al., 2016; Stefanovic et al., 2015; Park et al., 2015; Chen et al., 2015; Palmier et al., 2013; Ono et al., 2011; Lee et al., 2010; Narhi et al., 1999; Zhu et al., 2014).

Autosomal recessive mutations such as mutations in the GBA, parkin, PINK1, ATP13A2 and DJ-1 genes are likely linked to an early onset PD. Mutations in the parkin gene are the most common form of autosomal recessive PD. Parkin and Pink1 work together in regulating mitophagy, and in cases of mutations in parkin and PINK1, but also in ATP13A2, GBA and DJ-1, aberrant mitophagy is observed that leads to cell death. (Lesage et al., 2016; Hanagasi et al, 2016; Vilageliu and Grinberg, 2016; Song et al., 2016; Park et al., 2015; Noelker et al., 2015; van der Merwe et al., 2015).

The LRRK2 autosomal dominant mutation (G2019S) with gain of function is the most common known PD-associated gene and is also found in cases of idiopathic PD (Ramírez et al., 2016; Kalinderi et al., 2016). This mutation increases the risk to develop PD with 80% and it is linked to late onset PD. Curiously, the pathology in this case is independent of Lewy body formation (Kalia et al., 2015; Gaig et al., 2009).

1.4. Alpha-synuclein

Duplication of the *SNCA* gene leads to PD with a similar age of onset as sporadic PD (Ibanez et al., 2004; Chartier-Harlin et al., 2004). Triplication of the *SNCA* gene, however, is associated to an earlier onset of PD, indicating that high levels of wild-type (WT) alpha-synuclein via oligomerization and consequently multi-oligomer fibrillar protein aggregation, leads to cell

death (Figure 4D) (Olgiati et al., 2015; Ikeuchi et al., 2008; Johson et al., 2004; Singleton et al., 2004). Interestingly, in PD patients with a triplicated *SNCA* gene, the amount of alpha-synuclein mRNA in the brain is doubled, but the amount of soluble protein is not; aggregates, however, do emerge, suggesting that the increased alpha-synuclein amount is sequestered by intracellular aggregates, as exemplified in Figure 4C and D (Miller et al., 2004; Ahn et al., 2008). Apart from gene overexpression, mutations in the alpha-synuclein gene can also be linked to formation of oligomers. It has been demonstrated that human cell lines expressing point-mutated A30P or A53T alpha-synuclein contain high levels of oligomers (Marmolino et al., 2016).

Changes in alpha-synuclein expression may also be linked to sporadic PD. It is known that with normal healthy aging alpha-synuclein in the dopaminergic neurons of the substantia nigra can be redistributed from the synaptic terminals to the soma and the dendritic compartment (Figure 4B) (Xuan et al., 2011; Chu and Kordower, 2007). However, some investigators found unaltered levels of alpha-synuclein expression in the post-mortem midbrain of patients with PD and they claimed that alpha-synuclein did not play a role in these cases. More research is needed to elucidate the role of alpha-synuclein in the etiology sporadic PD, especially because other researchers have found indications that alpha-synuclein expression was changed in sporadic cases of PD: Studies revealed increased (Chiba-Falek and Nussbaum, 2001; Grundemann et al., 2008) or decreased (Kingsbury et al., 2004; Dachsel et al., 2007) levels of alpha-synuclein in midbrain of patients with sporadic PD (Tagliafierro and Chiba-Falek, 2016; Beyer et al., 2004; Tan et al., 2005).

It is clear that overexpression of alpha-synuclein plays a role in neurodegeneration; on the other hand, knock down of the alpha-synuclein gene can also lead to neurodegeneration, specifically in neurons located in the substantia nigra (Figure 4E) (Collier et al., 2016; Khodr et al., 2011; Gorbatyuk et al., 2010; Kanaan and Manfredsson, 2012). Interestingly, Vallortigara and colleagues (2015) demonstrated decreased levels of monomeric alpha-synuclein in the post-mortem brains of PD and Alzheimer patients who both had suffered from dementia; they correlated the low

levels of monomeric alpha-synuclein with synapse depletion and with the duration of dementia. These studies taken together strongly suggest that monomeric alpha-synuclein has an important role in the maintenance of healthy neurons, and that a decrease in the level of monomeric alpha-synuclein levels may induce mechanisms leading to dementia in PD and Alzheimer's disease.

Alpha-synuclein is a small protein composed of 140 amino acids predominantly cytosolic and can be found in both neuronal and glial cells of the thalamus, neocortex, hippocampus and substantia nigra. When first identified, alpha-synuclein was described as the precursor of non-amyloid-ß peptide. This protein belongs to the synuclein family that also contains beta- and gamma-synuclein. In physiological conditions, the N-terminal of the protein binds to lipid membranes and may go into a tetrameric helix form which has been reported to be resistant to fibril and aggregation formation (Tolmasov et al., 2016; Li et al., 2007; Kaganovich et al., 2008 Woods et al., 2007; Maiti et al., 2004; Sharma et al., 2001; Davidson et al., 1998).

The human *SNCA* gene is located on chromosome 4 (4q21.3-q22) (Chen et al., 1995) and in specific regions of brain, this gene can generate alpha-synuclein with 140 or with 126, 112 or 98 amino acids, when alternative splicing in exons 3 and 5 occurs (McLean et al., 2012; Beyer et al., 2006; Uéda et al., 1994). Alpha-synuclein has 3 main domains: the N-terminal, the central portion and the C-terminal as depicted in Figure 3 (Fusco et al., 2014). The N-terminal is highly conserved and allows the alpha helix secondary structure, two anti-parallel alpha helices and the formation of contiguous alpha helix interacting with lipid membranes (Surguchov, 2008; Jakes et al., 1994; Jao et al., 2004; Bussel and Eliezer 2003; Chandra et al., 2003; Davidson et al., 1998). The central portion of alpha- synuclein is prone to aggregate. Several studies investigated which specific characteristics of this portion determined its liability for aggregate formation. The first link between the central portion of alpha-synuclein and aggregation was made in a study of post mortem brains from patients with Alzheimer's disease. Investigators found that this central portion of the alpha-synuclein protein was highly hydrophobic suggesting that this is likely the main characteristic that

makes alpha-synuclein prone to aggregate (Pfefferkorn et al., 2012; Uéda et al., 1993). The C-terminal portion of alpha-synuclein plays a major role in the synaptic function of alpha-synuclein and the interaction with SNARE complex proteins. This portion exhibits a huge variability between species, most likely because post-translation modifications predominantly occur in this portion. These dynamic changes in the structure of alpha-synuclein may be dependent of environment and suggests that alpha-synuclein natively is an unfolded protein (Burré et al., 2012; Krumova et al., 2011; Oueslati et al., 2010; Burré et al., 2010; George 2001; Hasegawa et al., 2002; Weinreb et al., 1996; Uversky 2002).

Alpha-synuclein can interact with many lipids membranes and proteins, for instance with those related to calcium and dopamine homeostasis (Burré et al., 2010; Woods et al., 2007; Fountaine and Wade-Martins 2007; Founatine et al., 2008; Tehranian et al., 2006). Furthermore, alpha-synuclein can form multimers by self-assemblage forming insoluble aggregates in an irreversible process (Dettmer et al., 2015; Dettmer et al., 2013; Serpell et al., 2000; Bartels et al., 2011; Uversky and Eliezer 2009; Conway et al., 1998).

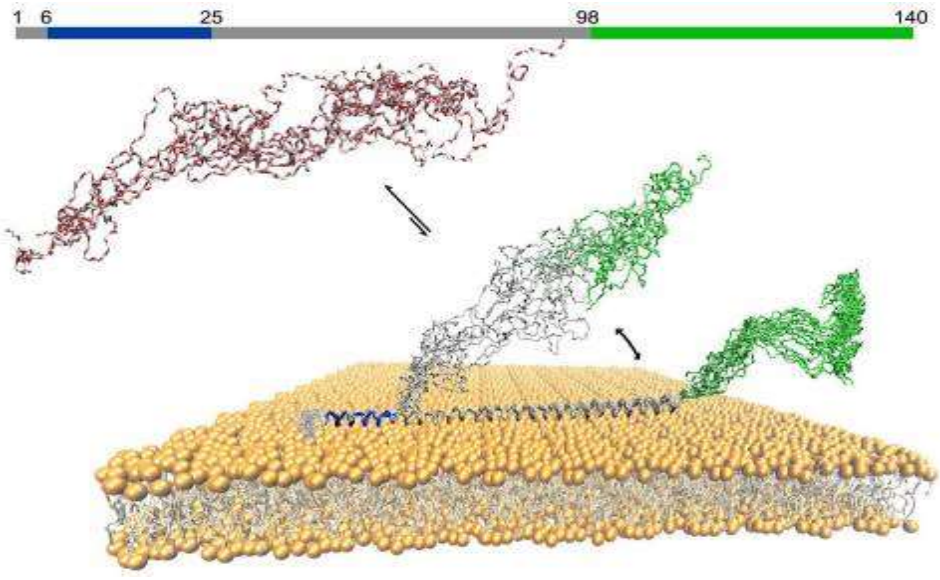


Figure 3.: Illustration of membrane affinity of alpha-synuclein. In blue: N- terminal portion;in grey: central region; In green: C-terminal fragment (Fusco et al., 2014).

The physiological function of alpha-synuclein remains unclear. It is known that, as a predominantly presynaptic protein, it participates in the process of trafficking, docking and endocytosis of vesicles in the presynaptic membrane (Vargas et al., 2014; Burré et al., 2012; Burré et al., 2010; Maroteaux et al., 1998). Alpha-synuclein can also be found in the soma, dendrites and axons, where the levels of this protein are very low (Figure 4A). Alpha-synuclein has been shown to be essential for the regulation of neurotransmitters release and in the cell response to cellular stress (Iwai et al., 1995; Maroteaux et al., 1988). Furthermore, alpha-synuclein can interact with TH (tyrosine hydroxylase), inhibiting dopamine biosynthesis, or with DAT (dopamine transporter), modulating dopamine transport. All these findings suggest that alpha-synuclein is crucial for normal/ healthy dopaminergic signal transfer (Khan et al., 2012; Bellucci et al., 2011; Perez and Hastings 2004).

Insoluble alpha-synuclein formation occurs as a consequence of genetic mutations, of deficits in degradation and under oxidative conditions (Follmer et al., 2015; Fredenburg et al., 2007; Narhi et al., 1999; Conway et al., 1998; Hashimoto et al., 1999). Some studies suggested that the elimination of alpha-synuclein could be crucial to prevent insoluble protein formation (Oshima et al., 2016; Gustafsson et al., 2016; Dehay et al., 2016). The monomeric form of the protein is predominantly degraded by chaperone-mediated autophagy (CMA); there are a few cases in which alpha-synuclein can be degraded by proteasomes. The oligomeric form of the protein is degraded by autophagy, independent of CMA (Vogiatzi et al., 2008; Webb et al., 2003). Mechanisms which favor the formation of oligomeric and insoluble alpha-synuclein during neurodegeneration in PD are not well understood. However, some studies have demonstrated deficits in degradation systems, linked to neurodegeneration. (Ritz et al 2016; Martella et al., 2016 Tanner et al., 2014; Marques et al., 2011).

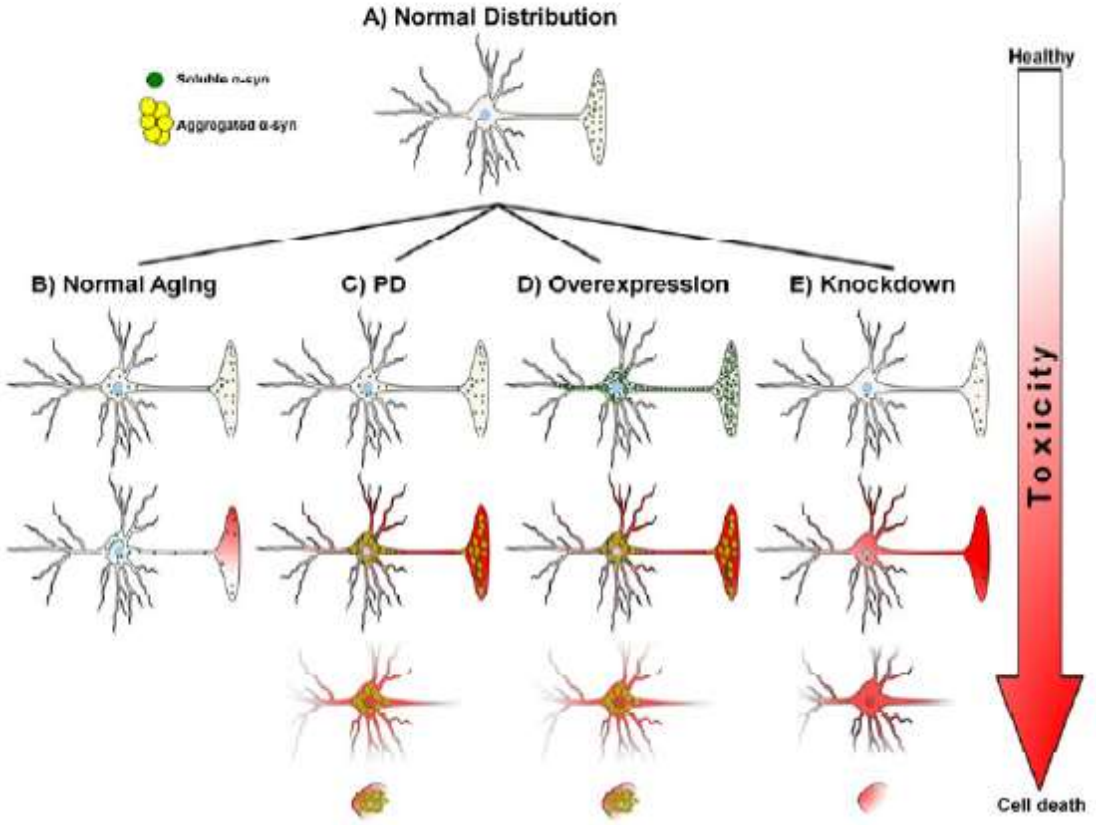


Figure 4.: Scheme of the function and localization of alpha-synuclein in dopaminergic neurons: (A) under normal conditions, where alpha-synuclein is mainly found at the synaptic terminal; (B) during normal aging, where alpha-synuclein can be found spread throughout the neuron; (C) in early onset PD, where alpha-synuclein is prone to aggregate; (D) in case of overexpression of alpha-synuclein,, leading to the spread out of the protein in the synaptic terminal and the soma; (E) when the gene is knocked down, leading to cell death (Benskey et al., 2016).

The degradation of alpha-synuclein has been extensively investigated. It has been demonstrated that small inclusions of the protein and puncta aggregates are ubiquitinated and driven to the ubiquitin proteasome system. However, proteasomes do not degrade big cargos such as aggregates or organelles. The degradation of ubiquitinated or non-ubiquitinated cargos, including alpha-synuclein, occurs preferentially through lysosomes by the autophagy process (Pallauf and Rimbach, 2013; Lynch-Day et al., 2012; Nagatsu and Sawada, 2006; Ciechanover et al., 2000).

In the autophagy process, cargos can be delivered in lysosomes via different pathways that are characteristic for microautophagy, chaperone-mediated autophagy or macroautophagy. In microautophagy, cargos that have been taken up from the cytoplasm and directly delivered in a lysosome membrane, are invaginated into the lysosomal lumen and promptly degraded. In chaperone-mediated autophagy, the cargo enters a lysosome through signaling of a chaperone, which binds to a receptor located in lysosome membrane. Macroautophagy is the best-studied autophagy process. In this type of autophagy, macromolecules and organelles are isolated in the cytosol by a double membrane forming a vesicle called autophagosome, which gets mature and fuses with a lysosome (Miller- Fleming et al., 2014; Klionsky, 2005; Majeski and Dice, 2004; Kunz et al., 2004). P62 is the protein which recognizes ubiquitinated cargos to initiate autophagy; it requires and binds to LC3 (Atg8 yeast ortholog), which drives vesicle formation and is present in both autophagosome membranes, as depicted in Figure 5. LC3 is found in two forms: LC3-I, present in immature autophagosome (or also called early endosome), and LC3-II, present in mature autophagosome (or also called late endosome). When the autophagosome becomes mature, LC3-I is converted into LC3-II. During the fusion process, LC3-II from the outer membrane fuses with the lysosome and is degraded with P62 and the vesicle. Several studies analyzed the autophagic flux and the removal of cargos by measuring LC3-II and P62 levels, respectively (Bang et al., 2016; Klionsky et al., 2012; Germain et al., 2011; Mizushima et al., 2010; Mizushima and Yoshimori, 2007).

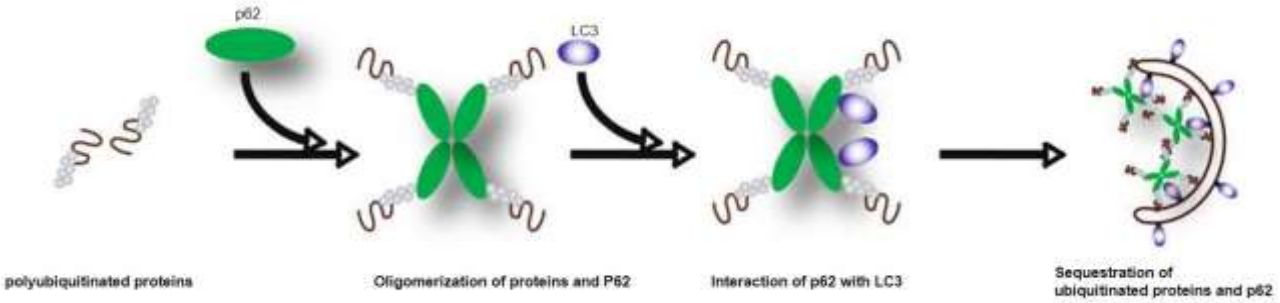


Figure 5.: Illustration of ubiquitinated proteins being recognizes by P62, that binds to LC3 that drives vesicle formation. Adapted from Komatsu and Ichimura (2010).

Mutated alpha-synuclein and large amounts of alpha-synuclein, due to overexpression, have been reported to disturb the ubiquitin-proteasome system and autophagy; interestingly, dysfunction of degradation pathways also leads to accumulation and aggregation of alpha-synuclein. During aging and in the presence of large amounts of alpha-synuclein or mutated A53T alpha-synuclein, chaperone-mediated autophagy and macroautophagy is inhibited. Curiously, autophagy inducers decreased alpha-synuclein levels, indicating that autophagy is crucial for alpha-synuclein degradation and for the prevention of aggregate formation (Shruthi et al., 2016; Ottolini et al., 2016; Ebrahimi et al., 2013; Dagda et al., 2013; Wu et al., 2013; Song et al., 2013; Decressac et al., 2013; Lee and Lee, 2002). On the other hand, aggregates can cause lysosome rupture, which might lead to mitochondria dysfunction. Moreover, especially in the presence of mutated A53T alpha-synuclein, endoplasmic reticulum homeostasis is disturbed inducing increased ROS levels and mitochondria dysfunction. Since autophagy has a global role of degradation in the cell, inhibition of this process can lead to high levels of ROS by impairment of the endoplasmic reticulum and reduced mitochondria removal. Especially in dopaminergic neurons, defects in the degradation of non-functional mitochondria causes a fast increase of ROS levels inducing neurodegeneration (Redmann et al., 2016; Lehtonen et al., 2016; Freemand et al., 2013). Together, these studies demonstrate that alpha-synuclein toxicity involves the impairment of degradation pathways, worsening the

consequences of protein accumulation and aggregation (see Figure 6).

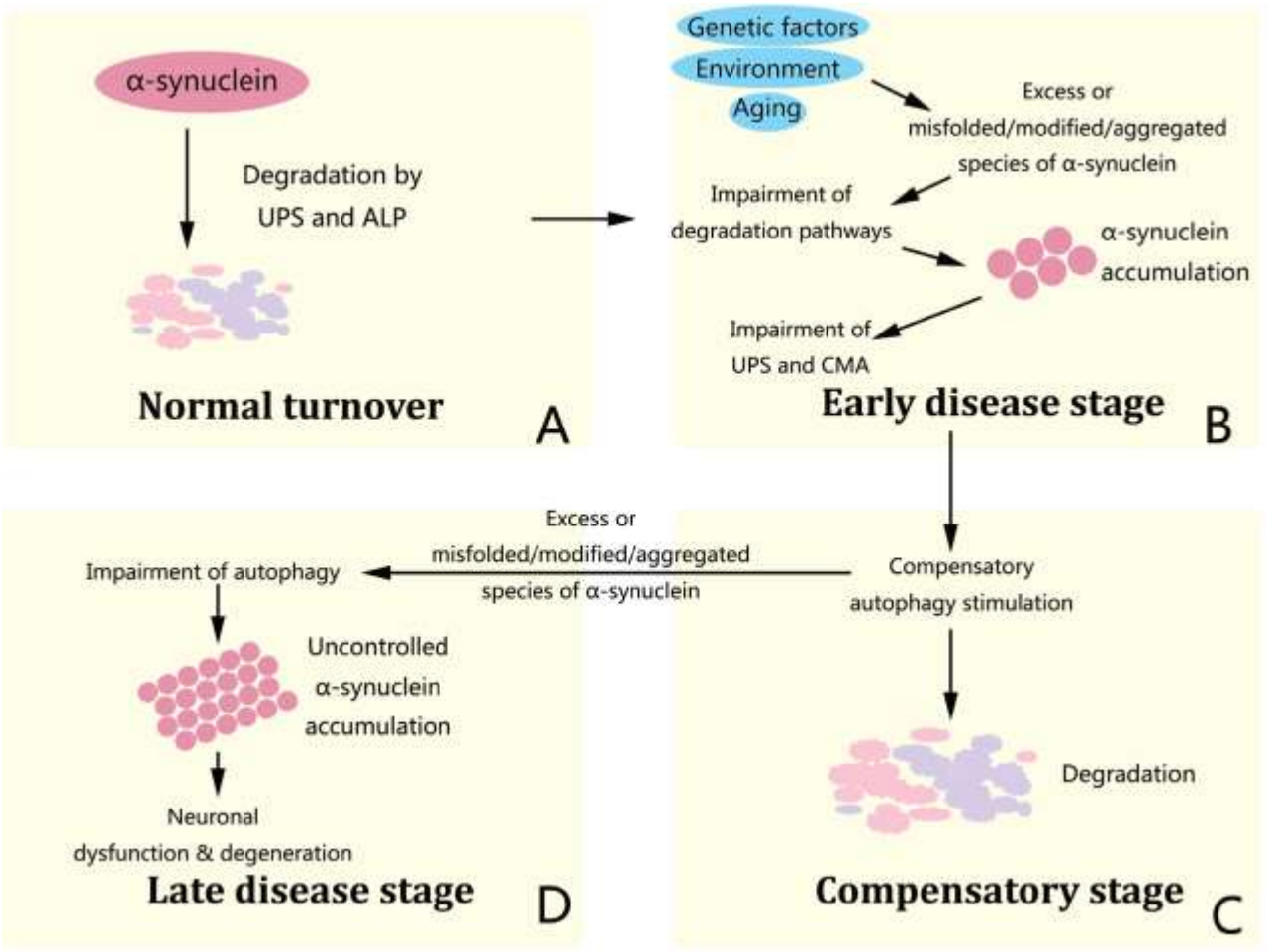


Figure 6: α-synuclein pathology in PD. The successive dysfunction of protein degradation pathways is crucial in α-synuclein pathology, neuronal dysfunction and degeneration. A. α-synuclein is regularly degraded via both UPS (ubiquin -proteasome system) and ALP (autophagy-lysosomal pathway). B. In the early disease stage, primary impairment of degradation pathways induced by genetic, environmental and age-related factors further prevents the degradation of α-synuclein by CMA (chaperone-mediated autophagy) and UPS and leads to its toxic aggregation in the cytoplasm. C. Afterwards, crosstalk among degradation pathways drives the induction of autophagy and temporarily compensate for degradation impairment. D. In the late disease stage, α-synuclein accumulation inactivates autophagy, leading to complete dysfunction of all protein degradation pathways and uncontrolled accumulation of α-synuclein, which consequently contributes to neuronal dysfunction and degeneration featured in PD (Li et al., 2015).

1.5. Dopaminergic degeneration

The best known symptom of PD is the presence of a resting tremor which seems to be one of the consequences of the degeneration of the dopaminergic neurons in the substantia nigra in the ventral midbrain (see Figure 7). These neurons are involved in the control of important functions of brain such as the initiation of voluntary movement, reward processing and working memory. Dopaminergic neurons can be classified into A8, A9 and A10 subtypes; the A8 dopaminergic neurons originates retrorubal field, the A9 subtype in the substantia nigra (SN) pars compacta in the ventral mesencephalon and the A10 neurons in ventral tegmental area (VTA). The A9 dopaminergic neurons in the SN are the most studied since they appear to be the main/firstly affected dopaminergic neurons in PD. Presently, much research is focusing on elucidating the pathways and molecules involved in the generation and the development of dopaminergic neurons, such information is of importance for the in-vitro generation of dopaminergic neurons from stem cells, a new approach in the development of cell replacement therapies to treat PD (Blesa, 2016; Roeper, 2013; Toulousse and Sullivan, 2008; Dahlstrom and Fuxe, 1964).

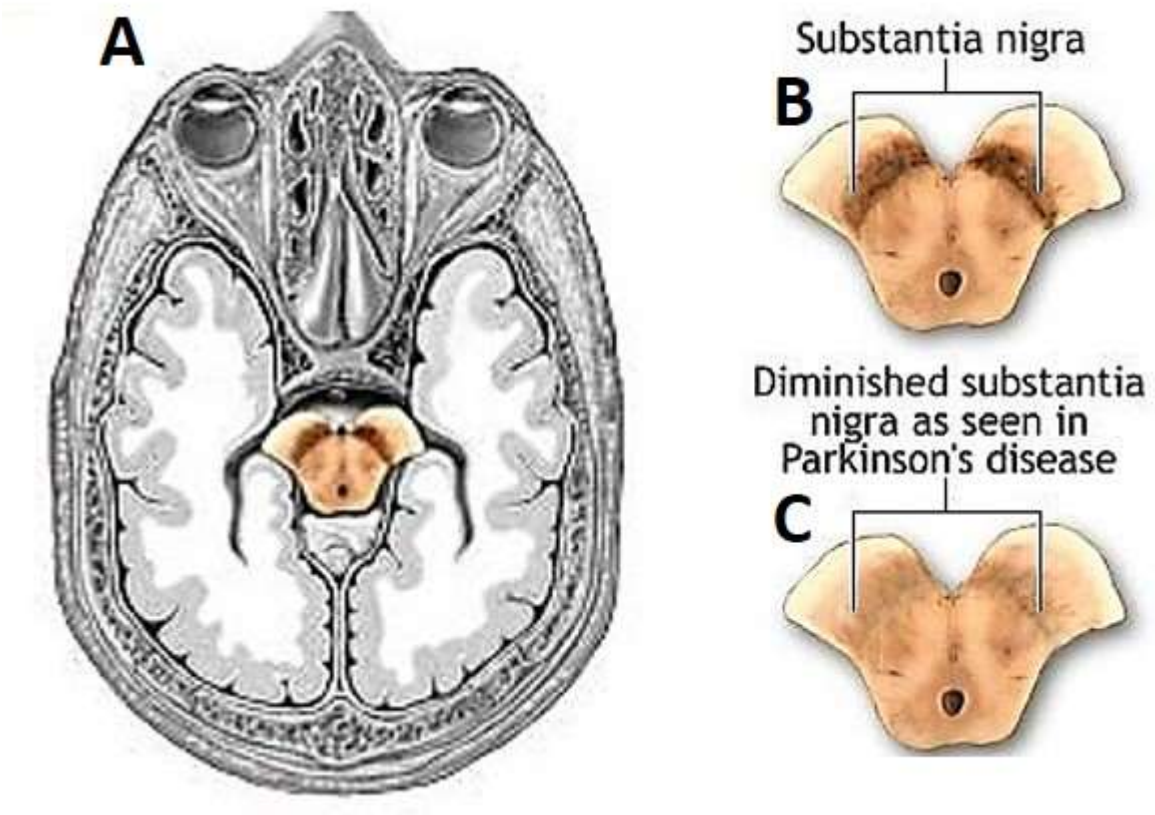


Figure 7.: Illustration of the SN in the healthy brain (A). Comparison of the healthy SN (B) with the SN of a patient with PD (C) clearly shows the loss of the black stained dopaminergic neurons in this region. "Substantia Nigra and Parkinson's Disease." A.D.A.M. Editorial Team. 5 May 2006. [Cited 10 December 2008] Available at <http://medicalimages.allrefer.com/large/substantia-nigra-and-parkinsons-disease.jpg>.

Mechanisms that underlie the process of neurodegeneration in the substantia nigra, are not well understood. It is known that the degeneration of the SN dopaminergic neurons coincides with the process of alpha-synuclein accumulation and aggregation, not only in the dopaminergic neurons but also in glia cells. Studies investigating the post-mortem brain of PD patients revealed the presence of cellular inclusions called Lewy bodies or Lewy neurites, which mainly contains alpha-synuclein. Therefore, alpha-synuclein aggregation has been considered a hallmark of both sporadic and familial PD in post-mortem analysis (Hurtig et al., 2000; Ince et al., 1998), suggesting that alpha-synuclein is a biomarker of PD (Atik et al., 2016; Barker and Williams-Gray 2016; Aarsland 2016; Koga at al., 2015).

Overexpression of wild-type (WT) alpha-synuclein or expression of the mutated A53T form of alpha-synuclein has been reported to reduce the release of dopamine through its covalent binding to dopamine and/or modulation of tyrosine hydroxylase; this interference in dopamine metabolism will consequently lead to cell toxicity (Orth et al., 2004; Perez et al., 2002; Xu et al., 2002; Tabrizi et al., 2000). Mutant A53T alpha-synuclein appears to be more prone to aggregate (including with dopamine) in comparison to A30P or WT alpha-synuclein (Marmolino et al., 2016; Winner et al., 2011; Moussa et al., 2008).

Although formation of protein aggregates can be related to mutations in proteins, this process also occurs naturally during aging and these aggregates can be found distributed throughout the central nervous system (Dayan, 1970; Johri and Bergman, 2003). These aggregates can lead to aging-related neuronal death but also to a variety of neurodegenerative diseases such as PD (Gibb and Lees, 1998). During aging, cells may be more vulnerable to oxidative stress leading to the damaging of proteins, lipids and nucleic acids. These physiological changes are worsening for a set of environmental factors and genetic susceptibility, facilitating neurodegenerative diseases development (Mattson and Magnus, 2006).

Alpha-synuclein aggregates have been found spread in brains affected by other neurodegenerative diseases such as Alzheimer's disease, Lewy bodies dementia, multiple system atrophy and others, so called under the term synucleinopathies (Kawakami and Ichikawa, 2015; Gassowska et al., 2014; Spillantini and Goedert, 2000; Spillantini, 1999). Although the function of alpha-synuclein function is still unclear, it is known that in dopaminergic neurons alpha-synuclein is a pre-synaptic protein with an important role in the synthesis, regulation, storage and release of dopamine; it is a crucial protein for synapses plasticity (Calo et al., 2006; Sidhu et al., 2004). Besides that, an increased number of copies of the *SNCA* gene is linked to PD since it has been shown to lead to accumulation of alpha-synuclein and Lewy's bodies formation (Zharikov et al., 2015; Fuchs et al., 2007).

It is known that some proteins are folded to form three-dimensional structures in cells. However, problems may occur in this folding process and some proteins can be misfolded or folded incompletely. In these cases, proteins may be prone to aggregate and spread (Mahul-Mellier et al., 2015). Besides that, it has been shown that the neurotoxic effect of alpha-synuclein is caused by the propensity of alpha-synuclein to form multiple oligomers (Pantusa et al., 2016; Outeiro et al., 2007).

The role of the protein aggregation process in the cell is unclear. Some studies showed that aggregates could lead to cell death (Ysselstein et al., 2015; Stefani and Dobson, 2003; Bence et al., 2001; Pike et al., 1991). In contrast, others studies showed that oligomers of alpha-synuclein (pre-aggregates) are more toxic than aggregates (Kaufmann et al., 2016; Lam et al., 2016; Deas et al., 2016; Lorenzen and Otzen 2014). In addition, it has been suggested that aggregate formation is a protective mechanism to prevent cell death (Mahul-Mellier et al., 2015; Arawaka et al., 2014; Danzer and McLean, 2011; Kaganovich et al., 2008).

The toxicity of alpha-synuclein oligomers has been studied in vivo, using animal models, and in vitro, using cells with induced overexpression of alpha-synuclein or with induced expression of A53T or A30P alpha-synuclein. Yeast or mammalian cells have been used to attempt to understand the oligomerization and aggregation process. These cell models have shown that aggregation is well-regulated process to prevent cell death (Rusmini et al., 2015; Rubinsztein, 2006; Chiti and Dobson, 2006; Sherman and Goldberg, 2001). Experiments with both mutated forms of alpha-synuclein revealed that the oligomeric ß-sheet-rich secondary structure of alpha-synuclein is highly toxic and is crucial for the development of PD pathology (Sharon et al., 2013; Conway et al., 2010; Lee and Trojanowski, 2006; Lee et al., 2002).

During PD pathogenesis, soluble oligomers, with high membrane-binding affinities, are thought to spread among neurons and glia cells with the seeding oligomers acting like prions (Miklya et al., 2014). In this theory, PD is considered a prion-like disease, comparable to prion

diseases such as bovine transmissible spongiform encephalopathy in animals and Creutzfeld-Jacob disease in humans. Interestingly, studies on post-mortem brains of PD patients that did receive a fetal tissue graft 10-15 years ago, revealed that Lewy body-like structures were present in the graft tissue, suggesting that alpha-synuclein, in a prion-like way, had entered the graft cells from the surrounding host cells (Helwig et al., 2016; Rey et al., 2016; Dikiy and Eliezer, 2012; Danzer et al., 2009; Li et al., 2008).

Experiments using animal models to study prion-like property of alpha-synuclein revealed that animals treated with alpha-synuclein fibrils showed the formation of alpha-synuclein aggregates and the development of synucleinopathy, including concomitant deficits in synaptic function. In addition, cultured cells internalize alpha-synuclein added to the culture media which appeared to promote aggregate formation. These aggregates can be secreted from cells via exocytosis and subsequently internalized by other neurons in culture via endocytosis, thus initiating a spreading cycle of seeding alpha-synuclein and aggregate formation. Holmqvist and collaborators (2014) revealed that monomeric, oligomeric or fibrillar alpha-synuclein can be transported from the enteric system to the brain via the vagus nerve using the slow and fast intracellular microtubule transport system. These results suggest an intimate link between the translocation of alpha-synuclein and the formation of aggregates. The above described propagation of alpha-synuclein seems to be essential for aggregate formation in familial as well as sporadic PD (Lööv et al., 2016; McCann et al., 2016; Paumier et al., 2015; Luk et al., 2012a; Luk et al., 2012b; Luk 2009; Volpicelli-Daley et al., 2011; Sidhu et al., 2004).

Neurodegeneration is a complex and multifactorial process in PD. The presence of aggregates and oligomers as cited above, can lead to cell death, but the mechanisms underlying alpha-synuclein toxicity remain unclear. Investigations concerning the toxicity of alpha-synuclein showed that the disturbance of the ubiquitin-proteasome system and the function of lysosomes also are involved (Yerbury et al., 2016; Su and Niu, 2015; Xu et al., 2015; Korczyn and Hassin-Baer,

2015; Chu et al., 2011). Moreover, concomitant impairment of mitochondria function generates oxidative stress (Guerra et al., 2016; Ghio et al., 2016; Qian et al., 2008). In PD, the activity of the mitochondrial complexes I and IV is preferentially affected in substantia nigra, leading to an increase of ROS (Reeve et al., 2015; Subramaniam et al., 2014; Binukumar et al., 2010; Przedborski, 2007).

1.6. The role of ROS in PD

ROS represent a set of molecules derived from the partial reduction of oxygen. Among ROS molecules there are the superoxide anion, hydrogen peroxide (H_2O_2), the hydroxyl radical and others. In physiological conditions, the role of ROS is thought to maintain cellular homeostasis since it can participate in modulating the cell cycle, in apoptosis, as well as in the signaling of NGF (nerve growth factor) and EGF (epidermal growth factor). Therefore, ROS also modulates growth, differentiation and cell survival (Chen et al., 2016; D'Autreaux and Toledano, 2007; Droge, 2002; Burhans and Heintz, 2009). However, in high levels, ROS are toxic and the response by anti-oxidants is not enough to maintain a normal redox homeostasis in the cell. Consequently, RNA/DNA (including mitochondrial DNA) oxidation and destabilization of replication and repair processes can occur, affecting cell-signaling, the ubiquination and proteasome system, leading to oxidative stress and cell death (Fischer et al., 2016; Kharel et al., 2016; Zhang et al., 2016; Gosh et al., 2012).

The term "oxidative stress" has been widely discussed. Halliwell and Gutteridge (2006), defined oxidative stress as a disequilibrium between the production of ROS or reactive nitrogen species (RNS), and the efficiency of removal of ROS and/or RNS including reparation of cellular damage. Alternatively, in the same year, Jones (2006), defined oxidative stress as a perturbation in the control and signaling involved between ROS and RNS and antioxidants. This last definition

seems more appropriate, since it recognizes the role of H_2O_2 and antioxidants, such as GSH, and their signaling pathways.

H_2O_2 is a less toxic form of ROS than the superoxide anion, and both catalase and glutathione in the oxidized form (GPx) can degrade H_2O_2. Alternatively, deficits of H_2O_2 degradation can lead to a reaction of this molecule with ferrous iron, generating a hydroxyl radical that is very toxic (Gammela et al., 2016; Labunskyy and Gladyshev, 2013). ROS can be generated as a consequence of various cellular processes such as the accumulation of alpha-synuclein or the presence of mutated proteins in cellular compartments such as the endoplasmic reticulum (ER) during stress. Notwithstanding, ROS are produced mainly in the electron transport chain at the inner mitochondrial membrane (Chen et al., 2016; Penke et al., 2016; Blesa et al., 2015; Santos et al., 2014; Santos et al., 2009; Turrens, 2003).

Mitochondria are crucial for the maintenance of healthy neurons, since these organelles are responsible for the producing energy (ATP); moreover, mitochondria DNA encodes for 7 essentials proteins of the respiratory chain (Devi et al., 2008; Mootha et al., 2003). Mitochondria are involved in the regulation of apoptosis, and the homeostasis of calcium and ROS levels. In normal functioning mitochondria, the electron chain generates a molecular cascade through respiratory chain. This cascade induces a gradient of protons over the mitochondria inner membrane, which is used for ATP synthesis. Electrons are extracted from reduced substrates and are transferred to molecular oxygen (O_2), through a chain of enzymatic complexes (I and IV). In the last step of the electron transport chain, cytochrome c oxidase (complex IV) completely reduces O_2 in water, without any formation of oxygen radicals. However, mainly in cases of mitochondria dysfunction, partial reduction of O_2 is observed, generating the radical superoxide anion. It is estimated that about 0.1%-0.5% of consumed O_2 by mitochondria are partially reduced, consequently forming the radical superoxide anion. This radical can be dismuted in H_2O_2 and O_2 by the Cu/Zn-SOD1 enzyme in the intermembrane space, or in the mitochondria matrix by Mn-SOD2 (Quirós et al., 2016; El-

Hattab and Scaglia, 2016; Arun and Donmez, 2016; Migdalska and Schapira, 2016; Lopert and Patel, 2016).

Mitochondria dysfunction is present during normal aging, leading to higher levels of ROS that accelerate alpha-synuclein aggregation and dopamine depletion, and so contributing to triggering neurodegeneration (Coxhead et al., 2016; Khan, 2016; Macconi et al., 2016; Fang et al., 2016; Kuter et al., 2016; Pandia et al., 2015; Kong et al., 2014; Navarro and Boveris, 2010). Especially dopaminergic (DA) neurons from the substantia nigra compacta (SNc) are susceptible to oxidative stress present during aging. In order to try to explain this susceptibility to ROS, some investigators proposed a theory, the free radical theory, based on a hypothesis formulated in 1950 by Denham Harman. According to this hypothesis, accumulated ROS causes damage in macromolecules which consequently leads to neurodegeneration (Zhao et al., 2016; Harman, 1956). During aging, the redox state of the brain is disturbed by a decreasing of antioxidants such as glutathione (GSH), contributing to ROS toxicity leading to all effects described above including genetic mitochondrial mutations, protein damage and ultimately the development of neurodegenerative diseases such as PD (Yuan et al., 2015; Currais and Maher, 2013; Zuo and Motherwell, 2013; Kudo et al., 1990).

1.7. Dopaminergic neurons

DA neurons from the SNc seem to be more vulnerable than other neurons (from other regions) to increased ROS levels that occur during normal aging. This specific property might be explained by their unique high metabolism. The reason that these neurons show a high metabolism is related to their autonomous activity as a kind of pacemaker, including during the absence of synapses. In the majority of neurons, Na^{+} channels present in the membrane are responsible for changing the membrane potential, but in DA neurons this process involves Ca^{++} channels. As a

consequence, DA neurons have a huge influx of Ca^{++}. To compensate this high amount of intracellular Ca^{++}. DA neurons increases their metabolism and endoplasmic reticulum (ER) and mitochondria start storing this "excessive" amount of Ca^{++} in order to re-establish the homeostasis (Duda et al., 2016; Dragicevic et al., 2014; Yabuki et al., 2014).

Under normal conditions, DA neurons are already exposed to high concentrations of ROS inherent to the dopamine metabolism, that includes a high concentration of iron generating ROS through Fenton reactions. Furthermore, DA neurons show a low concentration of antioxidants (Reeve et al., 2014). Human DA neurons located in the SN have a thin unmyelinated axon and a huge number of synaptic terminals (about 1 million per cell) which consume a large amount of energy; therefore, a considerable number of mitochondria are needed locally to support proper synaptic activity (Matsuda et al., 2009; Orimo et al., 2011).

In general, cells have specific protectors against ROS, which can be enzymatic or non-enzymatic. Among enzymes that participate in the redox metabolism, SOD (superoxide dismutase), GPx (glutathione peroxidase), catalase and PRxs (peroxiredoxins) are the main ones studied. Non-enzymatic ones include vitamin C (ascorbic acid), vitamin E (tocopherol), GSH, N-acetyl-L-cysteine, among others. These non-enzymatic antioxidants act mainly as chelators of metals, as blockers of peroxidation, or as components of cycles of oxi-reduction (Borella and Varela, 2004; Bonnefoy et al., 2002; Gilgun-Sherki et al., 2001). Dopaminergic neurons also have their own antioxidants and/or specific pathways of antioxidant action.

The dopamine transporter DAT is considered a selective protector of dopaminergic neurons against ROS produced in the dopamine metabolism. Oxidized dopamine is taken up by DAT at the nerve terminal, where dopamine is repackaged into synaptic vesicles. However, the level and activity of DAT decrease during aging leaving SN consequently more vulnerable to oxidative stress (Shingai et al., 2014; Emborg et al., 1998; Harrington et al., 1996).

Neuromelanin is a pigment responsible for the characteristic coloration of the substantia

nigra, and it has antioxidant properties. This pigment is present in both DA neurons from the SN and noradrenergic neurons located in the locus coeruleus. In addition, to dopamine and dopamine metabolites, various other proteins and lipids can be found in neuromelanin. The antioxidant properties of intraneuronal neuromelanin are thought to be related to its capacity to store metal, protecting the SN against oxidative stress (Clewett et al., 2016; Reimão et al., 2016; Zucca et al., 2015). During aging, the level of neuromelanin decreases which is thought to contribute to the development of PD. Moreover, dying dopaminergic neurons are thought to release neuromelanin, activating neuroglia and starting neuroinflammation, which is another characteristic of PD. Some investigators claimed that neuromelanin is directly involved in PD pathogenesis, since its decrease *leads to GSH* levels decrease and collapses mitochondria membrane potential, consequently leading to dopaminergic neurons depletion (Zucca et al., 2014; Naoi et al., 2008; Naoi et al., 2009).

GSH (glutathione) is considered a nonselective agent against ROS and the main thiol antioxidant in the entire brain, including the SN. GSH can neutralize ROS and reactive nitrogen species. Its antioxidant mechanism involves the reduction of the reactive species using the GPx enzyme. In this reduction reaction, GSH is converted into glutathione disulfide (GSSG); subsequently GSSG is reduced into GSH by glutathione reductase (GR) which uses electrons from NADPH. Besides that, the conjugation of GSH with electrophilic compounds mediated by glutathione-S-transferase (GST) and the subsequent elimination of these conjugated compounds, protect cells against toxic products generated by the cellular metabolism. During neurodegeneration, the redox state of cells can be altered increasing the reduction of GSH into GSSG. Whence, the cellular ratio between GSH/GSSG is widely used to indicate the redox state of cells (Flohé, 2016; Fang et al., 2016; Dickinson and Forman, 2002; Drigen, 2000, Griffith, 1999).

Interestingly, most studies about GSH levels showed that during aging the GSH levels decrease. Furthermore, GSH levels are more decreased in PD patients' brains compared to healthy aged brain, contributing to exacerbated oxidative stress, damaging SN (Zhou et al., 2015; Smeye

and Smeye, 2013; Sian et al., 1994).

1.8. Mitochondria dysfunction in PD

The average number of mitochondria per synaptic terminal is about 10; therefore, in total, each dopaminergic neuron can have more than 10 million mitochondria. Thus, mitochondria dysfunction will inevitably contribute to an increase in the amount of ROS in dopaminergic neurons, especially because about 0.2-2% of total oxygen, consumed in normal conditions, is naturally converted into free radicals in the mitochondria (Maharjan et al., 2016; Richter, 1992).

During the process of energy production by mitochondria, unpaired electrons are formed, mainly in the complexes I and III, facilitating the production of ROS which can accelerate aging and activate antioxidant enzymes like SOD and GPx, and antioxidant components like DAT (dopamine transporter), VMTA2 (vesicular monoamine transporter 2, which drives dopamine from the intracellular medium to synaptic vesicles), among others (Scialò et al., 2016; Lenaz et al., 2016; Forkink et al., 2015; Oka et al., 2015). Several studies have claimed that complex I disruption increases ROS levels in the matrix of mitochondria and in the cytosol, leading to glutathione depletion and consequently cell death. In addition, it has been shown that the level of ROS increases quickly throughout tissues, including the PD brain and platelets of PD patients in cybrid models (Abeti et al., 2016; Arduíno et al., 2015; Bronstein et al., 2015; Bender et al., 2006; Orth and Schapira, 2002).

To analyze the production of ROS in relation to mitochondria dysfunction, several studies used experimental animals and administered drugs that specifically block complex I of the mitochondrial electron transport chain. Among these set of drugs, MPTP and rotenone are the ones that have been shown toxic for dopaminergic neurons in humans who were unintentionally chronically exposed to them and consequently developed PD (Blesa and Przedborski, 2014;

Weisskopf et al., 2010; Burns et al., 1983; Ballard et al., 1985; Ramsay et al., 1991; Manning-Bog et al., 2002; Tanner 1989; Di Monte et al., 2002). Moreover, the protection selectively offered in dopaminergic neurons by for instance DAT and VMAT2 and nonselectively by SOD and GPx is downregulated in PD. This promotes an unbalance between the prevention of ROS toxicity and the production of ROS, leading to oxidative damage of proteins, nucleic acids and lipids, which are hallmark of PD (Pan et al., 2015; Nordström et al., 2015).

The mitochondria electron transport chain is the source of 90% of ROS produced in cells and is localized close to mtDNA (mitochondrial DNA), facilitating the emerging of mutations at mtDNA; it should be noted that mtDNA is not protected by histones and its replication cycle since their propagation might cause somatic mosaicism. Consequently, mutated mtDNA needs to be repaired quickly, otherwise, these mutations could be propagated causing somatic mosaicism, which is increased with aging (Zhao et al., 2016; Leman et al., 2015; Wang et al., 1998; Fayet et al., 2002).

Compared to other tissues, the brain appears to be more susceptible to mtDNA mutations, especially the SN: more than 40% of all deletions in mtDNA has been observed to occur in the SN in healthy sixty-year-old subjects (Ameur et al., 2011; Bender et al., 2006). Mutations in mtDNA and/or impairment of electron transport chain lead to mitochondria dysfunction and consequently energy depletion. A decreased energy production compromises the repair of damaged mitochondria and the quality control of mitochondria, making mitochondria an easy target for degradation (mitophagy) (Lauri et al., 2014). Damaged or depolarized mitochondria cause electron leakage, generating excessive ROS and releasing pro-apoptotic factors, such as cytochrome C, to initiate cell death (Harischandra et al., 2015; Brustovetsky et al., 2002). Beyond, electron chain inhibitors, impairing complex I, and they can interact with alpha-synuclein, especially with mutated forms of the protein which implies in ROS increasing. In the presence of rotenone, damage caused by mutated alpha-synuclein at complex I is potentiated. Curiously, basal expression of alpha-synuclein has been shown to protect against excessive ROS generation (Byers et al., 2011; Choong et al., 2011;

Nguyen et al., 2011; Betarbet et al., 2000).

Mitochondria form a highly interconnected network throughout neuron and its dynamics involves continuous autophagic destruction, via a macroautophagy process called mitophagy. The maintenance of proper mitochondrial function involves fusion and fission processes that alter mitochondrial morphology (van der Bliek et al., 2013; Bereiter-Hahn and Voth, 1994). Deficits in the fusion or fission machinery cause aggregation and loss of directed movement impairing proper migration of mitochondria into neurites. Furthermore, investigations on the impairment of fusion/fission revealed the spontaneous generation of mtDNA mutations, as demonstrated in a set of neurodegenerative disorders (Chen and Chan, 2009; Chen et al., 2003; Becker et al., 2009).

During high metabolic activity or stress, the mitochondria fusion process leads to an increase in the density of cristae allowing maximization of ATP production (Westermann, 2012; Youle and van der Bliek, 2012). The fission process is important for the transportation of mitochondria to regions with a high energy demand. In addition, this process is also involved in the segregation of impaired mitochondria from the network enabling their degradation through mitophagy (Youle and van der Bliek, 2012; Otera et al., 2013). Therefore, the balance between fission and fusion and mitophagy processes are essential for mitochondrial homeostasis. Particularly, aberrant fission has been shown to be involved in reduced mitochondria respiration, consequently leading to ATP (energy) depletion, increased ROS production and the release of pro-apoptotic factors during neurodegeneration (Archer, 2013; Jheng et al., 2012; Jahani-Asl et al., 2011; Detmer and Chan, 2007; Costa et al., 2010; Chen and Chan, 2009; Knott and Bossy-Wetzel, 2008; Burté et al., 2015; Yu et al., 2008; Chen et al., 2005).

Alpha-synuclein has been shown to interact with mitochondria membranes causing damage and changes in mitochondria fission and morphology (Nakamura et al., 2008; Nakamura et al., 2011). Alpha-synuclein plays a role in the maintenance of synaptic functionality, in the ubiquitin-proteasome system and in the regulation of oxidative stress (Clayton and George, 1998; Nemani et

al., 2010; Perez et al., 2002). In order to understand alpha-synuclein toxicity and to analyze the consequences for mitochondria dynamics, several models have been created in which alpha-synuclein was overexpressed or mutated forms of this protein were transfected. It was revealed that overexpression of alpha-synuclein inhibited the fusion of mitochondria membranes, disturbing the mitochondria cycle, which leads to fragmented or swollen mitochondria containing laminated bodies. Moreover, alpha-synuclein overexpression increased the colocalization of autophagosomes and mitochondria. Interestingly, siRNA-mediated knockdown of alpha-synuclein was able to prevent changes in mitochondria morphology resulting in elongated mitochondria (Hsu et al., 2000; Martin et al., 2006; Kamp et al., 2010; Ryan et al., 2015; Rostovtseva et al., 2015).

Mutations in alpha-synuclein may amplify mitochondria dysfunction. A53T alpha-synuclein colocalizes to the mitochondria membrane, disrupting complex I and interfering with the fission process and the autophagy machinery. However, mitophagy is blocked, leading to the appearance of fragmented mitochondria. Damaged mitochondria DNA and dysmorphic mitochondria were found in transgenic mice carrying A53T alpha-synuclein (Choubey et al., 2011; Chinta et al., 2011; Martin et al., 2006). These findings may have been a consequence of the altered affinity to membranes of mutated alpha-synuclein, since alpha-synuclein has been shown to interact mainly with outer mitochondria membrane. But, in specific conditions involving enough ATP supply and pH changes, alpha-synuclein can migrate to inner mitochondria membrane quickly changing mitochondria membrane potential, inhibiting complex I and leading to aggregation and fragmentation of the organelle (Cole et al., 2008; Liu et al., 2009; Shavali et al., 2008; Zhang et al., 2008; Devi et al., 2008; Mootha et al., 2003).

A proper mitochondria membrane potential is important for maintaining a normal ER morphology. In case of an alteration in the membrane potential of mitochondria, ER fragments have been shown to release calcium, causing high intracellular calcium levels, that leads to increased ROS levels. These findings suggest that both ER stress and mitochondria dysfunction contribute to

dopaminergic degeneration. Interestingly, mitochondria morphologic changes, caused by interaction of alpha-synuclein with the mitochondria membrane, are exacerbated in the presence of mutated alpha-synuclein like A53T alpha-synuclein. On the other hand, A30P alpha-synuclein does not exacerbate these changes, since this protein does not interact with mitochondria membrane (Bao et al., 2016; Ghio et al., 2016; Nakamura et al., 2011).

As cited above, mitochondria and ER are affected by alpha-synuclein toxicity. More research is needed on the crosstalk between both organelles which may lead to a better understanding of their dysfunction. Mitochondria and ER possess membrane contact sites (Figure 8), which allow a direct contact between organelles and exchange of metabolites, of signalization of organelle dynamics, of ATP metabolism, of protein folding and autophagy (van Vliet et al., 2014; Vance, 2014; Vishnu et al., 2014). Moreover, it has been shown that both organelles form contacts in synapses, promoting the calcium flow and synaptic activity (Krols et al., 2016; Mironov and Symonchuk, 2006). Together these studies strongly suggest that contact between mitochondria and ER are essentials to neuron survival.

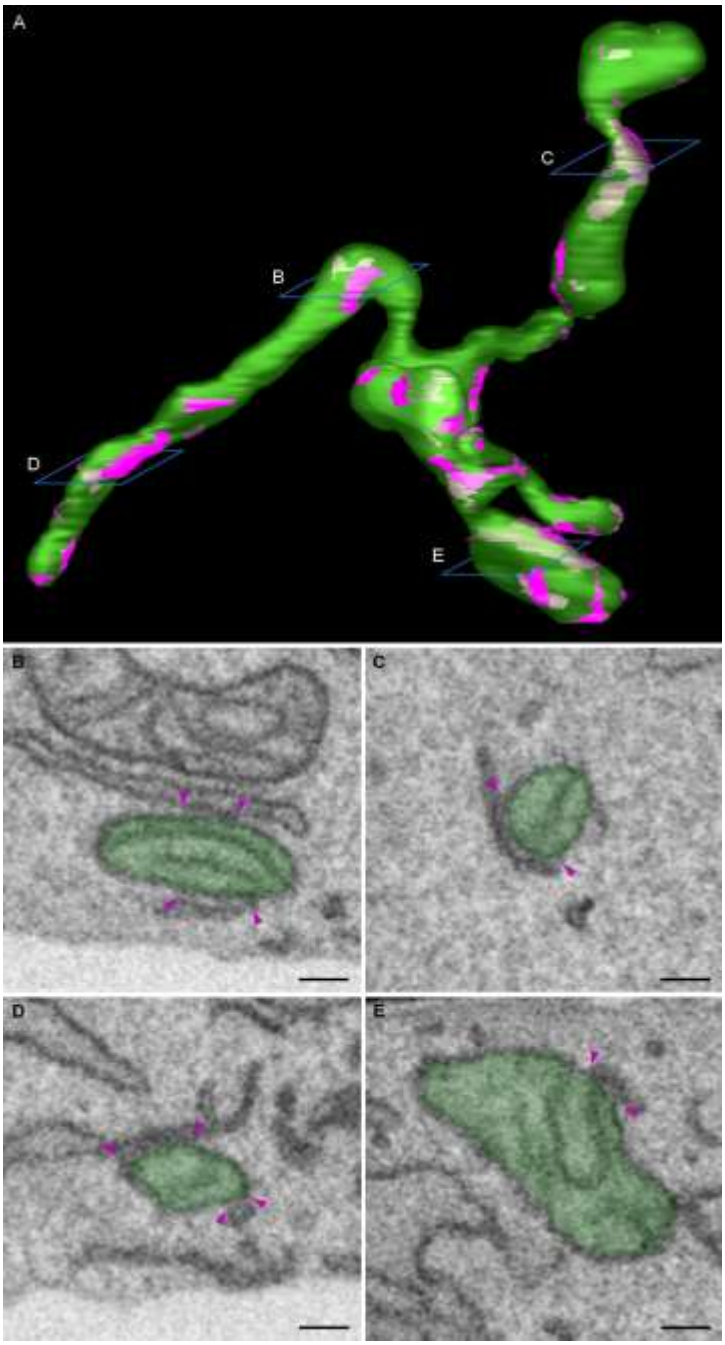


Figure 8: 3D morphology of ER-mitochondria contact sites. Mouse embryonic fibroblasts were imaged using a Zeiss Auriga Crossbeam focused ion beam scanning electron microscope (FIB-SEM). 3D reconstruction of a large cellular volume. The complete reconstruction of two mitochondria (transparent green) and their ER-mitochondria contact sites (magenta) is shown. A contact site is defined as a region where the ER and the mitochondrial membranes are in closer proximity than 30 nm. It is clear that a single mitochondrion makes multiple contacts with the ER and that these contacts are diverse in size, ranging from punctate sites to large patches of the outer mitochondrial membrane being juxtaposed to the ER. b–e Represent different examples of scanning electron micrographs extracted from the volume illustrating a section of the mitochondria and their contacts with the ER. The reconstructed mitochondria depicted in a are shown in transparent green. Magenta arrowheads mark the borders of the ER-mitochondria contact sites. The position of these slices is depicted in blue in a. Scale bar 200 nm (Krols et al., 2016).

It has been reported that mitochondria fission processes occur near the contact sites with the

ER even in the absence of mitochondria fission factors. Besides that, the mitofusins MFN1 and MFN2, which are proteins related to mitochondria fusion, depend for their activity on Miro1, a crucial protein associated with mitochondria trafficking and dynamics. Interestingly, Miro 1 is localized in sites of contact between ER and mitochondria. In addition, the yeast Miro ortholog, Gem, has been shown to participate in mitochondrial and endoplasmic division through interaction with membrane contact sites between organelles (Rowland and Voeltz, 2012; Misko et al., 2010; Fransson et al., 2006). The mitophagy process (mitochondria autophagy) also seems to be dependent of mitochondria and ER membrane contact sites. Several ATG proteins, including the ATG8 mammalian ortholog LC3, are found at mitochondria and ER contact sites. Moreover, it has been demonstrated that in yeast or mammalian cells, mitochondria and ER contact sites form a platform allowing mitophagosome biogenesis and mitochondria degradation (Böckler et al., 2014; Hamasaki et al., 2013).

Investigations focusing on mitochondria and ER contacts in PD, revealed that alpha-synuclein is located at mitochondria and ER contact sites. Furthermore, overexpression of alpha-synuclein appeared to increase mitochondria and ER contacts, affecting calcium transfer between them. However, in the presence of the A30P or A53T mutated types of alpha-synuclein, contacts between both organelles are inhibited, even more when Lewy bodies are present. (Guardia-Laguarta et al., 2014; Calì et al., 2012). This diminished contact between both organelles induces defective mitochondria fission. Since the presence of overexpressed or mutated alpha-synuclein also leads to blockage of autophagy, accumulations of mitochondria can be found (Manor et al., 2015). Therefore, inhibition of autophagy caused by alpha-synuclein, also increases in ROS levels by damaged mitochondria and ER stress, which will eventually lead to cell death.

Mitochondria dysfunction can cause, but also can be caused by, ER stress. Post-mortem brains from patients with PD and PD animal models both showed signs of ER stress. Overexpressed or mutated alpha-synuclein accumulates in the ER impairing protein folding and provokes stress in

this organelle. It has been reported that A53T alpha-synuclein increases levels of ROS by impairing mitochondria and ER function, causing cell death (Colla et al., 2012 Smith et al., 2005).

Stressed ER also generates ROS, decreasing GSH levels and transferring excessive calcium to mitochondria that also start to generate more ROS. GSH is the main molecule responsible for maintaining the redox state in ER and mitochondria. In case of accumulation of alpha-synuclein in the ER, GSH has been shown to oxidize activating the unfold protein response (UPR). In order to restore ER homeostasis, the protein Ire1α (inositol-requiring enzyme 1 alpha) activates UPR, which in turn activates chaperons such as Pd1 (protein disulfide isomerase) that increase the folding and secretion of proteins to be degraded and thus reestablishing the ER redox state. However, some proteins such as alpha-synuclein disrupt both the ubiquitin proteasome system and autophagy leading to ER stress and UPR activation. Secretion of alpha-synuclein promotes accumulation of the protein, contributing to formation of Lewy bodies (Labbadia and Morimoto, 2015; Brigelius-Flohé and Maiorino, 2013 Vembar and Brodsky, 2008; Scrhoder, 2008).

Pd1 is the main protein involved in the folding protein machinery of ER. The protein is found overexpressed in cases of ER stress, therefore, being one of the main markers of ER stress. During the protein folding process, Pdi oxidizes the protein generating disulfide bonds in proteins that become reduced. The enzyme Ero1 (endoplasmic reticulum oxidoreductase 1) oxidizes Pdi, reactivating this protein that becomes ready for another cycle of protein folding. Once reduced, the protein Ero1 transfers oxygen to molecular oxygen generating H_2O_2. In addition, accumulated protein in ER favors calcium leakage to cytosol (Feissner et al., 2009; Malhotra and Kaufman, 2007; Tu and Weissman, 2004; Hayne et al., 2004).

Mitochondria also play a role in calcium buffering; mitochondrial uptake of excessive calcium released from the ER, consequently increases its metabolism as well as the production of ROS. Further, folding process (in ER) requires high levels of ATP. Thereby, prolongated UPR activation promotes high levels of ROS (Malhotra and Kaufman, 2007).

It has been demonstrated that in DA neurons UPR activates *XBP1*, yeast ortholog *Hac1*, which plays a role in activating gene expression to promote neuron survival. Alternatively, in the long-term presence of excessive or misfolded proteins, Hac1 can activate apoptotic genes such as CHOP that drives neurons to death. Besides that, Ire1α promotes the alternative splicing of HAC1 mRNA, which is also considered one of the main markers of ER stress. Therefore, UPR plays a paradoxical role in neurons: initially it activates mechanisms to ameliorate ER stress, however in long term UPR activation leads to cell death (Zeeshan et al., 2016; Krols et al., 2016; Mercado et al., 2016; Grimm, 2012; Delic et al., 2012; Szegezdi et al., 2006; Nikawa et al., 1996).

All these reports make clear that the crosstalking between mitochondria and ER is important to maintain homeostasis in these organelles, but that it can also trigger cell death.

Alpha-synuclein harms mitochondria in several ways. Emerging studies regarding the first steps of neurodegeneration have claimed that alterations in intracellular trafficking are essential to neuron survival. Experiments in cells with different levels of alpha-synuclein expression in the absence of aggregates, revealed that high levels of this protein can lead to hyperphosphorylation of tau protein, causing destabilization of microtubules and so impairing intracellular trafficking of vesicles and organelles. These findings suggest that alteration in intracellular trafficking is one of the first steps of neurodegeneration and can favor aggregates formation. It has been demonstrated that the disrupted trafficking of mitochondria impairs ATP supply at specific sites such as synaptic terminals; moreover, it impairs the generation of new/healthy mitochondria by fusion and fission processes at the soma (Creedle et al., 2015; Nguyen et al., 2014; Kaul et al., 2011; Billingsley e Kincaid, 1997).

1.9. Impaired intracellular trafficking in PD

Mitochondria quality control is essential to neuronal survival. This process involves the

trafficking of mitochondria towards neuron regions demanding much energy and the return of the mitochondria to the soma for recycling/repair, since that is where fusion and fission processes preferentially occur. Axons of dopaminergic neurons of the SN account for 95% of the cell volume and recruit a significant portion of its energy. Anterograde mitochondria trafficking concerns the axonal transportation of mitochondria from the cell body to the synaptic terminals. For recycling or in cases of mitochondria damage and dysfunction, the mitochondria need to be retrogradely transported to the cell body, where mitophagy involves, lysosomes and the ubiquitin -proteasome processes to degrade damaged mitochondria (Lehmann et al., 2016; Gumeni and Trougakos, 2016; Florenzano, 2012; Cheng et al., 2010; De Vos et al., 2008; Brookes et al., 2004).

Since neurons are polarized cells and possess long axons, intracellular trafficking is crucial to neuronal survival, morphology and function (Hirokawa et al., 2010). Motor proteins from the kinesin family (KIFs), and other proteins like dynein, dynactin are responsible for the maintenance of intracellular trafficking along the microtubules (Hirokawa 2010; Hirokawa, 1998). The direction of trafficking is dependent of the polarity of specific sites. In the axons endings and distal dendrites microtubule polarity is positive. However, in the proximal dendrites the polarity is mixed between positive and negative, while in the soma microtubular polarity is positive at the distal portion after microtubule organizing center (MTOC) (Xiao et al., 2016).

Injuries in the cytoskeleton are responsible for the changes in the rearrangement and movement of organelles in several neurodegenerative diseases such as PD. Microtubules participate in diverse cellular functions like motility, cell division, transportation of organelles, vesicles and proteins, in the maintenance of cellular morphology and in the general organization of the cytoplasm. Microtubular dynamics are regulated by the concentration of free tubulin. Intriguingly, in PD, alpha-synuclein and Lewy bodies were found to be colocalized with free tubulin and with the tubulin polymerization promoting protein (TPPP), suggesting that alpha-synuclein might be involved in the harming of intracellular trafficking through microtubules stabilization impairment

(Szunyogh et al., 2015; Oláh et al., 2011; Lee et al., 2006; Wade, 2009; Morris and Hollenbeck, 1995).

A growing body of literature has suggested that disrupted axonal transport is intimately involved in triggering PD (Hunn et al., 2015; Cheng et al., 2010). It has been suggested that alpha-synuclein impairs mitochondria axonal transport by disturbing the expression of motor proteins, like the kinesins/dynein and the miro/milton complex. In addition, alpha-synuclein also seems to disrupt their interaction with microtubules (Gilley et al., 2012; Sterky et al., 2011; Yang et al., 2010; Bueler, 2009). Other studies demonstrated that during neurodegeneration, alterations in motor proteins might occur with consequences for proper mitochondria trafficking (Cooper et al., 2012; Cai et al., 2005). In our previous studies, we showed that, before protein aggregation, expression of the anterograde motor proteins KIF1Bα and KIF5 as well as of the retrograde motor proteins dynein, dynactin and syntaphilin were altered in cell cultures and in animals treated with rotenone. In addition, we demonstrated that mitochondria trafficking was synchronized with the expression of anterograde motor proteins and that alterations in these proteins lead to specific changes in mitochondria trafficking. We have also found that the expression in the expression of Rabs 1 ,3, 4, 5, 6, 11 and 32 was altered before protein aggregation in aged rats treated with rotenone (manuscript in preparation). Together, these studies strongly suggest that altered intracellular trafficking is an important part of PD pathogenesis.

To transport cargos such as mitochondria, motor proteins associate with the adaptor proteins Trak (drosophila ortholog Milton) and Miro, which are attached to the outer mitochondria membrane, as illustrated by Figure 9 (Devine 2016).

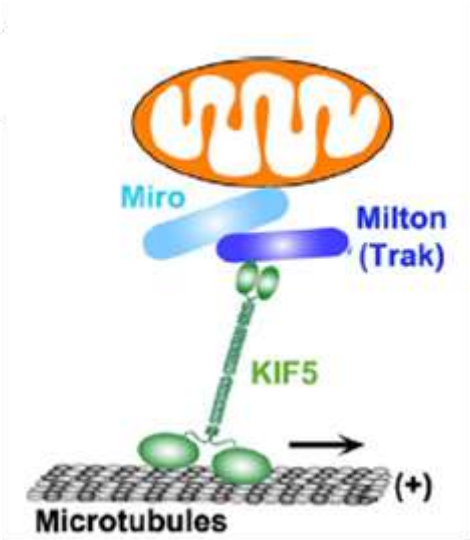


Figure 9: The Miro- Trak (Milton) adaptor complex mediates KIF5-driven mitochondrial transport. Adapted from Sheng (2014).

Interestingly, experiments in which the expression of Miro was modified demonstrated that increased Miro expression leads to increase in mitochondria transport, indicating that this protein can regulate mitochondria dynamics (Chen and Sheng, 2013). In addition, loss of Miro caused defective trafficking in both directions, suggesting that Miro is an adaptor for both anterograde transport (via interaction with KIF5) and retrograde transport (via interaction with dynein). (Russo et al., 2009; Guo et al., 2005). Furthermore, studies on mitochondria fragmentation and interconnectivity showed that non-functional Miro led to mitochondria trafficking impairment and fragmentation, while overexpression of Miro increased mitochondria trafficking and interconnectivity, resulting in an increased mitochondria length in neurons (MacAskill et al., 2010; MacAskill et al., 2009; Fransoon et al., 2006).

Miro is a calcium (Ca^{+2}) sensor containing 4 calcium-binding EF-hands. It has been shown that increased levels of calcium dissociate motor proteins from Miro and Trak, blocking mitochondria trafficking (see Figure 10). This process is crucial for the anchoring of mitochondria at specific sites where much ATP is required, for instance at the synapses. When ADP slows down,

stationary mitochondria move to another site with low ATP levels. However, the impairment of mitochondrial trafficking can lead to dysfunction of mitochondria at the site where they anchored, causing their increased generation of ROS (Klosowiak et al., 2013; Liu et al., 2009; Wang and Schwarz, 2009; Saotome et al., 2008; Mironov, 2007).

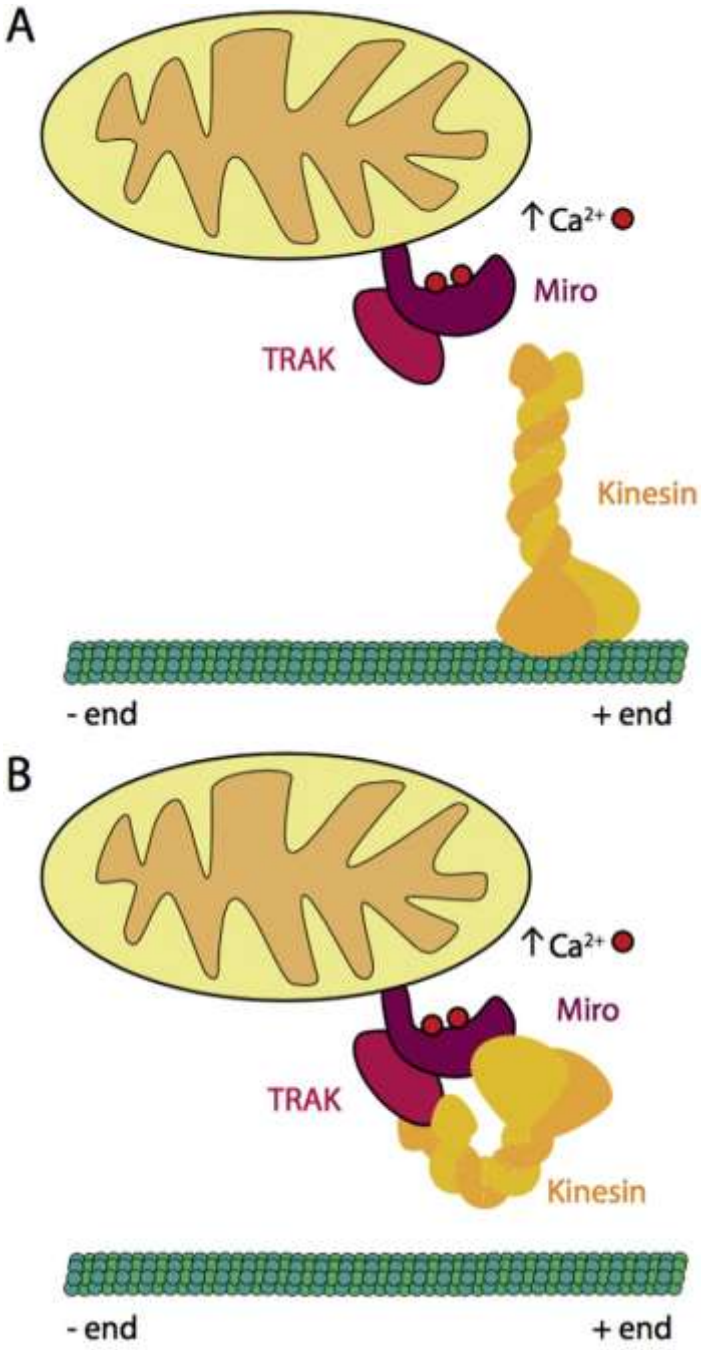


Figure 10: Models of Ca^{2+}-dependent mitochondrial arrest. According to the first model (A), calcium binding to Miro EF-hands induces the release of the protein from the kinesin motors, determining the detachment of the mitochondrion from the microtubule tracks. In the second model (B), calcium binding to Miro promotes the detachment of the kinesin motors from the microtubules and the interaction of their motor domains with Miro, causing mitochondrial arrest. Adapted from Devine (2016).

It has been reported that Miro also interacts with Mfn (mitofusin) proteins, which play a role in mitochondria fusion. Intriguingly, mitochondrial trafficking is decreased in neurons in which MFN2 was knocked-out, suggesting that Miro and MFN work together in the regulation of

mitochondria trafficking (Saotome et al., 2008). It is known that Miro is associated with the mitochondrial outer membrane; it coordinates the transport of mitochondria moving together with ER and it takes care that mitochondria stay close enough for the initiation of fusion or fission processes (Friedman et al., 2011). Once in contact, Mfn2 and 1 interact with Miro and both are required for proper axonal transport (Misko et al., 2010), suggesting association of these proteins and balanced trafficking are essential for the fusion process. Absence of Miro has been shown to exacerbate mitophagy. Curiously, transgenic Mfn2 knockdown mouse showed blocked mitophagy (Liu et al., 2012). It has been shown that ER provides lipids to the formation of membrane vesicles in autophagy (Axe et al., 2008). These lipids are transferred and accumulated at the outer mitochondria membrane before they are transported to the site of vesicle formation and mitophagy initiation (Hailey et al., 2010). However, alterations in Miro or MFN proteins can disturb these processes, revealing that both proteins are needed for proper fusion and mitophagy processes.

Damaged mitochondria are targeted to mitophagy via PINK1 signaling. Parkin forms a complex with PINK1 in the process of mitophagy and ubiquitinates substrates at the outer mitochondria membrane, including Miro, which triggers the mitophagy process. It has been suggested that Miro works like a receptor for both proteins, since Miro interacts with PINK1 and Parkin allowing their association with the outer mitochondria membrane. Moreover, damaged mitochondria require fast Miro ubiquitination mediated by Parkin. In addition, fibroblasts of patients carrying parkin mutations showed altered Miro turnover, suggesting that Miro is intrinsically involved in the regulation of fusion/fission and mitophagy events (Birsa et al., 2014; Kazlauskaite et al., 2014; Hardy, 2010).

As cited previously, alpha-synuclein toxicity in the ER and mitochondria can change calcium levels in cytosol. However, the mechanisms underlying this process in PD are poorly understood. Furthermore, Dučić (2015) demonstrated that alpha-synuclein plays a role in the regulation of intraneuronal calcium levels. Together these findings suggest that alpha-synuclein

impairs mitochondria dynamics and mitophagy, leading to disrupted mitochondria trafficking via the Miro signaling.

The specificity of intracellular trafficking of organelles among cellular compartments is strictly regulated by small GTPases (Rabs) from the Ras super family of proteins. Besides that, Rabs are responsible for the correctly attach of motor proteins and cargos, for the motility of cargos and their delivery to the correct destination. Furthermore, Rabs can be involved in alpha-synuclein toxicity. It has been shown that extracellular alpha-synuclein also contributes to the formation of Lewy bodies. Moreover, Sung and collaborators (2001) revealed that alpha-synuclein added to the culture medium is internalized by cells via an endocytic pathway that is dependent of Rab5. Mechanisms behind alpha-synuclein propagation are unclear. However, a recent study demonstrated that alpha-synuclein can be propagated by the traveling of lysosome vesicles along tunneling nanotubules from cell to another cell (Abounit et al., 2016).

Rab 5 is a multifunctional protein that regulates the first steps in endocytic pathways, in anchoring, trafficking, and fusion of endosomal membranes and in recycling through autophagy (Olchowik and Miaczynska, 2009). In addition, mutated Rab5 leads to an accumulation of enlarged early and late endosome/phagosomes and defects in the regulation of trafficking of endosomes/phagosomes to lysosomes involving Rab7 (Girard et al., 2014; Wegner et al., 2010; Torres et al., 2010). Rab 5 has been suggested to be involved in trafficking of early endosomes/phagosomes. After vesicle maturation, Rab 7 coordinates the fusion of late endosomes with autophagosomes and LC3 requirement (Wang et al., 2016). Together, these findings indicate that Rab 5 is involved in the formation and transportation of immature endosomes/phagosomes and LC3 signaling, contributing to the first steps of autophagy (Figure 11).

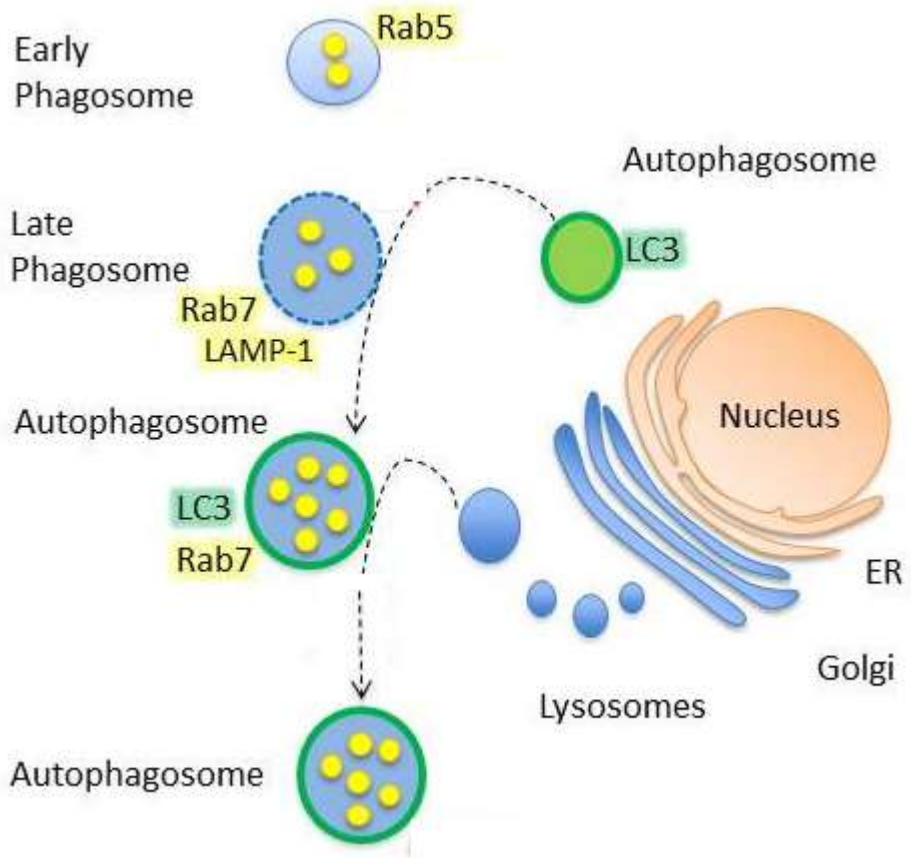


Figure 11: Involvement of Rab5 and Rab7 in vesicular trafficking associated with autophagy. Adapted from Lòpez de Armentia (2016).

Investigations on intracellular trafficking and autophagy dysfunction in neurodisorders, revealed that degradation via lysosomes is crucial for the proper, balanced axonal trafficking of vesicles and lysosomes. Further, impairment in intracellular trafficking leads to the accumulation of lysosome vesicles causing axonal swelling and neurite dystrophy. Other studies have shown that alterations in the endocytic pathway lead to an accumulation of endolysosomes (endosomes driven to lysosome) impairing autophagy and causing accumulating of alpha-synuclein (Magalhaes et al., 2016; Peric and Annaert, 2015; Lee et al., 2011).

Intracellular trafficking is essential to neuronal survival. Changes in mitochondria or lysosome vesicles can lead to autophagy dysfunction and ultimately cell death. The dysfunction of alpha-synuclein and mitochondria plays a major role in trafficking impairment. Rab5, LC3 and

Miro play unique roles in endocytosis and trafficking of earlier endosomes, in autophagy and in the dynamics of mitochondria and ER. However, alpha-synuclein toxicity related to trafficking and Rabs, Miro or LC3 are poorly understood.

1.10. Models to study PD

In order to investigate PD pathology, a variety of models have been created to address the clinical, tissue, cellular and molecular characteristics of PD.

Animal models and primary cell cultures are widely used. Primary cell culture is a fast way to study single neurons. Animal models may provide insight in the systemic toxicity of alpha-synuclein. Interestingly, investigations on alpha-synuclein trafficking showed that alpha-synuclein can be transported from the enteric system until the SN, where this protein accumulates and aggregates (Holmqvist et al., 2014). Obviously, this alpha-synuclein propagation could only be demonstrated in animal models with experimentally induced PD since animals do not develop naturally neurodegenerative diseases like PD.

SH-SY5Y cell line (neuroblastoma) is one of the most frequently used cellular models to study PD. It is a neuronal human cell line which can be quickly and inexpensively differentiated into neuron-like cells. In addition, chronic exposure to neurotoxins or overexpression of different types of alpha-synuclein can mimic a PD phenotype. Nevertheless, the line is derived from a malignant tumor and, therefore, its basic physiology is altered. In the analyses of experiments with the SH-SY5Y cell line, there should be awareness that the SH-SY5Y derived neurons are incomparable to true, mature human mature neuron (Kovalevich and Langford, 2013).

S. cerevisiae (budding yeast) have been considered an important model to study the cellular biology, biochemistry and genetics of eukaryotes. This organism shows cellular pathways, proteins and genes that are well conserved during evolution (Smith and Snyder, 2006). Approximately 30% of yeast genes have known human ortholog genes, allowing studies on them with respect to the

development of human diseases (Walberg, 2000). Furthermore, neurodegenerative diseases such as PD comprise the formation of protein aggregates, most often formed by protein misfolding processes. Mechanisms related to protein folding, oligomerization and aggregation can be studied in yeasts since protein quality control is conserved in these organisms (Ciaccioli et al., 2012; Khurana and Lindiquist, 2010). Therefore, yeast humanized models to study PD are also very well accepted (Franssens et a., 2013).

Ten years ago, Yamanaka demonstrated that somatic cells could be reprogrammed into pluripotent stem cells (Takahashi and Yamanaka, 2006). The possibility to generate these induced pluripotent stem cells (hiPSC) from patients and to differentiate them into any cell type (so also the cell type that is specifically affected in that patient) has presented a unique tool for studying the development of neurodegenerative disorders apart from other applications (Fig.12). In order to address mechanisms underlying PD, researchers have started to differentiate hiPSC from PD patients into dopaminergic neurons for the analysis of pathogenesis of PD; it is obvious that PD iPSC derived dopaminergic neurons provide a much more appropriate cell model than any of the PD cell models used so far. However, still a number of hurdles have to be taken related to the genetic and epigenetic signatures still present in iPSC-derived cells. Moreover, much more efficient differentiation protocols for DA neurons and particularly for their purification still have to be developed (Kang et al., 2016; Jacobs et al., 2014; Devine et al., 2011; Marchetto et al., 2010).

It is clear that most experimental models for PD have their limitations and draw-backs. The researcher needs to choose the model that is the most appropriate and accurate to answer his specific detailed research questions.

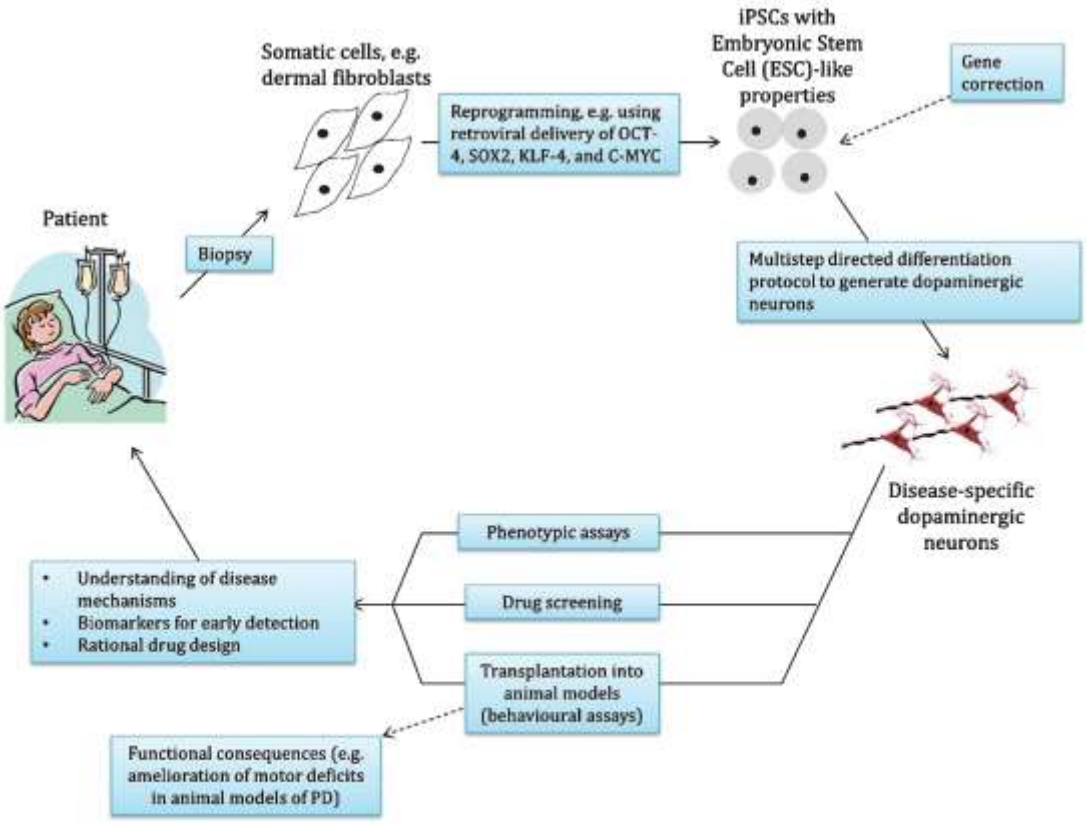


Fig. 1. The iPSC paradigm for modelling PD and other neurodegenerative diseases.

Figure 12: The iPSC paradigm for modeling PD and other neurodegenerative diseases (Jacobs et al., 2014).

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2. Objectives

2. Objectives

The objective of this study is to analyze mitochondria trafficking and functions of mitochondria and autophagy after expression of WT, A30P or A53T alpha-synuclein in the following cellular models: neuron-like cells derived from SH-SY5Y line, humanized yeast cell and hiPSC-derived dopaminergic neurons from patients containing triplicated alpha-synuclein gene ($SNCA^3$).

2.1. Specific objectives

1- To analyse mitochondria trafficking, distribution and connectivity in neuron-like cells derived from SH-SY5Y, overexpressing WT, A30P or A53T alpha-synuclein.

2- To analyse mitochondria-mediated apoptosis and autophagy in neuron-like cells derived from SH-SY5Y, overexpressing WT, A30P or A53T alpha-synuclein.

3- To compare the effects of NAP (microtubule stabilizer) upon mitochondria trafficking, distribution, connectivity, mitochondria-mediated apoptosis and autophagy in neuron-like cells derived from SH-SY5Y, overexpressing WT, A30P or A53T alpha-synuclein.

4- To evaluate A30P and A53T alpha-synuclein toxicity through viability and sensitivity assays in yeasts Δ (knockout) for Gem, Ypt53 and Atg8, during fermentation or respiration metabolisms.

5- To quantify H_2O_2 produced by mitochondria in yeasts Δ for Gem, Ypt53 and Atg8, cultivated in intermediary medium (cells are able to respirate and fermentate), expressing A30P and A53T alpha-synuclein.

6- To analyse GSH/GSSG ratio in yeasts Δ for Gem, Ypt53 and Atg8, cultivate in respiratory medium expressing A30P and A53T alpha-synuclein.

7- To analyse ER stress, UPR activation and apoptosis signalization through ratio between unspliced HAC1and spliced HAC1, PDI and CHOP protein levels.

8- To reprograme fibroblasts from control and $SNCA^3$ patients into hiPSC.

9- To differentiate hiPSC into dopaminergic neurons.

10- To analyse mitochondria trafficking and lysosome vesicles in human $SNCA^3$ dopaminergic neurons.

11- To analyse viability and vulnerability of human $SNCA^3$ dopaminergic neurons through exposure to rotenone.

12- To analyse mitochondria localization in human $SNCA^3$ dopaminergic neurons after exposure to rotenone.

3.Results

In order to, facilitate visualization of results this section was divided into 3 parts. Part I shows results obtained using SH-SY5Y model, Part II shows results obtained using humanized yeast model and Part III shows results obtained using hiPSC-derived dopaminergic neurons.

Part I

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3. Results

3.1. Impairment of mitochondria dynamics by human A53T α-synuclein and rescue by NAP (davunetide) in a cell model for Parkinson's disease.

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Abstract

The formation of oligomers and aggregates of overexpressed or mutant α-synuclein play a role in the degeneration of dopaminergic neurons in Parkinson's disease by causing dysfunction of mitochondria, reflected in their disturbed mobility and production of ROS. The mode of action and mechanisms underlying this mitochondrial impairment is still unclear. We have induced stable expression of wild-type, A30P or A53T α-synuclein in neuronally differentiated SH-SY5Y neuroblastoma cells and studied anterograde and retrograde mitochondrial trafficking in this cell model for Parkinson's disease. In contrast to wild-type and A30P, A53T α-synuclein significantly inhibited mitochondrial trafficking, at first retrogradely and in a later stage anterogradely. Accordingly, A53T α-synuclein also caused the highest increase in ROS production in the dysmobilized mitochondria in comparison to wild-type or A30P α-synuclein. Treatment with NAP, the eight amino acid peptide identified as the active component of activity-dependent neuroprotective protein (ADNP), completely annihilated the adverse effects of A53T on mitochondrial dynamics. Our results reveal that A53T α-synuclein (oligomers or aggregates) leads to the inhibition of mitochondrial trafficking, which can be rescued by NAP, suggesting the involvement of microtubule disruption in the pathophysiology of Parkinson's disease.

Introduction

Parkinson's disease (PD) is the most common neurodegenerative movement disorder affecting 1% of people above 65 years of age (Tanner 1992). The pathology of PD is characterized by the degeneration of dopaminergic neurons in the substantia nigra. This neurodegeneration is thought to be associated with the formation of aggregates containing α-synuclein (Siderowf and Stern 2003) and with increased oxidative stress (Qian et al. 2008; Gruden et al. 2011; Simcox et al. 2013). The vast majority of PD patients have the sporadic form of this disease which appears to be age related. A small percentage of PD is familiar and caused by specific mutations. The α-synuclein

gene (*SNCA*) was the first gene definitely associated with familiar PD, and there are three known missense point mutations, A53T and A30P and E46 K, besides duplication and triplication of *SNCA*, that all lead to an early disease onset (Polymeropoulos et al. 1997; Kruger et al. 1998; Singleton et al. 2003; Chartier-Harlin et al. 2004). Various genome-wide association (GWAS) studies have shown that SNPs in *SNCA* (and *MAPT*) are also common risk factors for sporadic PD (Edwards et al. 2010; Lill et al. 2012; Nalls et al. 2014).

The molecular mechanisms underlying the neurodegenerative effects of α-synuclein oligomers and aggregates seem to involve most prominently the mitochondria. Although direct damaging effects of α-synuclein oligomers and aggregates on mitochondria have been described, indirect effects on the processes of autophagy and trafficking of mitochondria may be involved as well. Alterations in intracellular degradation pathways, such as macro-autophagy, have been observed in many studies linking protein aggregation mechanisms with neurodegeneration (Victoria and Zurzolo 2015). It has been demonstrated that overexpression of α-synuclein can lead to inhibition of autophagy and concomitant α-synuclein accumulation, whereas the knockdown of α-synuclein resulted in autophagy enhancement, suggesting that α-synuclein may have a regulatory role in autophagy (Winslow et al. 2010) The presence of damaged mitochondria by the direct action of (mutant) α-synuclein oligomers, on the contrary, appears to stimulate excessive mitophagy leading to mitochondrial fragmentation (Wang et al. 2012; Perfeito et al. 2014).

Normal mitochondrial turnover depends on a proper balance between anterograde and retrograde trafficking (Arnold et al. 2011); in PD, this mitochondrial turnover appears to be impaired (Simcox et al. 2013; Hunn et al. 2015). In anterograde trafficking, mitochondria are transported from the soma to the axon up to the synaptic terminals; by retrograde trafficking, the mitochondria return to the cell soma for breakdown and re-entering the biogenesis cycle (Amiri and Hollenbeck 2008). Retardation in anterograde transport can result in an abnormal cellular distribution of mitochondria and a decrease in ATP levels at the synapses (Cai et al. 2005);

impairment of retrograde transportation leads to an accumulation of mitochondria in the synaptic terminal interfering with proper synapse formation and function (Van Laar and Berman 2009).

Alpha-synuclein oligomers and aggregates seem to interfere directly with normal mitochondrial turnover (Qian et al. 2008; Celardo et al. 2014). Alterations in axonal transport and in the level of motor proteins have been observed in transgenic *Drosophila* co-expressing tau and α-synuclein, in postmortem brain tissue of sporadic PD patients, in animal and cellular models of sporadic PD and in rats overexpressing α-synuclein (Chu et al. 2012; Chaves et al. 2013; Melo et al. 2013; Roy and Jackson 2014). Studies have shown the interaction between α-synuclein and tau (Credle et al. 2015; Magdalinou et al. 2015). In neurons, tau is essential for stabilizing microtubules and so for enabling proper motor transport (Wade-Martins 2012). In case of overexpression of α-synuclein, tau is phosphorylated, leading to its loss of function and the subsequent impairment of trafficking (Magen et al. 2014; Credle et al. 2015). Esteves et al. (2014) demonstrated that α-synuclein oligomers are able to disrupt microtubules, leading to abnormal axonal trafficking and consequently mitochondrial dysfunction. In particular, the α-synuclein gene mutation A53T is able to form oligomers and aggregates more easily and faster than other types of α-synuclein (Giasson et al. 2002). Accordingly, it was demonstrated that in particular A53T α-synuclein significantly reduced mitochondrial motility in cellular models for PD in which human A53T α-synuclein was expressed, i.e., in mouse hippocampal neurons and SH-SY5Y neuroblastoma cells (Xie and Chung 2012) or in mouse cortical neurons (Li et al. 2013).

Activity-dependent neuroprotective protein (ADNP) is essential for brain formation and provides neuroprotection throughout the entire adult brain; ADNP mRNA and protein expression responds to brain injury and a variety of cytotoxic insults. Structure-activity studies have identified a short eight amino acid peptide in ADNP, NAPVSIPQ (abbreviated to NAP) that appears to be responsible for neuroprotection (Bassan et al. 1999; Zamostiano et al. 2001; Gozes 2007). Treatment with NAP has been shown to restore microtubule integrity and to rescue microtubules-

dependent axonal trafficking, and, with that, mitochondrial function (Bassan et al. 1999; Zamostiano et al. 2001; Gozes 2007; Esteves et al. 2014). NAP also contributed to functional recovery in mice overexpressing α-synuclein by reducing hyperphosphorylated tau levels (Magen et al. 2014).

In the present study, we aimed to analyze in more detail the effects of A30P or A53T α-synuclein on anterograde and retrograde mitochondrial trafficking in SH-SY5Y neuroblastoma cells in which we managed to induce a stable expression of wild-type, A30P or A53T α-synuclein. In addition, we have studied the effect of NAP treatment on

the mitochondrial mobility and function in these cells.

Materials and methods

Cell culture

SH-SY5Y cells (passage 17), obtained from ATCC cell culture, were not used above passage 35 as these cells are reported to lose their neuronal phenotype after repeated passaging. Cells were maintained in DMEM (1×) supplemented with 15% FBS, 1% Pen/Strep, 100 mM Napyruvate and 2 mM Glutamax (DFCS) in tissue culture treated dishes or flasks. At 70–80% confluence, cells were passaged using trypsin/EDTA (Lonza) following general cell culture procedures. Split ratios ranging from 1/20 to 1/40 were used to ensure similar densities among transgenic lines. To initiate differentiation into neuron-like cells, SH-SY5Y cells were plated at a density of 2 × 104 cells/ cm2 in wells pre-treated with poly-d-lysine (PDL). After 1 day, cells were exposed to 10 μM retinoic acid (Sigma) in DMEM (1×) supplemented with 10% FBS for 5 days, after which medium was replaced to DMEM 1× (High Glucose) containing 10 ng/ml BDNF (Peprotech), in order to promote the outgrowth of neural extensions.

Construction of viral vectors

Viral vectors empty or containing wild-type α-synuclein and mutant α-synuclein A30P or A53T (pENG1-3, a generous gift by Ellen Nollen) were constructed by PCR amplification (Phusion High-

Fidelity DNA Polymerase, ThermoScientific) with overhangs for SpeI and NsiI. PCR amplicons and pSin- EF2-Nanog-Pur (Adgene plasmid 16578) were restricted using BcuI (SpeI, Thermo Scientific) and Mph1103I (NsiI, Thermo Scientific), and subsequently ligated using T4 ligase (ThermoScientific) after which the product was transformed in DH5α competent cells. PCR screening was performed to select positive colonies, which were checked by restriction analysis. Correct plasmids were sent for sequencing and transfected in HEK293T to validate expression. Vector constructs are shown in Supplemental Fig. S1.

SH-SY5Y viral transduction and transfection

Lentiviral particles were generated using a modified protocol based on the protocol of Trono et al. (http://tcf.epfl.ch/page-6764-en.html). Briefly, HEK-293T cells were transfected when cells were 70–80% confluent. A mixture containing 100 µl OPTIMEM (Gibco) 1.4 µg viral vector, 0.4 µg pMD2- VSV-G and 1 µg pCMV-D8.91, was supplemented with 6 µl FUGENE HD (Lonza) and incubated for 15 min at room temperature (RT) to generate transfection complexes. The next day medium was changed with 2 ml OPTIMEM, and viral particles were harvested between 36 and 48 h. Viral supernatant was collected and sterilized using a 0.45 µm filter (Nalgene), mixed with 10% DFCS in a 1:1 ratio and supplemented with polybrene (8 µg/ml). This mixture was added to a 6-well plate, and 1 × 105 cells were added to be transduced in suspension. The next day media were changed to DFCS 15%, and cells were placed on puromycin (2–4 µg/ml, Sigma) selection three days after transduction. Puromycin selection was continued during cell culture of the lines.

Characterization of SH-SY5Y by immunofluorescence

Transgenic cell lines cultured on PDL-coated coverslips were fixed in paraformaldehyde 4% for 20 min at RT and rinsed in PBS 3 times. Samples were permeabilized and blocked with PBS containing 0.1% Triton, 1% BSA and 5% normal goat serum for 60 min at RT. Samples were incubated with rabbit anti MAP2 (1:1000, Abcam), mouse anti α-synuclein (1:500, Invitrogen) or rabbit anti TOM20 (1:1000 Santa Cruz FL-145) antibodies at RT for 1 h followed by incubation of

fluorescent secondary antibodies (1:400) and Hoechst for 1 h at RT. Images were acquired using a Leica AF-6000 fluorescent microscope.

Mitochondria mobility

SH-SY5Y were transfected with pDsRed2-Mito (Clontech Cat. nr: 632421) in OPTIMEM (Gibco) using Lipofectamine 2000 (Invitrogen), following manufacturer's instructions. After 1 day of transfection, cells were exposed to 200 μg/ml of G418 for 2 weeks in order to select for cell lines containing the plasmid. SH-SY5Y were differentiated as described above, and mitochondrial mobility was measured in live cells after 4, 6 or 8 days of differentiation, using spinning disk confocal microscopy at 63× objective in a climate controlled chamber. The track was calculated by image comparison of the same field every 10 s for 20 min. Single-cell image stacks were analyzed using the ImageJ difference tracker plugin. Kymographs were generated using ImageJ (FIJI) Multi Kymograph plugin. The experiment was repeated three times independently.

ROS measurements

After 8 days of differentiation, ROS production was measured by incubating cells with CM-H2DCFDA probe (Invitrogen) at 10 μM for 1 min. Images were recorded using spinning disk confocal microscopy at a 63× objective, and analyzed using ImageJ. We have used CM-H2DCFDA [5-(and-6)-chloromethyl-2′,7′-dichlorodihydrofluorescein diacetate, acetyl ester], an improved, more stable, derivative of DCFDA, as a detector of ROS; this dye is not fluorescent when chemically reduced, but after cellular oxidation and removal of acetate groups by cellular esterases it becomes fluorescent. We measured fluorescence intensity of the transgenic neurons (i.e., expressing A53T, A30P or a surplus of WT α-synuclein) and the control (transfected with an empty vector).

Mitochondria morphology and connectivity measurement

After 8 days of differentiation, neurons were fixed and stained for mitochondria using TOM20 antibody. Mitochondria morphology and connectivity among fluorescent mitochondria were

observed and compared through stained neurons or neurons expressing pDsRed2-Mito. Total amount of neurons containing normal mitochondria morphology was analyzed and quantified.

Treatment with NAP

NAP (Santa Cruz, sc361778A) was dissolved in DMSO (Sigma Aldrich) at a stock concentration of 5 mM. Cells were treated with 5 nM NAP or vehicle, DMSO, for 24 h to evaluate rescuing of mitochondria trafficking.

Western blotting

At 8 days of differentiation, protein was extracted using protein lysis buffer. Protein lysates were sonicated and centrifuged at 13,000 rpm for 10 min. The supernatant was fractionated by SDS-PAGE (25 or 50 μg protein/lane) using a 12.5% tris–HCl gel at 100 V for 1.5 h. Proteins were transferred to immobilon membrane (FL-Millipore IPFL 00010) for 1 h at 100 V. Membranes were blocked using PBS with 0.5% Tween 20 and 5% milk for 30 min. The following antibodies were used directed against: β-actin (1:1000, 37 kDa, Abcam, #ab6276) and α-synuclein (1:1000, 14 kDa, Invitrogen, LB509). The blotted protein bands were visualized using Odyssey scanner (Li-Cor Biosciences, Lincoln, NE). Band density was quantified by computer-assisted image analysis software (ImageJ). For reliable quantification of the Western blot data, the defined methodology was followed as described by Taylor et al. (2013).

Real-time PCR

Total RNA and genomic DNA were collected using an AllPrep DNA/RNA/Protein Mini Kit. CDNA was reverse transcribed from 1 μg of RNA using M-MLV Reverse Transcriptase. GAPDH mRNA and genomic GAPDH were used as reference for normalization. For quantitate real-time PCR, primers were acquired from Biolegio and reactions were run on a Biorad C1000 Touch thermal cycler and analyzed with Biorad CFX manager software.

Statistical analysis

Mitochondria trafficking, immunofluorescence, Western Blotting and real-time PCR results were

analyzed by one- way or two- way ANOVA followed by Tukey's post hoc test. Neurons containing normal mitochondria morphology were analyzed by Student's t test. A p value ≤ 0.05 was considered significantly different, using GraphPad Prism software (GraphPad Software Inc., version 5.00, CA).

Results

Characterization of differentiated, α-synuclein transgenic SH-SY5Y cells

After 4 days in vitro (DIV) in the presence of retinoic acid and BDNF, the transgenic SH-SY5Y cells (i.e., expressing A53T, A30P or a surplus of WT α-synuclein) and the control SH-SY5Y cells (transfected with an empty vector) differentiated into neuron-like cells and formed small neurite-like extensions; at 6 DIV, these cells started to express the neuronal marker MAP2 and continued to grow their neurite- like extensions reaching stable maturity at 8 DIV (Fig. 1a– c). No differences in the neuronal differentiation pattern were observed between the control cells and the ones expressing the α-synuclein variants (supplemental Fig. 2). Selective survival based on puromycin resistance resulted in a neuronally differentiated SH-SY5Y cell culture of which all cells expressed a high level either of A53T, A30P or WT α-synuclein; in the control SH-SY5Y cell cultures, expression of α-synuclein could not be detected through immunofluorescence (Fig. 1d). The expression of the α-synuclein variants in the differentiated transgenic and control SH- SY5Y cells was quantified at 8 DIV using qPCR (Fig. 1e). Results of qPCR showed that, although each of the transgenic cell lines contained a similar number of α-synuclein gene copies, transcriptional activity was lower in the cell lines containing mutant α-synuclein in comparison to those expressing wild-type α-synuclein, suggesting interference of these α-synuclein mutants with transcription, nuclear shuttling, or cytoplasmic mRNA processing.

Analysis of α-synuclein linked to YFP corroborated the results of immunofluorescence, suggesting that A53T is more prone to aggregation since its pattern of labeling resembles that of small aggregates (Fig. 2). The expression of α-synuclein was quantified in Western blots

demonstrating increased expression of A53T isoforms (Fig. 2). Our findings suggest a reduction in degradation, possibly due to the aggregated state of A53T α-synuclein.

Mitochondrial trafficking in differentiated, α-synuclein transgenic SH-SY5Y cells

We transfected the various neuronally differentiated SH-SY5Y cell cultures with the pDsRed2-Mito plasmid: approximately 50% of the cells showed red staining in the cytosol that appeared to be confined to mitochondria and completely co-localized with the green immunostaining by the mitochondria-specific TOM20 antibody (Fig. 3). The strong specific staining by pDsRed2-Mito allowed us to investigate mitochondrial mobility in the neurite-like extensions in the neuronally differentiated SH-SY5Y cells and the consequences of aberrant α-synuclein expression for mitochondrial trafficking. In order to examine if and when mitochondria traffic was impaired by α-synuclein in SH-SY5Y cell cultures, we analyzed mitochondria mobility in the different transgenic and control SH-SY5Y cells at 4, 6 and 8 DIV in the presence of retinoic acid and BDNF representing different stages in differentiation. At 4 DIV, when the neuron-like

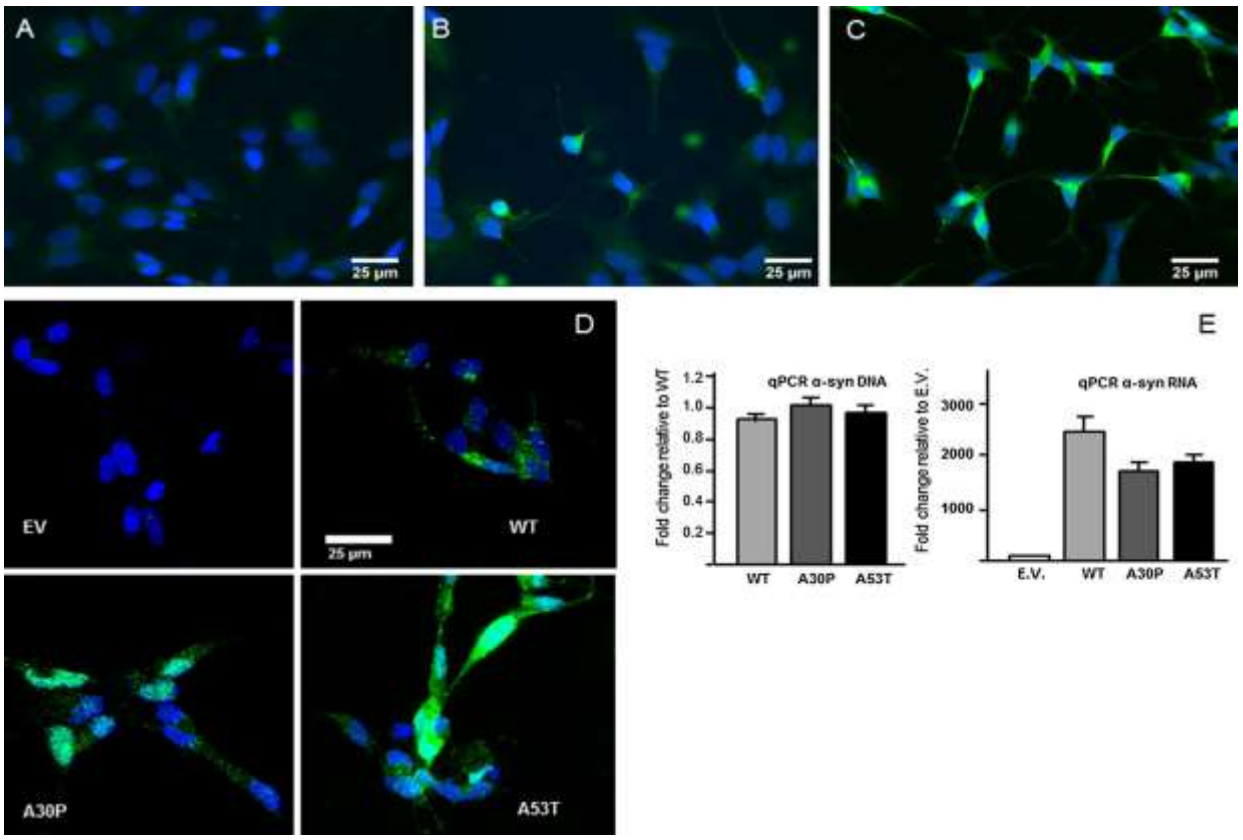

Fig. 1 Characterization of differentiated, α-synuclein transgenic SH- SY5Y cells. Immunofluorescence shows a positive stain for microtubule associated protein (MAP2) (*green*) at 4 days of differentiation (**A**), at 6 days of differentiation (**B**) and at 8 days of differentiation (**C**). Immunostaining for α-synuclein shows its expression in the α-synuclein transgenic SH-SY5Y cells (WT, A30P and A53T) but not in the control cells (transfected with an empty vector, EV) (**D**). *Blue* staining in **a**–**d** is Hoechst nuclear staining. Quantification of α-synuclein with q-PCR after 8 days of differentiation confirms the increased expression of α-synuclein (E).

cells were still immature and proper mitochondria trafficking was crucial for the outgrowth of the young “neurites,” a similar pattern of mitochondrial trafficking activity was observed in all the groups of SH-SY5Y-derived neuron-like cells. The first sign of disturbed mitochondrial mobility was observed at 6 DIV only in the A53T α-synuclein expressing SH-SY5Y cells where retrograde trafficking was significantly decreased in comparison to the control. Only later, at 8 DIV, also the anterograde trafficking of mitochondria was significantly decreased to almost 30%, again only, in the A53T α-synuclein expressing SH-SY5Y derived cells. No changes were observed in the other transgenic SH-SY5Y cell lines or in the control cells (Fig. 4). So, Fig. 4 presents evidence for the presence of mitochondria trafficking during differentiation and shows that the disturbance in mitochondria trafficking is specific for neuronal cells, since it does not occur in undifferentiated SH-SY5Y cells.

Effect of NAP on mitochondrial trafficking

To examine the mechanisms by which the A53T α-synuclein-induced impairment of mitochondrial trafficking in the neuronally differentiated SH-SY5Y cells, we treated the SH-SY5Y cell cultures with NAP since it has been shown to restore microtubule integrity (Gozes 2007; Esteves et al. 2014). Adding NAP, indeed, appeared to completely restore retrograde as well as anterograde mitochondria trafficking in the A53T α-synuclein expressing SH- SY5Y derived cells (Fig. 4). Although both motor proteins, dynein and kinesin, have clear structural distinctions and wander across the microtubule surface with different speed, step sizes and in opposite directions, apparently the A53T α-synuclein-induced microtubules destruction, restorable by NAP, affects the interaction between the motor proteins and the microtubules in a similar mode.

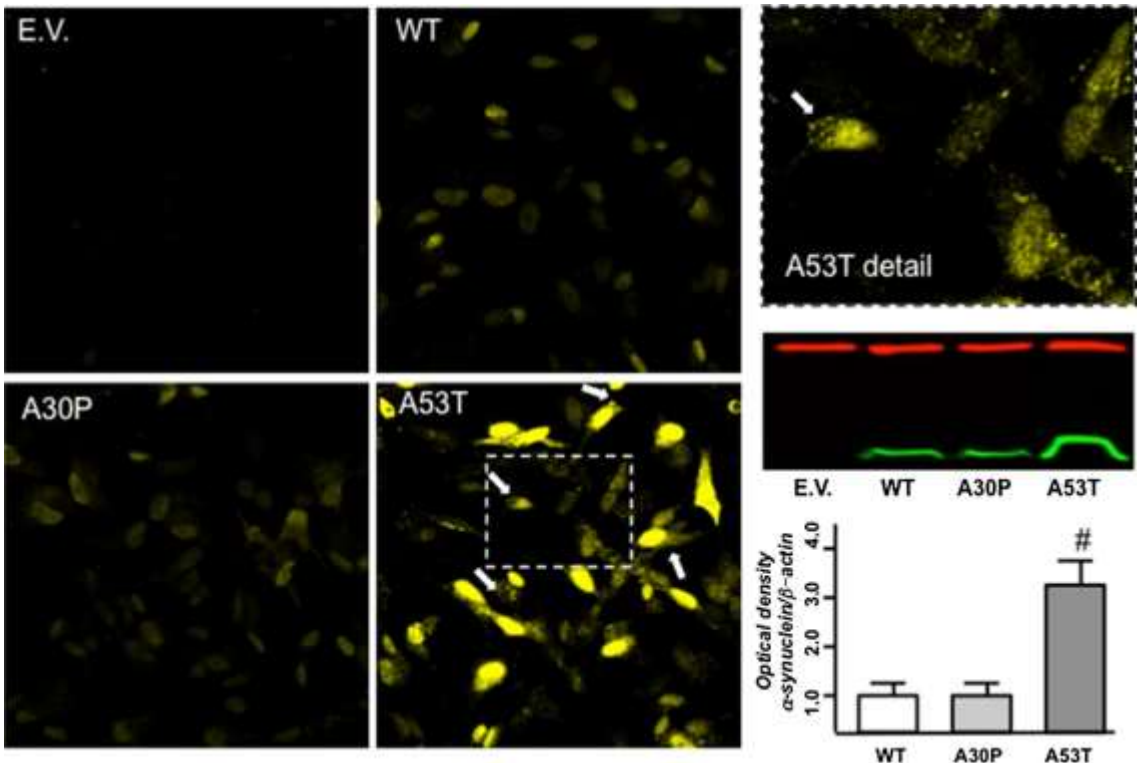

Fig. 2 α-Synuclein expression. Photomicrographs show homogeneous α-synuclein staining in neurons expressing WT or A30P YFP- α-synuclein. Neurons expressing A53T YFP-α-synuclein showed puncta accumulation of the protein at soma and neurites (*arrows* in magnification of *boxed area*). Blottings of insoluble protein fraction (*red bands* = β-actin; *green bands* = α-synuclein) reveal significantly higher levels of A53T α-synuclein compared to WT and A30P α-synuclein. Experiments were repeated 3 times. #$p < 0.001$ as compared with WT according to one-way analysis of variance (ANOVA) followed by Tukey post-test.

Effect of NAP on mitochondrial membrane potential and morphology

Impairment of mitochondrial dynamics due to the overexpression of α-synuclein or the expression of its mutants A30P and A53T may involve an increase in the production ROS and a disruption of the mitochondrial membrane potential. We found a significant increase in ROS levels in the α-synuclein expressing cells in comparison to the controls, with the highest level in the A53T mutant (Fig. 5a, c). Adding NAP to the culture, completely annihilated the ROS increase observed in all the α-synuclein expressing cell lines (Fig. 5b, c). Living neuronal cells transfected with pDsRed2-Mito or those fixed and stained for TOM20 showed the same pattern of mitochondria morphology when expressing WT or A30P alpha-synuclein compared to control (transfected with empty vector). About 65% of neurons expressing A53T alpha-synuclein showed small spherical mitochondria clusters. After NAP treatment, only 20% of DA neurons expressing A53T alpha-synuclein showed fragmented mitochondria (Fig. 6).

Discussion

Our studies show that, in contrast to wild-type and A30P α-synuclein, A53T α-synuclein significantly inhibited mitochondrial trafficking in the SH-SY5Y cell model for Parkinson's disease. Retrograde trafficking appears to be the first to be disturbed, followed in a later stage by anterograde trafficking. Accordingly, A53T α-synuclein also caused the highest increase in ROS production in these apparently demobilized and fragmented mitochondria in comparison to wild-type or A30P α-synuclein. Treatment with the active peptide (NAP) of activity-dependent neuroprotective protein (ADNP) completely annihilated the adverse effects of A53T on mitochondrial dynamics.

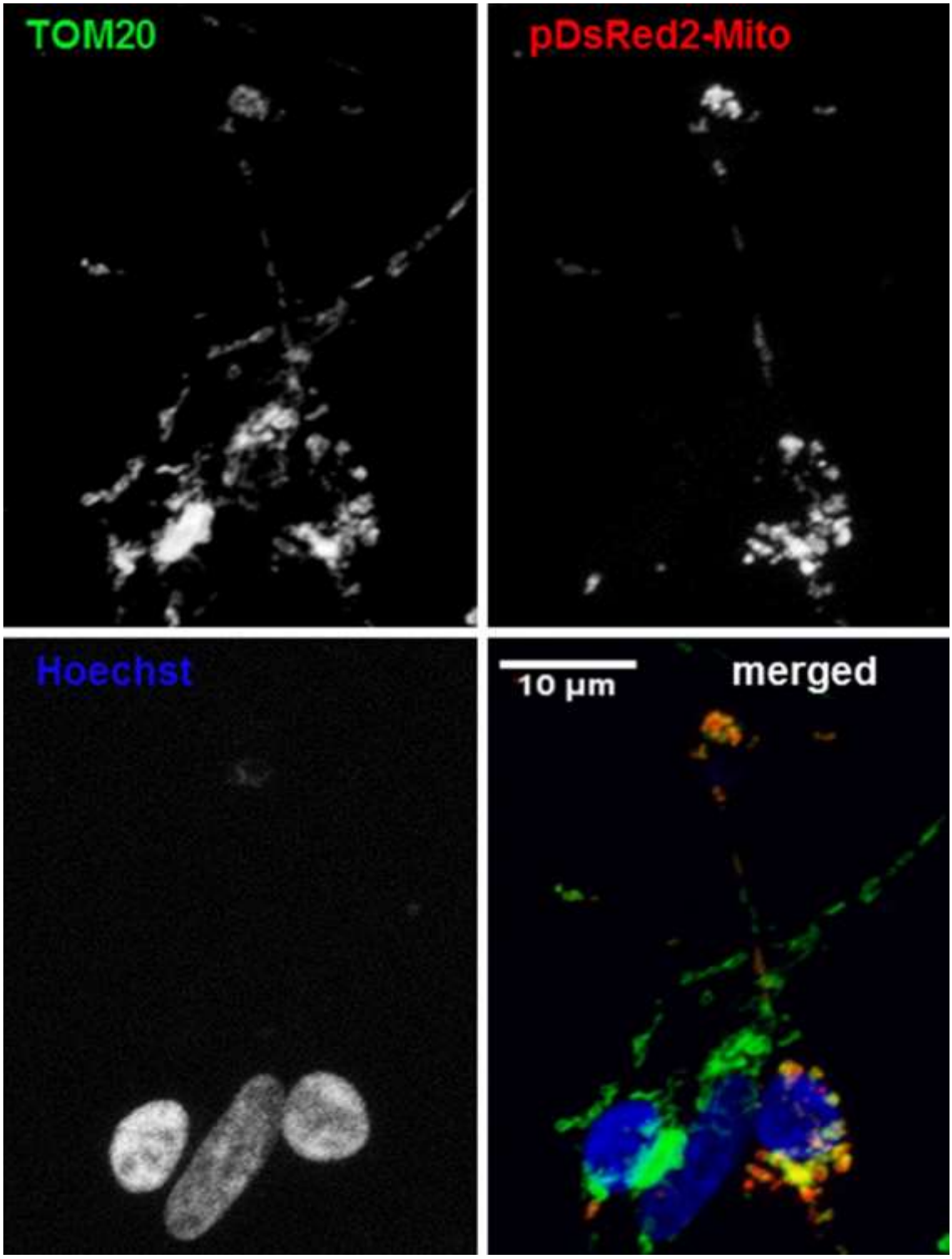

Fig. 3 Mitochondrial labeling. Immunofluorescence imaging shows the co-localization of fluorescent staining for the mitochondria-specific TOM20 (*green*) and pDsRed2-Mito (*red*). In the merged picture, the nuclei are *blue* due to the Hoechst nuclear staining.

Our findings regarding the effect of A53T α-synuclein on mitochondrial trafficking not only confirm but also extend previous data (Xie and Chung 2012; Li et al. 2013) since we also studied the time frame of mitochondria trafficking disturbance: we wanted to determine which type of trafficking was disturbed first, anterograde or retrograde. Impairment of anterograde or retrograde trafficking can lead to different injuries in organelles in neurons. In our previous studies (Chaves et al. 2013; Melo et al. 2013), we revealed that the expression of proteins involved in anterograde or retrograde trafficking was differentially affected. It has been reported that disrupted anterograde mitochondria trafficking leads to fragmentation of mitochondria, abnormal mitochondria distribution and biogenesis, depletion of ATP and high ROS levels, resulting in cell death (Matenia et al. 2012), while disrupted retrograde trafficking leads to aging and swollen mitochondria, depletion of ATP, higher ROS levels and cell death (Morris and Hollenbeck 1993). Although the outcome (cell death) is the same, determining the direction of trafficking that is impaired first enables elucidation of the actual mechanisms responsible for neuronal death; it may lead to a more specific therapeutical approach to prevent the phenotype of the disease. In the present study, we showed that A53T first impairs retrograde trafficking in neurons derived from the SH- SY5Y cells. In addition, we showed that NAP is able to recover mitochondria trafficking. The prominent effect on mitochondrial trafficking of A53T α-synuclein in comparison to A30P and wild- type α-synuclein most likely reflects the fact that A53T

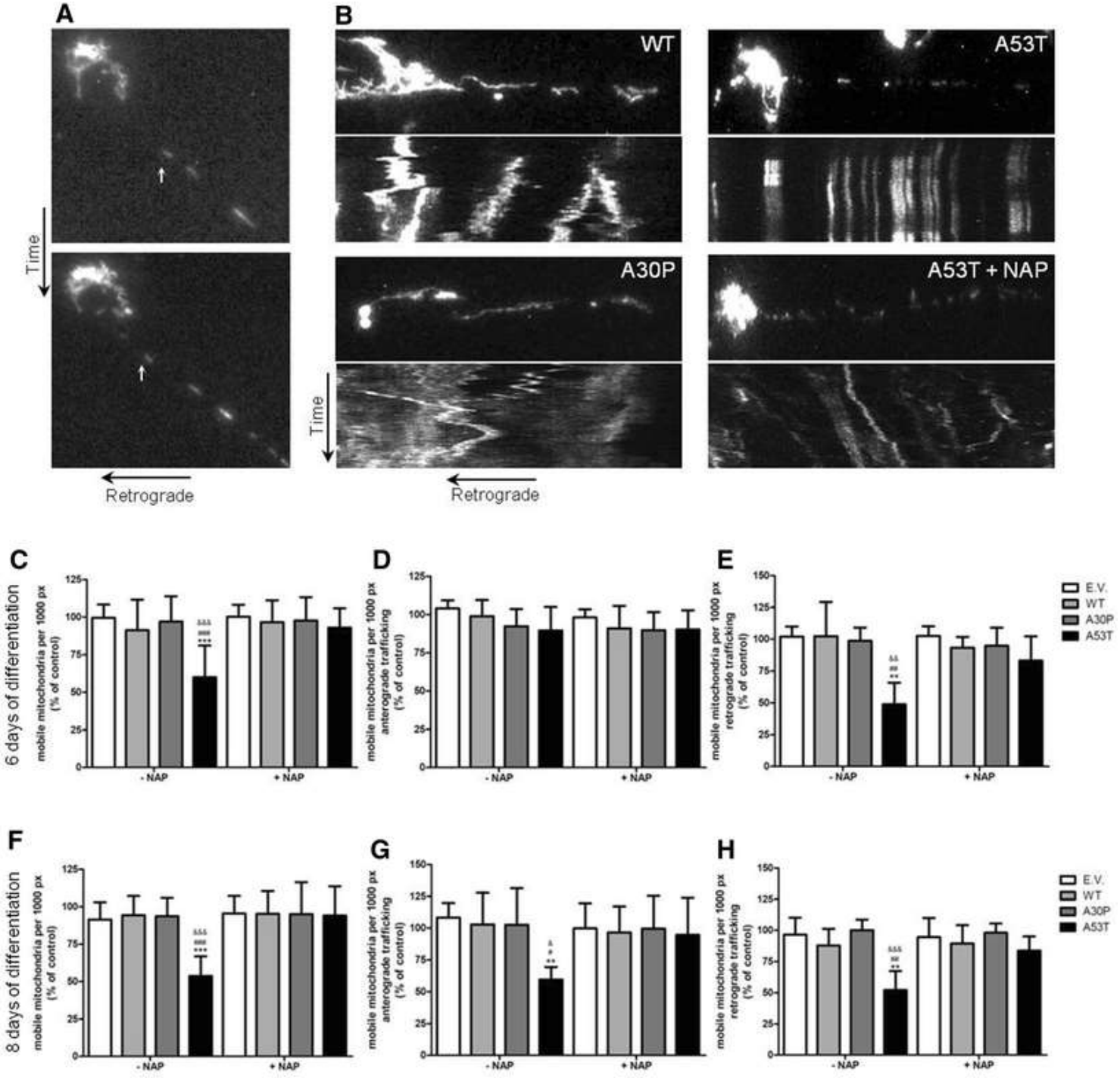

Fig. 4 Mitochondria mobility analysis in SH-SY5Y. **A** Time-lapse recordings show the movements of mitochondria retrogradely in the neurite-like extension of a SH-SY5Y cell after 8 days of differentiation. **B** Kymographs represent mitochondria movement in neurons derived from SH-SY5Y cells after 6 days of differentiation expressing either wild-type, A30P or A53T α-synuclein (in the absence and presence of NAP). Analysis of movement reveals that trafficking is decreased at 6 and 8 days of differentiation (**C**, **F**, respectively). Separate analysis of retrograde and anterograde trafficking shows unaltered anterograde trafficking but decreased retrograde trafficking at 6 days of differentiation (**D**, **E**); at 8 days of differentiation both anterograde and retrograde trafficking appears to be reduced (**G** and **H**, respectively). Neurons treated with NAP at 5 nM for 48 h show a rescued mitochondria trafficking at 6 and 8 days of differentiation (**C**, **E**–**H**). Data of moving mitochondria per 1000 pixels are expressed as percent relative to control (E.V.) ± SD. Two-way ANOVA following Bonferroni posttest were the statistical tests employed *$p \leq 0.05$ compared to

control. #$p \leq 0.05$ compared to cells expressing WT. &$p \leq 0.05$, compared to cells expressing A30P α-synuclein. Data are expressed as mean of three independent experiments.

α-synuclein is more prone for oligomer or aggregate formation. It has been shown that the interaction of wild- type α-synuclein and α-synuclein variants with molecular motors, tubulin, and the microtubules-associated proteins, MAP2 and Tau, is stronger for oligomers than for monomers (Prots et al. 2013). There appears to be differential effects between seeds and oligomers on (Tau-promoted) microtubules assembly and on the microtubules gliding

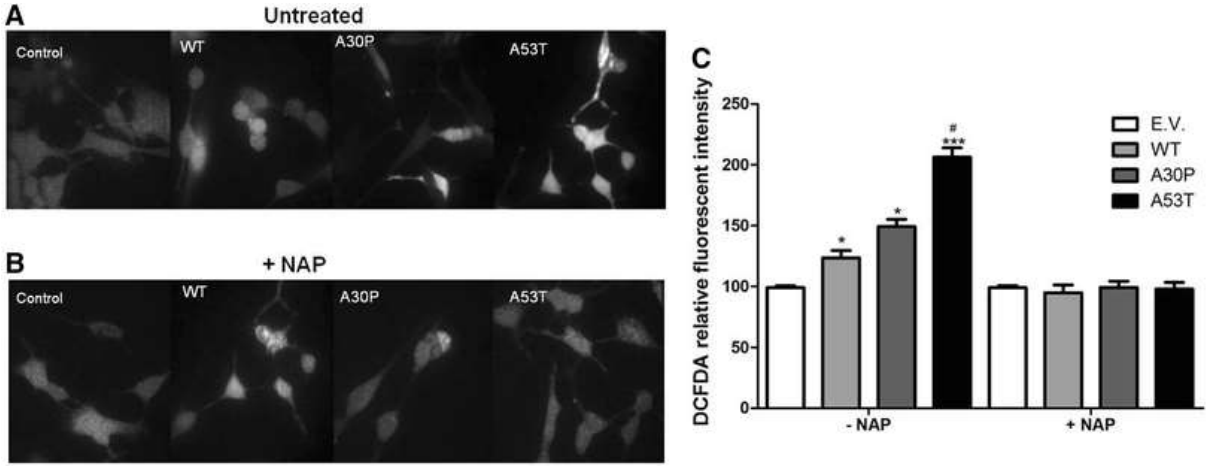

Fig. 5 ROS production. Photomicrographs show neurons incubated with green fluorescent probe DCFDA untreated (**A**) or treated with NAP (**B**). Differentiated SH-SY5Y cells at 8 DIV expressing WT, A30P or A53T α-synuclein or controls (E.V. = empty vector) were incubated with CM-H2DCFDA, a fluorescent ROS detector, untreated (**A**) or treated with NAP (**B**). Fluorescence intensity measurements (**C**) show significantly higher levels of ROS compared to control (E.V.); treatment with NAP restored levels of ROS to basal levels of control cells. Data are expressed as percentage of control (E.V.) ± SD. Two- way ANOVA following Bonferroni posttest was the statistical test employed *$p \leq 0.05$; **$p \leq 0.01$; ***$p \leq 0.001$ compared to control. #$p \leq 0.05$ compared to cells expressing WT. Data are expressed as mean of three independent experiments.

velocity across kinesin-coated surfaces (Prots et al. 2013). Due to the fact that NAP, a protein known to repair microtubules integrity, restores the A53T α-synuclein-induced impairment of mitochondrial trafficking, may point to a disruption of microtubules assembly by A53T α-synuclein disturbing the interplay between microtubules and kinesin (Prots et al. 2013). The earliest effects of A53T α-synuclein was observed in the retrograde dynein-driven trafficking of mitochondria, indicating that also the dynein-microtubules interplay may be disturbed. The reason that retrograde mitochondrial trafficking is the first to be affected may be related to the lower velocity of retrograde transport in which even small disturbances leads to an actual stop in trafficking. Obviously, the machinery underlying mitochondrial trafficking along the microtubules in axons concerns a complex structure consisting, besides of kinesin and dynein, of Miro (also known as RhoT1/2) and Milton (for a review see Schwarz, 2013). Interactions between oligomers/ aggregates of α-synuclein and Miro and Milton are as yet unknown.

Although the best known action of NAP is upon microtubule stabilization, this peptide has also been reported to affect autophagy (Gozes 2016) and oxidative stress (Greggio et al. 2011; Sharma et al. 2011). In fact, we observed a decrease in ROS content after NAP treatment, which may be either the cause or the consequence of altered mitochondria trafficking.

A wide variety of studies have shown that changes in mitochondrial trafficking are the earliest events to occur in cell models for Parkinson's disease with abnormal α-synuclein expression (Coskun et al. 2012; Prots et al. 2013; Arduino et al. 2015; Keogh and Chinnery 2015; Franco-Iborra et al. 2016). The stressed, dysmobilized, mitochondria start to produce high levels of ROS, subsequently leading to other cytopathological processes. The annihilating effect of NAP on the ROS levels in our cell cultures expressing α-synuclein variants must be ascribed to the recovery of the mitochondrial motility by NAP (Esteves et al. 2014).

Summarizing, we propose that A53T α-synuclein leads to impairment of trafficking in SH-SY5Y cells, most likely by a disturbance of microtubule integrity. This in turn leads to impairment

of mitochondrial turnover, particularly in distal regions of the cell. NAP treatment rescues mitochondria trafficking and so proper mitochondrial function and turnover. After 8 days of differentiation of the SH-SY5Y cells, we observed aggregates only in neurons expressing A53T alpha-synuclein. So, likely we have oligomers of WT, A30P and A53T alpha-synuclein after 6 days of differentiation and of WT and A30P after 8 days of differentiation. As cited above, time to form oligomers of alpha-synuclein is dependent of its type, where A53T alpha-synuclein oligomerizes easier and faster than A30P alpha-synuclein, which in turn oligomerizes easier and faster than WT alpha-synuclein. Moreover, not only the levels of oligomers influence cellular damage, but also the type of oligomer is important for the type and amount of damage in neurons (Stefanovic et al. 2015). In view of this, our findings

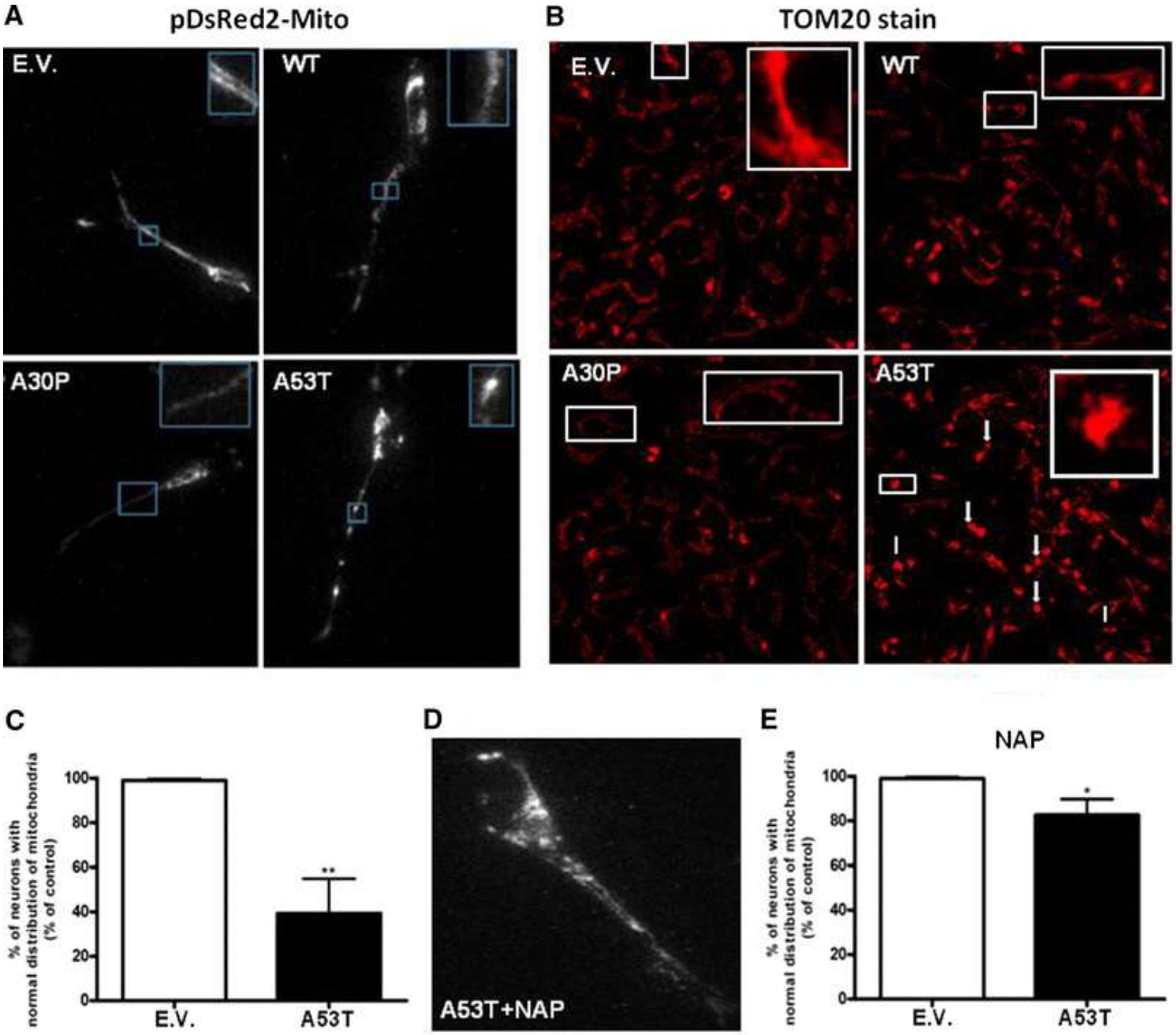

Fig. 6 Mitochondria distribution and connectivity. Photomicrographs illustrate mitochondria distribution and connectivity in neurons transfected with pDsRed2-Mito (63× objective) (A) or in neurons stained for mitochondria using TOM20 antibody (20× objective) (**B**). Quantification of neurons with normal mitochondria distribution reveals a significant decrease after expression of A53T α-synuclein (**C**). Treatment with NAP increases the amount of neurons with normal mitochondria distribution and connectivity to 70% (**D** and **E**). Data are expressed as percent relative to control (EV). *$p < 0.05$ and **$p < 0.01$ compared with EV according to Student's t test. $n = 5$. Experiments were repeated three times.

suggest that we have more oligomers of A53T alpha-synuclein and that they are more toxic than oligomers of A30P or WT alpha-synuclein. The increase in ROS production we observed may be related with lowered mitochondrial integrity at distal sites. We have shown that these effects can be rescued by using NAP, negating the effect of α-synuclein

expression and leading to increased mitochondrial turnover. Our data implies mitochondrial trafficking as an important mechanism relating mitochondrial damage to protein occlusions observed in Parkinson's disease. Early impairment of retrograde transport would lead to accumulation of damaged mitochondria near the axon terminal,

which has been proposed as an early event in clinical cases of Parkinson's disease (Cheng et al. 2010).

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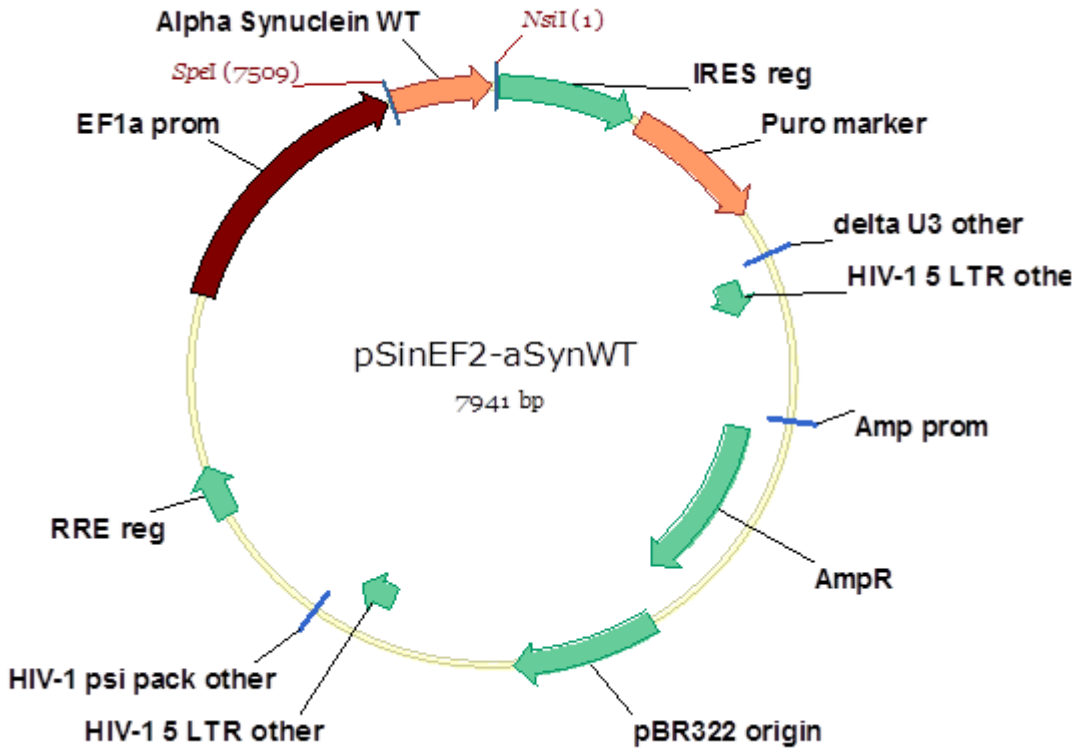
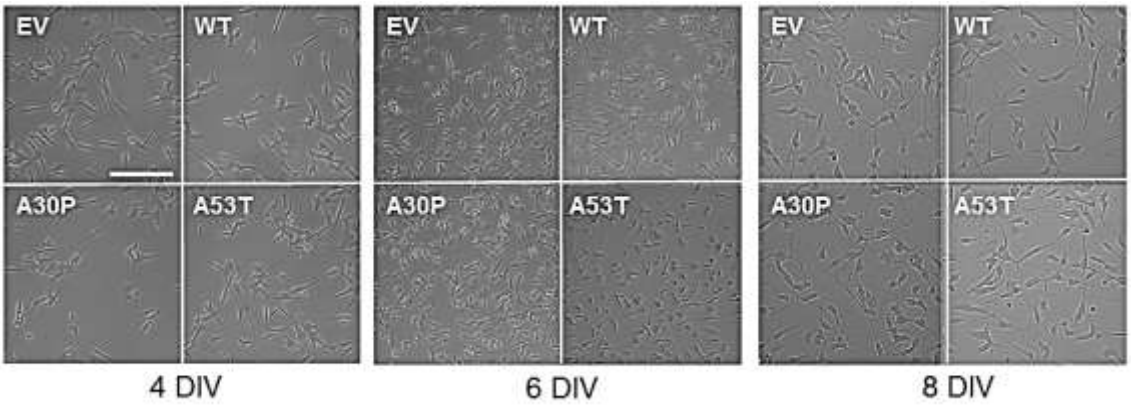
Supplementary figures:



Supplemental Figure 1. Example of the composition of the lentiviral vector used for the transfection of the α-synuclein (trans) genes.



Supplemental Figure 2. Neuronal-like differentiation of the various SH-SY5Y cell lines at 4, 6 and 8 days in vitro (DIV). No apparent differences in differentiation pattern were observed between the different cell lines. Phase-contrast microscopy, bar = 50 μm

Supplemental Table 1. List of primers used.

Target	Fw. Primer	Rev. Primer
GAPDH	TTTCTATAAATTGAGCCCGCAGC	TACGACCAAATCCGTTGACTCC
GAPDH DNA	AACCTGCCAAATATGATGACATCA	AGCCCAGGATGCCTTTGAG
α-synuclein	AAGAGGGTGTTCTCTATGTAGGC	GCTCCTCCAACATTTGTCACTT
Plasmid α-synuclein	AACTAGTATGGATGTATTCATGAAAGGAC	AATGCATTTAGGCTTCAGGTTCGTAG
Seq. Primer EF1a	TCAAGCCTCAGACAGTGGTTC	

Impairment of mitochondria dynamics by human A53T α-synuclein and rescue by NAP (davunetide) in differentiated SH-SY5Y after 6 days of culture

This study showed that, after 6 days of differentiation, only retrograde transport in dopaminergic neuron-like cells was impaired in the presence of A53T alpha-synuclein expression. As cited previously, overexpression of alpha-synuclein can lead to phosphorylation of tau and microtubule disassembly and block cellular traffic. Disturbances in mitochondrial retrograde trafficking disrupt mitochondrial biogenesis, causing mitochondrial fragmentation/dysfunction and increased ROS levels, leading to cell death (Devine et al., 2016; Levytskyy et al., 2016; Amiri and Hollenbeck, 2008).

It has been demonstrated that overexpression of alpha-synuclein can inhibits autophagy, which is concomitant to alpha-synuclein accumulation. On the other hand, knockdown of alpha-synuclein resulted in autophagy enhancement, suggesting that alpha-synuclein may have a regulatory role upon autophagy (Winslow et al., 2010). It is known that WT alpha-synuclein overexpression or the presence of A30P or A53T alpha-synuclein aggregates disturb autophagy systems, causing the accumulation of autophagosomes in the central nervous system (Koch et al., 2015)

LC3 is a key protein of autophagy initiation presented in two isoforms: LC3-I, which is present in immature autophagosomes (lysosome vesicles), and LC3-II, which is present in the mature autophagosome. LC3 is involved in phagosome membrane formation and is associated with autophagic membrane binding directly in ubiquitinated cargos. P62 is a protein that recognizes ubiquinated cargos, such as alpha-synuclein oligomers and aggregates, or damaged mitochondria. When the inner membrane of an autophagosome fuses with a lysosome, LC3-II is partially degraded, and P62 is totally degraded. In autophagy dysfunction, P62 and LC3 are commonly altered (Bjørkøy et al., 2009; Mishra et al., 2015).

Alpha-synuclein oligomers or mutant alpha-synuclein stimulate excessive fission of mitochondria, leading to mitochondrial fragmentation (Perfeito et al., 2014, Wang et al., 2012). Damaged mitochondria drive mitophagy; however, it is known that alpha-synuclein blocks autophagy in general. Specifically, A53T alpha-synuclein impairs macroautophagy, impeding the removal of damaged mitochondria. As a consequence, fragmented mitochondria accumulate in neurons (Chen et al., 2015; Winslow et al., 2011).

Alpha-synuclein overexpression toxicity is also related to the activation of apoptosis through mitochondrial dysfunction. It has been reported that accumulated alpha-synuclein activates p53, which can inhibit the anti-apoptotic BCL-XL protein. BCL-XL is related to maintenance of mitochondrial membrane potential. Its inhibition increases mitochondrial membrane permeabilization, causing cytochrome c release into the cytosol to activate caspase 3 and promote apoptosis (Nair et al., 2006; Dumitru et al., 2012).

Arduino and colleagues (2013) have demonstrated that mitochondrial dysfunction can lead to autophagy dysfunction through microtubule disassembly. It is clear that microtubule disassembly, mitochondria, and autophagy dysfunction play a role in Parkinson's disease (PD) pathology. Thus, we analyzed the consequences of the disruption of retrograde transport and treatment with NAP on mitochondria, autophagy function, and the apoptosis pathway.

MATERIAL AND METHODS

Mitochondrial morphology and connectivity measurements were performed as described previously.

Mitochondria membrane potential measurement with MitoTracker Orange CMTMRos

At 6 days of differentiation, live cells were incubated with MitoTracker Orange CMTMRos

(Invitrogen) at 75nM for 30 min. This probe labels only mitochondria with an intact membrane potential. Neurons were analyzed at confocal microscopy using 63x objective and the total number of labeled mitochondria in photos was quantified using ImageJ plugin Difference Tracker in order to quantify the amount of mobile mitochondria labeled by Mitotracker Orange.

ROS measurements were analyzed as cited previously.

Treatment with NAP was performed as cited previously.

Mitochondrial morphology and connectivity

Mitochondrial morphology and connectivity of neurons expressing pDsRed2-Mito were observed and compared. The total number of neurons containing mitochondria with normal morphology was analyzed and quantified.

Western blotting

After 6 days of differentiation of dopaminergic neuron-like cells, total protein was extracted using a solution containing PBS, 10% NP40, 5% sodium deoxycholate, 10% SDS, and 1% protease inhibitor. The cells were sonicated and centrifuged at 13000 rpm for 10 min. The supernatant was fractionated by SDS-PAGE (20 µg of protein/lane) using a 10% Tris-HCl gel at 100 V for 1 h. Proteins were transferred to a PVDF membrane (immobilon-FL, Millipore, IPFL 00010) for 1 h at 100 V. Membranes were blocked using PBS + 0.5% Tween 20 for 30 minutes. The following antibodies were used: β-actin (1:1000, 37 kDa, Abcam, #ab6276), LC3 (1:1000, 17-19 kDa, Novus Biologicals, NB600-1384), P62 (1:1000, 62 kDa, Enzo PML, BW9860-0100), Caspase 3 (1:1000, 35 kDa, Cell Signaling, #9665), BCL (1:1000, 29 kDa, Santa Cruz Biotechnology, #SC-634), p53 (1:500, 53 kDa, Santa Cruz Biotechnology, SC-126), Tau (1:1000, 50-80 kDa, Cell Signaling, #4019), and AT8 (1:1000, 79 kDa).

Statistical analysis

Mitochondrial trafficking, immunofluorescence, and Western blot results were analyzed by either Student's T-Test or one- way or two-way ANOVA followed by Tukey's post-hoc test. Differences were considered statistically significant at a p-value ≤ 0.05; analyses were conducted using GraphPad Prism software (GraphPad Software Inc., version 5.00, CA).

Results

Effect of NAP on mitochondrial membrane potential and morphology

Altered mitochondrial biogenesis leads to mitochondrial dysfunction and, consequently, increased ROS and altered mitochondrial membrane potential. We found gradually increased ROS levels in neurons expressing WT and A30P alpha-synuclein as compared to their levels in the controls. Neurons expressing A53T alpha-synuclein showed the highest levels of ROS (Figure 1A and C). NAP treatment rescued ROS levels in all cell lines expressing alpha-synuclein (Figure 1B and C). Additionally, alpha-synuclein led to lower mitochondrial membrane potential in all cell groups than in the control. However, NAP treatment rescued the mitochondrial membrane potential (Figure 1D).

Living neurons transfected with pDSRed-Mito expressing WT or A30P alpha-synuclein showed the same pattern of mitochondrial morphology as the control. Of the neurons containing A53T alpha-synuclein, 35% (Figure 2A and B) contained non-connected, small spherical mitochondrial clusters, suggesting the organelle was fragmented in the presence of A53T alpha-synuclein expression. As previously showed, mitochondrial trafficking was normalized after NAP treatment; however, neurons expressing A53T alpha-synuclein still showed small spherical non-connected mitochondria (10%) after 6 days (Figure 2C).

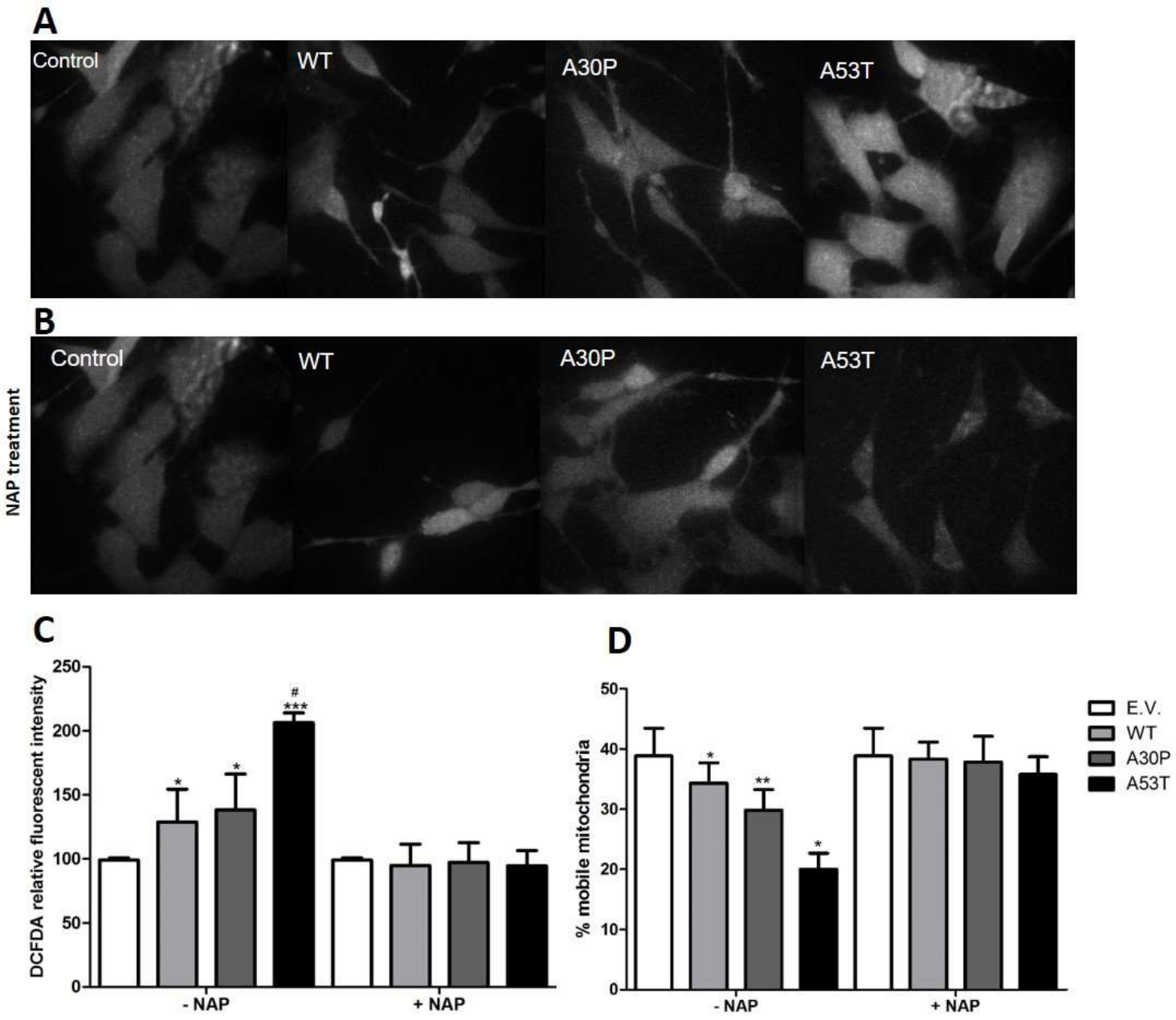


Figure 1: ROS production and mitochondrial viability. Photomicrographs show neurons incubated with green fluorescent probe DCFDA untreated (A) or treated with NAP (B). Differentiated SH-SY5Y cells expressing WT, A30P or A53T α-synuclein or controls (E.V.=empty vector) were incubated with CM-H2DCFDA, a fluorescent ROS detector, untreated (A) or treated with NAP (B). Fluorescence intensity measurements (C) show significantly higher levels of ROS compared to control (E.V.); treatment with NAP restored levels of ROS to basal levels of control cells. Differentiated SH-SY5Y cells expressing WT, A30P or A53T alpha-synuclein incubated with MitoTracker Orange show a decrease in the percentage of mobile mitochondria, with intact membrane potential, compared to control (E.V.); treatment with NAP restored the number of mitochondria labeled by MitoTracker Orange, (D). Data are expressed as percentage of control (C) or percent of moving mitochondria per 1,000 pixels (D) ± SD. Two-way ANOVA following Tukey post- test was the statistical test employed *$p \leq 0.05$; **$p \leq 0.01$; ***$p \leq 0.001$ compared to control. # $p \leq 0.05$ compared to cells expressing WT. Data are expressed as mean of 3 independent experiments.

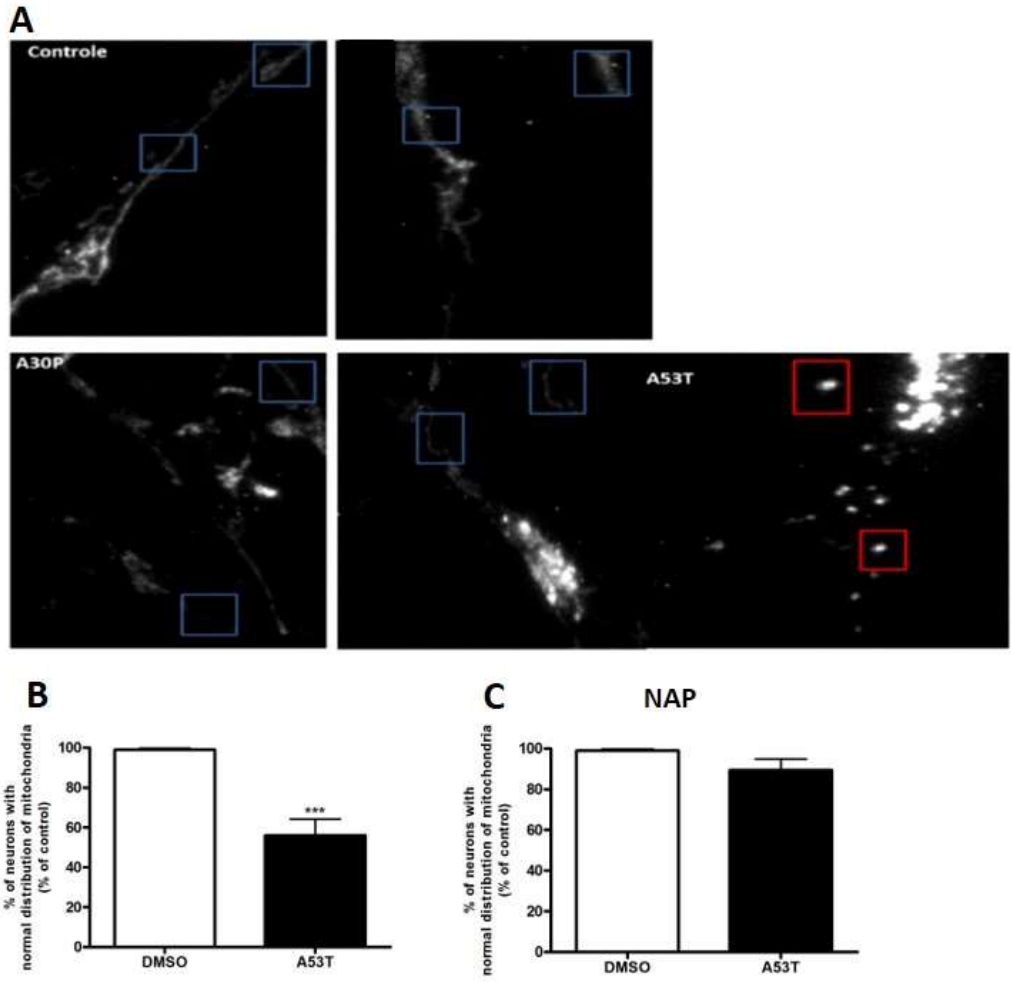


Figure 2: Mitochondria distribution and connectivity. Photomicrographs illustrate mitochondria distribution and connectivity in neurons transfected with pDsRed2-Mito (63x objective) (**A**). Quantification of neurons with normal mitochondria distribution reveals a significant decrease after expression of A53T alpha-synuclein (**B**). Treatment with NAP increases the amount of neurons with normal mitochondria distribution and connectivity to 90% (**C**). Data are expressed as percent relative to control (EV). *$p<0.05$ and **$p<0.01$ compared with EV according to Student's T-test. n = 5. Experiments were repeated 3 times.

Levels of phosphorylated tau are increased in DA neurons containing alpha-synuclein

Since our data showed that mitochondrial trafficking was disrupted, we analyzed the levels of tau and phosphorylated tau (Figure 3). We found decreased levels of tau protein in neurons containing WT, A30P, and A53T alpha-synuclein (Figure 3A and C). In addition, we found increased levels of hyperphosphorylated tau protein in neurons expressing A30P and A53T alpha-synuclein (Figure 3B and C). These data indicate that the deficit in mitochondrial transport is caused by destabilization of microtubules. NAP treatment increased tau levels in neurons expressing A30P or A53T alpha-synuclein (Figure3A and D). However, the treatment did not decrease the levels of hyperphosphorylated tau in neurons expressing A53T alpha-synuclein (Figure 3B and D).

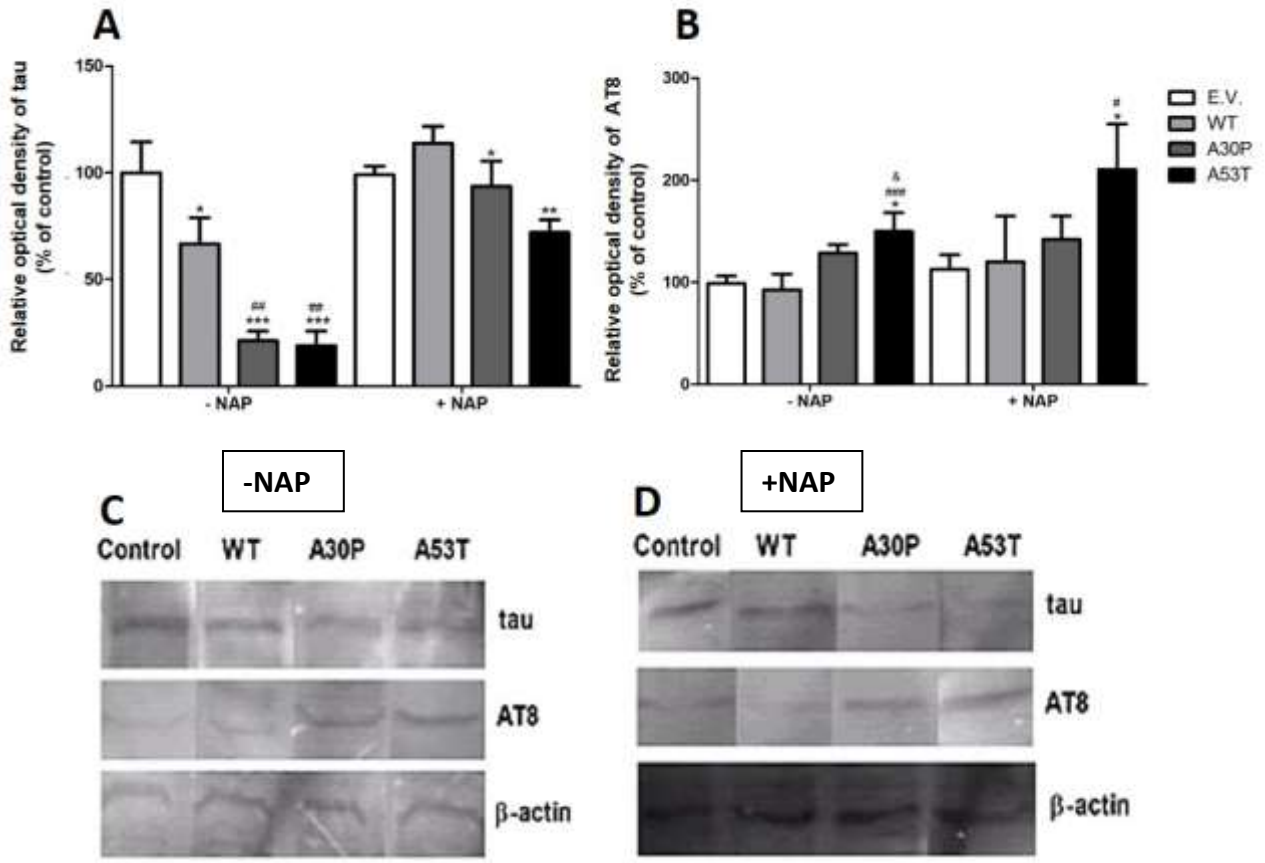


Figure 3: Tau expression. Quantification and representative blottings of tau protein showed decreased levels of tau in cells expressing WT, A30P or A53Talpha-synuclein compared to cells expressing E.V. (A and C). Treatment with NAP increased tau levels in all cell lines (A and D). Neurons expressing A53T alpha-synuclein showed higher levels of phosphorylated tau (AT8) compared to all others cell lines (B and D). Tretament with NAP was not effective in decrease phosphorylated tau in neurons expressing A53T alpha-synuclein. Data are expressed percent relative to control (E.V). Experiments were repeated 3 times*$p<0.05$, $^{\#}p<0.05$ and $^{\&}p<0.05$ as compared with E.V., WT or A30P, respectively, according to two-way analysis of variance (ANOVA) followed by Tukey post test.

NAP increases autophagic flux in neurons expressing alpha-synuclein

To measure autophagy flux, we analyzed the protein levels of LC3-II and P62. After 6 days of differentiation, we found decreased levels of LC3-II in neurons expressing WT and A30P alpha-synuclein (Figure 4A and C) and decreased levels of LC3-II and P62 in neurons expressing A53T alpha-synuclein (Figure A, B, and C), suggesting an induction of autophagy in these neurons.

After NAP treatment, we observed decreased levels of LC3 and P62 in neurons expressing A30P and A53T alpha-synuclein (Figure 4D, E, and F). These findings together suggest NAP treatment stimulates autophagy flux.

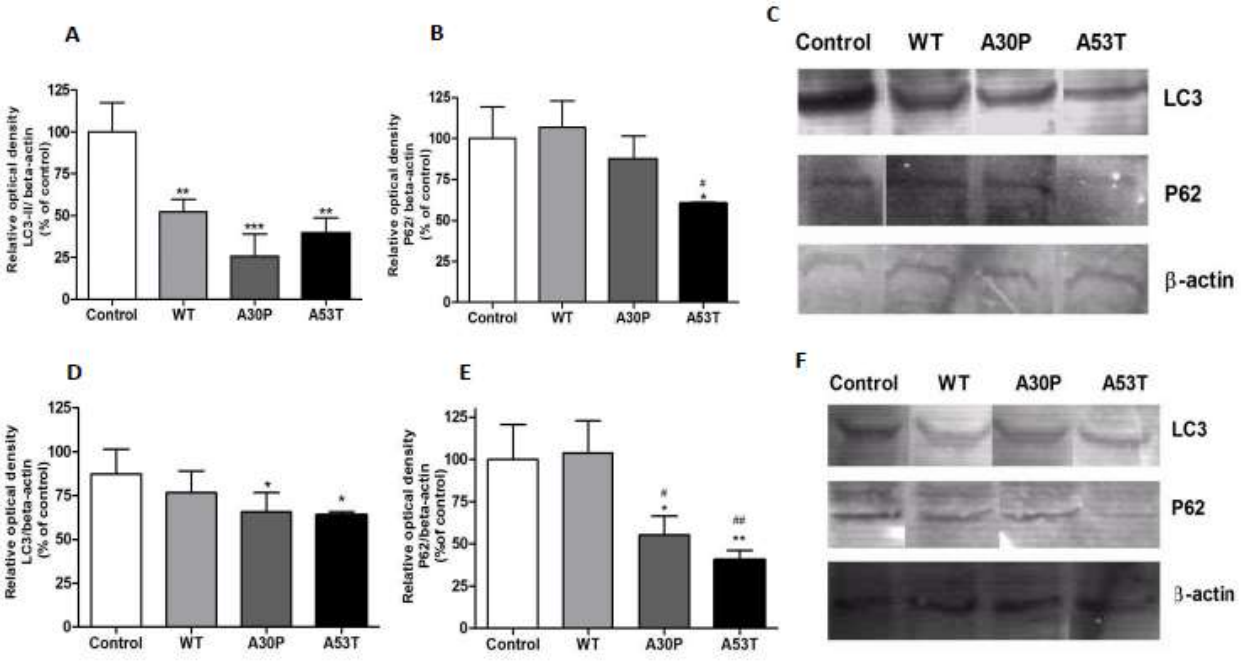


Figure 4: LC3-II and P62 expression. Quantification and representative blottings of both proteins showed decreased levels of LC3 in cells expressing WT, A30P or A53T alpha-synuclein compared to cells expressing E.V. (A and C), whereas neurons expressing A53T alpha-synuclein showed decreased levels of P62 (B and C). Treatment with NAP increased LC3-II (D and F) and P62 (E and F) compared to neurons expressing E.V. Data are expressed as absolute number. Experiments were repeated 3 times.*$p<0.05$, $^{\#}p<0.05$ as compared with E.V. or WT, respectively, according to one-way analysis of variance (ANOVA) followed by Tukey post test.

The apoptosis pathway is inhibited in neurons expressing alpha-synuclein after NAP treatment

Accumulation of alpha-synuclein activates the apoptosis pathway and damages mitochondria. We analyzed the apoptosis pathway by measuring the levels of p53, BCL-XL, and caspase 3 proteins. The levels of p53 increased in neurons expressing A53T (Figure 5A and D). Protein levels of BCL-XL were decreased in neurons expressing WT, A30P, or A53T alpha-synuclein (Figure 5B and D). Caspase 3 protein levels were increased in neurons expressing A53T alpha-synuclein (Figure 5C and D). These data strongly suggest activation of the apoptosis pathway in neurons overexpressing alpha-synuclein. After NAP treatment, we observed basal levels of p53 in all groups expressing alpha-synuclein (Figure 5E and H). Neurons expressing A30P or A53T alpha-synuclein showed increased levels of BCL-XL (Figure 5F and H) and decreased levels of caspase 3 (Figure 5G and H). These data suggest that treatment with NAP is effective in blocking apoptosis signaling cascades (Figure 5).

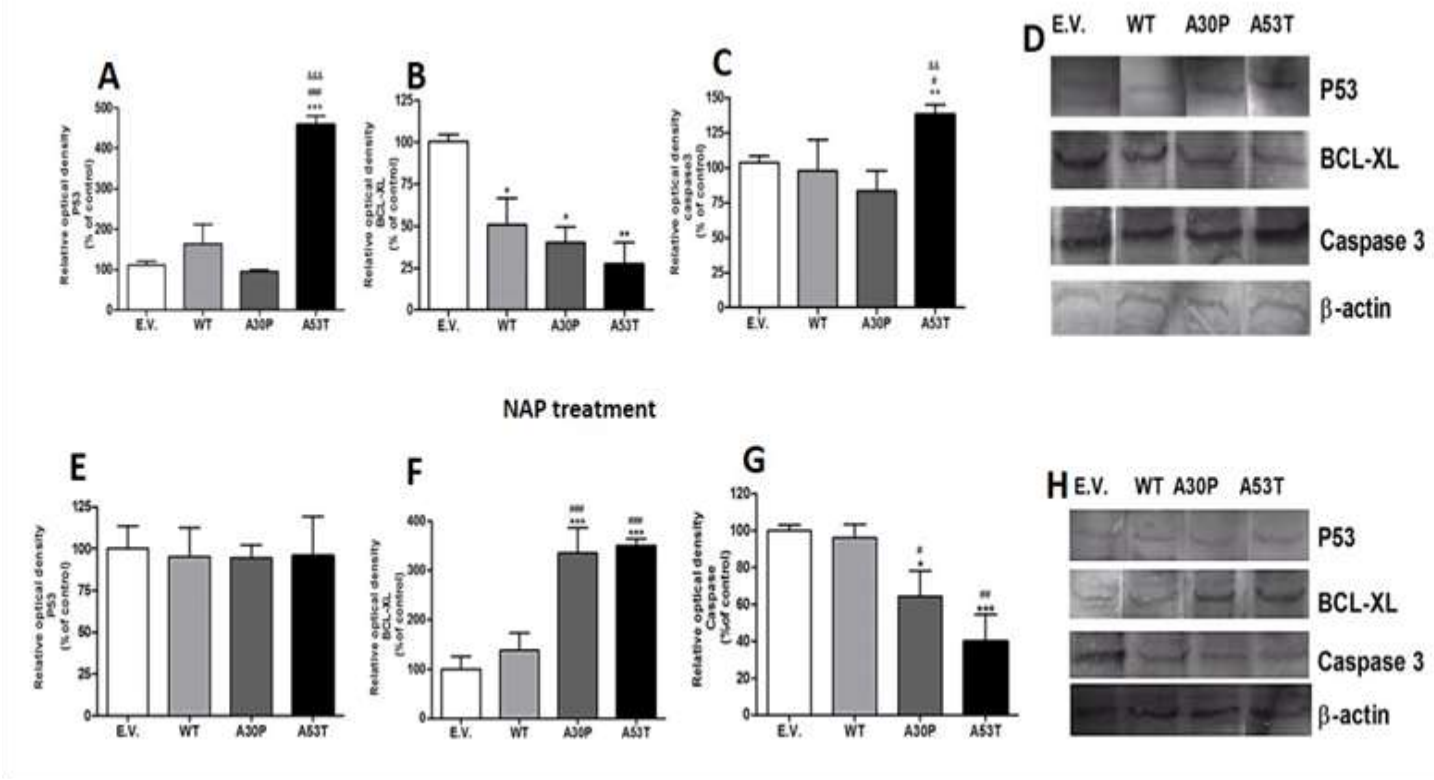


Figure 5: P53, BCL-XL and caspase 3 expression. Quantification and representative blottings showed increased levels of P53 (A and D) and caspase 3 (C and D) in cells expressing A53T alpha-synuclein compared to cells expressing E.V., whereas neurons expressing WT, A30P or A53T alpha-synuclein showed decreased levels of BCL-XL (B and D). Treatment with NAP normalized P53 levels (E and H), increased BCL-XL levels (F and H), whereas decreased caspase 3 levels (G and H) in neurons expressing A30P or A53T alpha-synuclein compared to neurons expressing E.V. Data are expressed as absolute number. Experiments were repeated 3 times.*$p<0.05$, $^{\#}p<0.05$ and $^{\&}p<0.05$ as compared with E.V., WT or A30P, respectively, according to one-way analysis of variance (ANOVA) followed by Tukey post test.

Discussion

Mitochondrial dysfunction and altered autophagy are common occurrences in PD and can be caused by changes in trafficking, which is a phenomenon observed in early stages of PD. Our study

focused on analyzing mitochondria, autophagy, and apoptotic pathway changes caused by different types of alpha-synuclein in early stages when only retrograde trafficking was impaired.

We used NAP at a concentration that rescued mitochondrial trafficking in order to check whether mitochondrial dysfunction, autophagy modulation, or apoptosis signaling could be consequences of altered trafficking. We observed that 35% of neurons expressing A53T alpha-synuclein contained fragmented mitochondria. Interestingly, neurons expressing both A30P and A53T alpha-synuclein had increased levels of phosphorylated tau and showed decreased numbers of mitochondria with the membrane potential intact. ROS levels were increased for neurons expressing WT, A30P, or A53T alpha-synuclein, demonstrating that overexpression of WT and A30P alpha-synuclein are both able to change the mitochondrial membrane potential and increase oxidative stress before changing mitochondrial trafficking. These data suggest that the expression of WT and A30P alpha-synuclein first changes the expression of phosphorylated tau and damages the mitochondrial membrane potential, whereas the impairment in mitochondrial trafficking and dysfunction are concomitant events in neurons expressing A53T alpha-synuclein.

It is known that alpha-synuclein can form oligomers before aggregates are actually observed, and oligomers are thought to be more toxic than aggregates, targeting mitochondria, impairing intracellular transport, and altering autophagy (Bart et al., 2010; Vekrellis et al., 2011; Chua and Tang, 2011; Iborra et al., 2015). Thus, we likely had oligomers of WT, A30P, and A53T alpha-synuclein after 6 days of differentiation. As cited above, the time to form oligomers of alpha-synuclein is dependent on alpha-synuclein type, where A53T alpha-synuclein oligomerizes easier and faster than A30P alpha-synuclein that oligomerizes easier and faster than WT alpha-synuclein. Not only do the levels of oligomers influence cellular damage, but the type of oligomer is also important in damaging neurons (Stefanovic et al., 2015). In view of this, our findings suggest we had more oligomers of A53T alpha-synuclein, and they were more toxic than oligomers of A30P or WT alpha-synuclein.

Mitochondrial dysfunction, altered intracellular trafficking, and autophagy alterations can be linked to PD. Arduíno and colleagues (2015) demonstrated that impaired mitochondrial function could modulate autophagy, leading to accumulation of autophagosomes. Su and Qi (2013) showed mitochondrial dysfunction led to overactivated lysosomes and altered autophagy flux. After 6 days of differentiation, we observed overactivated autophagy flux in neurons expressing WT, A30P, and A53T alpha-synuclein. Further, neurons expressing A53T alpha-synuclein showed decreased levels of P62, suggesting that A53T alpha-synuclein increases autophagy of ubiquitinated cargos. After NAP treatment, autophagy flux was restored in neurons expressing WT alpha-synuclein; however, neurons expressing A30P or A53T alpha-synuclein continued to express decreased levels of LC3. Further, these neurons showed decreased levels of P62, indicating increased autophagic flux of ubiquitinated cargos. NAP treatment increased removal of ubiquitination for both mutated alpha-synucleins. Taken together, these data suggest NAP restores autophagic flux and increases removal of ubiquitinated cargos generated in the presence of A30P or A53T alpha-synuclein.

It is known that overexpression of alpha-synuclein and mitochondrial dysfunction can lead to apoptosis. Our data showed increased p53 and caspase 3 levels in neurons expressing A53T alpha-synuclein, suggesting activation of the apoptosis pathway. Curiously, all groups expressing alpha-synuclein showed decreased levels of BCL-XL, revealing that mitochondria are susceptible to changes in the membrane potential in the presence of alpha-synuclein. Nevertheless, treatment with NAP was effective in blocking the apoptosis pathway.

These findings indicate that restoring mitochondrial trafficking can prevent mitochondrial dysfunction and cell death, suggesting that disturbed trafficking plays a crucial role in neurodegeneration in PD.

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Part II

Manuscript in preparation

3.2. Absence of Gem (Miro1 in mammals) ameliorates A30P and A53T alpha-synuclein toxicity, while absence of Ypt53 (Rab5 in mammals) and Atg8 (LC3 in mammals) contributes to A30P and A53T alpha-synuclein toxicity in a yeast (*S. cerevisiae*) model of Parkinson's disease

Abstract

Parkinson's disease (PD) is characterized mainly by alpha-synuclein aggregation and its toxicity involving mitochondrial dysfunction, oxidative stress, disturbed autophagy, and ER stress. A30P and A53T point mutations in alpha-synuclein are linked to familial PD and more severe cellular damage than WT alpha-synuclein. Alpha-synuclein has been reported to interfere/interact in direct or indirect ways with Miro1, Rab5, and LC3, which are the key proteins related to mitochondrial dynamics, endocytic and primary endosome trafficking, and formation and fusion of vesicles in lysosome, respectively. In this study, we created a model to study A30P and A53T alpha-synuclein toxicity related to Gem (Miro), Ypt53 (Rab5) and Atg8 (LC3) genes in *Saccharomyces cerevisiae* in which these genes were knocked down. We found that A30P alpha-synuclein toxicity was dependent on mitochondrial and autophagy dysfunction. A53T alpha-synuclein was more toxic than A30P alpha-synuclein, and its aggregation was dependent on Gem gene expression. A53T alpha-synuclein toxicity involving damaged mitochondrial and apoptotic signaling caused by ER stress was dependent on Gem and Atg8 genes, respectively.

Introduction

Parkinson's disease (PD) is the most common motor neurodegenerative disease and the second most common neurodegenerative disease (de Lau and Breteler, 2006). Alpha-synuclein protein accumulation/aggregation, mitochondrial dysfunction, oxidative stress, and degeneration of dopaminergic neurons in the substantia nigra are the main hallmarks of PD (Bose and Beal, 2016). Disturbed intracellular trafficking of organelles and vesicles in the presence of overexpressed alpha-

synuclein has been reported in models of PD (Schrinzi et al., 2016; Valadas et al., 2015). A30P or A53T point-mutated alpha-synucleins are linked to familial forms of PD and can exacerbate alpha-synuclein toxicity; moreover, A53T alpha-synuclein is more toxic than WT or A30P alpha-synuclein, causing more severe cellular damage (Lee et al., 2002; Chen et al., 2015). WT, A30P, or A53T point-mutated alpha-synuclein disrupt intracellular trafficking, increase endoplasmic reticulum (ER) stress, damage mitochondria, and disturb autophagy (Mazzulli et al., 2016; Bose and Beal, 2016). Mechanisms that have been suggested for alpha-synuclein toxicity involve Miro1 (Gem in yeast), Rab5 (Ypt53 in yeast), and LC3 (Atg8 in yeast) function.

Miro1 is a calcium-dependent motor/adaptor protein involved in the regulation of mitochondrial dynamics. In addition, Miro1 is a target of proteins responsible for controlling the fission and fusion processes, which are dysregulated by alpha-synuclein, contributing to mitochondrial dysfunction and increased hydrogen peroxide (H_2O_2) in PD (Devine et al., 2016; Chen et al., 2015; Wang et al., 2011).

Rab5 is a small GTPase involved in the endocytic pathway and primary endosome trafficking, playing a role in autophagy initiation. It has been reported that alpha-synuclein disrupts Rab homeostasis, leading to ER stress and impairment of early endocytic trafficking through association with Rab5. Moreover, alpha-synuclein is endocytosed by Rab5, changing endocytosis and lysosome dynamics (Sung et al., 2001; Sancenon et al., 2012).

LC3 is a protein crucial to autophagy and responsible for formation of endosomes and their fusion with lysosomes. Alpha-synuclein inhibits autophagy and increases ER stress. The unfolded protein response (UPR) is activated, increasing ER stress markers, such as *Hac1* (XBP1 in mamals) and chaperons like Pdi, in order to enhance protein-folding machinery and alleviate ER stress (Redmann et al., 2016; Winslow and Rubinsztein, 2011). However, UPR activation can also generate reactive oxygen species (ROS), deregulating the redox state of the ER, which releases excessive levels of calcium to mitochondria, leading to dysfunction of organelles generating ROS

and oxidized glutathione (GSSG) (Tomanek 2015; Colla et al., 2012; Scheuner and Kaufman 2008). Additionally, mutations in alpha-synuclein lead to accumulated misfolded proteins in the ER, activating apoptosis pathways through the transcription factor CHOP (Mizuno et al., 2016; Zhao et al., 2016; Colla et al., 2012).

Yeast (*Saccharomyces cerevisiae*) has been successfully used to investigate alpha-synuclein toxicity related to ER stress, mitochondria, and autophagy dysfunction (Tenreiro et al., 2016; Delorme-Axford et al., 2015; Ciaccioli et al., 2013; Sancenon et al., 2012). In other to investigate the role of Miro1, Rab5, and LC3 in alpha-synuclein toxicity, we create a model Δ (knockout) for each of these genes in yeast also expressing A30P or A53T alpha-synuclein.

MATERIAL AND METHODS

Transformation of *Saccharomyces cerevisiae*

The *Saccharomyces cerevisiae* strain used in this study was BY4741 (genotype: Matα; his3Δ1; leu2Δ0; met15Δ0; ura3Δ0; YHR104w: kanMX4). The Δ strains used were ΔGem1, ΔYpt53, and ΔAtg8, all derived from BY4741. Gem1, Ypt53, and Atg8 are homologous to human Miro, Rab5, and LC3 respectively. All cell lines were obtained from the Euroscarf collection (Frankfurt, Germany), kindly donated by professor Luis Eduardo Soares Netto from the Institute of Biosciences-University of Sao Paulo.

All strains were transformed using p426GPD empty plasmid (empty vector), or containing A30P or A53T alpha-synuclein, and they were donated by Professor Susan Lindquist (Outeiro and Lindquist, 2003). Both genes are under control of the constitutive promoter GPD, Glyceraldehyde-3-phosphate dehydrogenase. Selection markers of plasmids were ampicillin and uracil for bacteria and yeast, respectively.

Yeast cells were transformed with plasmids using the lithium acetate method based on Schiestl and Gietz (1989). Briefly, cells grew at 30 °C, shaking overnight at 200 rpm in 10 ml of

liquid minimal medium (SD), which consisted of 20% of glucose, 1.7% of nitrogenous base, 1.3% of dropout (1.25g of adenine; 1.2g of alanine; 0.9g of arginine; 1.2g of asparagine; 3g of aspartate; 1.2g of cysteine; 1.5g of phenylalanine; 1.2g of glycine; 3g of glutamate; 1.2g of glutamine; 1.2g of isoleucine; 0.9g of lysine; 0.6g of methionine; 1.2g of proline; 11.25g of serine; 0.9g of tyrosine; 6g of threonine; 4.5g of valine), and auxotrophic supplementation containing histidine, tryptophan, leucine, and uracil at recommended concentrations in 1 liter of ultrapure water. Next, 1 ml of cells was transferred to 29 ml of new SD medium to grow for 5 h. The samples were centrifuged at 1000 × *g* for 5 min, and the supernatant was discarded. Pellets were resuspended using TEL buffer composed of TE buffer (Tris-HCl 10 mM, EDTA 1 mM at pH 8.0) and lithium acetate 1×. Samples were centrifuged, pellets were resuspended in TEL buffer mixed with 5 µl of *carrier* salmon DNA and incubated for 20 min at room temperature. Subsequently, 700 µl of 40% PEG (polyethylene glycol) was added to the samples. After 35 min, cells were heated in a water bath at 42 °C for 10 min, followed by addition of 500 µl of TE. Next, samples were centrifuged at 16000 × *g* for 1 min, and half of the supernatant was discarded. Pellets were resuspended using supernatant, and 100 µl of the cells was plated on solid SD medium (containing 1% agar) without uracil.

Expression of A30P or A53T alpha-synuclein were verified through western blotting techniques after incubation of inoculum in SD medium for 8 h.

Viability and sensitivity essay

In general, all cell strains grew better in fermentation medium, which is synthetic defined minimal medium containing glucose (SD). In analyses when mitochondrial activity was necessary, respiratory medium was used (SGE). SGE medium is composed of the same components as SD medium, but glucose was replaced by glycerol and ethanol. However, all cell strains had more than 24 h to grow in liquid SGE medium, so we also used an intermediary medium (SG) in which glucose was replaced by galactose.

Viability of cells was analyzed through growth curves and serial dilution in SD or SG media in the absence and presence of H_2O_2.

Pre-inoculum of yeast was prepared in SD liquid medium (1 colony in 300 ml of medium) shaken at 200 rpm for 12 h at 30 °C. Next, samples were centrifuged at 1000 × *g* for 5 min, resuspended, and inoculum was incubated in 300 ml of liquid SD, SG, or SGE medium at 0.2 OD_{600} (optical density$_{600}$ = 1= 3×10^7 cell/ml) shaken at 200 rpm. To construct a growth curve, the OD_{600}, was measured every 2 h for 12 h and again after 12 h, i.e., for 24 h of growth. To analyze yeast growth through a serial dilution or sensitivity to H_2O_2, cells were plated from 1.0 OD_{600} in 4 diluted concentrations (1/10, 1/100, 1/1000, 1/10000) in solid SD or SG medium for 4 days or SGE medium for 8 days. Cells cultured in SGE did not grow. To analyze the sensitivity of lines to H_2O_2, cells were plated in SD solid medium containing 0 mM, 0.5 mM, 1.0 mM, 1.5 mM, 2 mM, or 3 mM of H_2O_2. After 8 days, yeast growth was observed and registered through photomicrographs.

H_2O_2 levels in isolated mitochondria

Mitochondrial isolation

Pre-inoculum was prepared in SD medium as described above, and inoculum was incubated in 1 liter of SG medium and shaken at 200 rpm at 30 °C for 24 h. Mitochondrial isolation followed the protocol of Glick & Pon (1995) with adaptions. Briefly, cells were centrifuged at 1000 × *g* for 5 min at 4 °C, washed in 1.2 M sorbitol, and centrifuged. Subsequently, the of the pellet was weighted, and cell walls were lysed using 3 ml/g of the pellet and digest buffer (1.2 M sorbitol, 60 mM sodium phosphate buffer pH 7.5, 1 mM EDTA, 1% β-mercaptoethanol, and 1 mg/ml zymolyase) at 37 °C. After 2 h, spheroplasts (yeast without cell walls) were washed twice with buffer A (1.2 M sorbitol and 20 mM sodium phosphate buffer pH 7.5) and centrifuged for 10 min at 4 °C, 1000 × *g*. Spheroplasts were resuspended in SHE buffer (0.6 M sorbitol, 20 mM HEPES pH 6.0, 1 mM EDTA, and 0.5 mM PMSF) and homogenized in an agitator potter for 30 cycles on ice. Samples were centrifuged at 1000 × *g* for 10 min at 4 °C, and the supernatant was centrifuged at

27000 × *g* for 10 min at 4 °C. The pellet containing membranous organelles was resuspended in 20 ml of SH buffer (0.6 M sorbitol and 20 mM HEPES pH 6.0) and centrifuged as cited previously. Pellet was resuspended in 500 µl of SH buffer. Protein levels were analyzed using the NanoDrop system1000 Spectrophotometer (Thermo Fischer Scientific).

H_2O_2 measurement through Amplex Red™ oxidation

Samples with 100 µg/ml of suspension containing mitochondria were incubated with 50 µM of Amplex Red (Molecular Probes) in the presence of 1.0 U/ml of horseradish peroxidase (HRP, Sigma-Aldrich) for 10 min in a shaker at 30 °C inside a fluorimeter (Cary 100 Bio, Varian). The reaction of Amplex Red oxidation generates resorufin, which has an excitation peak at 563 nm and an emission peak at 587 nm. The quantitative values of H_2O_2 in arbitrary units of fluorescence were calculated from a calibration curve with different concentrations of H_2O_2 diluted 1/1000 from 30% of the chemical (v/v).

GSH/GSSG ratio

The ratio between glutathione in reduced form (GSH) and oxidized form (GSSG) is commonly used as a parameter to estimate intracellular redox (reduction/oxidation reactions) state.

To measure the GSH/GSSG ratio, pre-inoculum grew overnight in SD medium, and inoculums were prepared using the total sample volume, which was incubated overnight in SGE medium. Next, samples were centrifuged at 1000 × *g* for 5 min, the volume of the pellet was estimated, and cells were resuspended in sulfosalicylic acid at the same volume of the pellet. To lyse cells, glass beads were added to samples that were vortexed for 20 min at 4 °C. Extracts of samples were centrifuged at 16000 × *g* for 40 min, and the supernatant, which contained peptides of

low molecular weight, was collected for analysis. The pellet was resuspended in Tris base for protein quantification in a NanoDrop.

HPLC (High Performance Liquid Chromatography) with electrochemical detection

To measure the GSH/GSSG ratio, the Coulochem III HPLC-ECD system (ESA, Inc.) equipped with one guard cell (model 5020) and analytic cell with electrode BDD (boron doped diamond, model 5040) was used. In this system, samples were separated by reverse phase chromatography using M.S. GEL C18 (EP-DF-5-120A, 4.6 × 150 mm, MC Medical, Inc.) at 35 °C in an isocratic flow of 0.7 ml/min with 25 mM monobasic sodium phosphate, 1.4 mM l-octanesulfonic acid, 6% acetonitrile (v/v) at a final pH 2.65 (adjusted with phosphoric acid). Component elution was monitored by applying a potential of +1400 mV in the analytic cell and +900 mV in the guard cell, finalizing loading by applying a potential of +1900 mV for 30 s followed by rebalancing for 5 min. GSH was used at 0, 0.5, 1, 2.5, 5, 7.5, and 10 mM and GSSG at 5, 10, 25, 50 mM, 75, and 100 mM as quantitative standards to construct a calibration curve.

Supernatants were filtered (using filter membranes with pore sizes 0.22 µm), and 50 µl of each sample was injected in the machine. Chromatograms of total thiols and disulfide detections were analyzed, and the GSH and GSSG peaks were identified and quantified.

Evaluation of UPR (unfolded protein response) and endoplasmic reticulum (ER) stress through alternative splicing of *Hac1* and expression of Pdi and CHOP

Overexpression of the *Hac1* gene is a hallmark of UPR and endoplasmic reticulum stress. Hac1 alternative splicing is dependent on Ire1 in a non-canonical way. Thus, it is possible to identify the presence of large forms (^{U}Hac1) of 651 base pairs and processed forms (^{S}Hac1) of 450 base pairs (present in situations of ER stress).

Reverse transcription PCR

To evaluate *Hac1* levels, total RNA was extracted using hot phenols following a protocol published by Scherrer & Darnell (1962). Briefly, inoculum grew in SD or SGE medium to achieve

1.0 OD. Next, cells were centrifuged at 1000 × *g* for 2 min at 4 °C. The supernatant was discarded, and the pellet was washed with 1 ml of DEPC (diethylpyrocarbonate) water. Cells were centrifuged at 16000 × *g* for 5 min at 4 °C, and the superior aqueous phase was mixed with 500 µl of acid phenol. Cells were incubated at 65 °C in a dry bath and vortexed every 10 min. Cells were incubated on ice for 5 min then centrifuged at 16000 × *g* for 5 min at 4 °C. The superior aqueous phase was mixed with 500 µl of acid phenol again and vortexed followed by incubation on ice for 5 min. Cells were centrifuged at 1000 × *g* for 5 min at 4 °C, and the superior aqueous phase was separated and mixed with 500 µl of chloroform, vortexed, and centrifugation at 1000 × *g* for 5 min at 4 °C. The superior phase was mixed with 50 µl of 3 M sodium acetate pH 5.2 and 1 ml of cold absolute ethanol. Samples were incubated at -80 °C for 2 h followed by centrifugation at 16000 × *g* for 10 min at 4 °C to precipitate RNA. RNA was washed twice with 70% cold ethanol, dried, and resuspended in 50 µl of DEPC water. Total RNA was quantified in NanoDrop and used for cDNA synthesis. The kit SuperScript II RT (Thermo Fisher Scientific) was used for cDNA synthesis following the manufacturer's instructions. The cDNA was treated with 1 ml of RNase H from *E. coli* at 37 °C for 20 min.

Hac1 levels were evaluated through cDNA amplification using 2 µl of cDNA at 1 µg/µl, 1 µl of 10 µM forward primer (TACAGGGATTTCCAGAGCACG), 1 µl of 10 µM reverse primer (TGAAGTGATGAAGAAATCATTCAATTC), 1.5 µl of 10 mM dNTP mix, 5 µl of 10× PCR buffer, 1.5 µl of $MgCl_2$ at 50 mM, 0.5 µl of Taq DNA polymerase at 5 U/L, and ultrapure water to dilute the solution to 50 µl. Samples were denatured at 94 °C for 2 min, followed by 22 denaturation cycles at 94 °C for 30 seconds, an annealing cycle at 54 °C for 30 seconds, and an extension cycle of 72 °C for 10 min. Samples were loaded into a 1% agarose gel, and images were analyzed in ImageJ (National Institutes of Health, Bethesda, Maryland, USA). cDNA of ADH4 (1369 base pairs) was amplified using the proper forward primer (TCACGACAATGCTAAGGCA) and reverse primer (AACACCATGAGGCAAGTGGT) and was used as a control to normalize

Hac1 expression, followed by the calculation of the ratio between the spliced form of ^{S}Hac1 (processed form of 450 base pairs) and the unspliced form of ^{U}Hac1 (large form of 651 base pairs).

To analyze Pdi and CHOP expression, inoculum grew in SD medium for 8 h, followed by protein extraction and Western blotting.

Western blotting

Three OD_{600} of cells were centrifuged at 1600 × *g* for 1 min at 4 °C. Cells were resuspended in 1 ml of ultrapure water followed by the addition of 160 µl of 2 M NaOH (sodium hydroxide) and 7.4% β-mercaptoethanol. Subsequently, cells were incubated on ice for 10 min, then mixed with 160 µl of 50% trichloroacetic acid (TCA), followed by 10 min of incubation on ice. Cells were centrifuged at 16000 × *g* for 2 min at 4 °C, and the pellet was washed with 500 µl of 1 M Tris-Base. Samples were centrifuged at 16000 × *g* for 10 seconds at 4 °C. Next, the pellet was resuspended in 150 µl of 5× Laemmli sample buffer (60 mM of Tris-HCl pH 6.8, 20% glycerol, 2% SDS, 14.4 mM β-mercaptoethanol, and 0.1% bromophenol blue). DTT (1 mM) was added, and samples were incubated at 95 °C for 5 min followed by incubation on ice.

Thirty µg/lane of each sample was fractioned in 12% Tris-HCl SDS-PAGE gels at 200 V for 1 h in Tris-Glycine buffer (25 mM of Tris, 192 mM glycine, and 0.1% SDS). Next, proteins were transferred to nitrocellulose membranes through the i-BLOT™ Gel Transfer System (Invitrogen) at 24 V, 650 mA for 30 min according to the manufacturer's recommendations. Membranes were blocked with 5% milk (Blotting Grade Blocker Non-Fat Dry Milk, BioRad) in TBS-T for 1 h at room temperature followed by 3× washing with TBS-T for 10 min. To verify alpha-synuclein, Pdi, and CHOP expression, membranes were incubated overnight in TBS-T with the following antibodies: anti-alpha-synuclein (sc-7011R, Santa Cruz Biotechnology; 18 kDa) at 1/1000, anti-PDI (sc-20132, Santa Cruz Biotechnology; 55 kDa) at 1/1000, and anti-CHOP (sc-7351, Santa Cruz Biotechnology; 30 kDa) at 1/500. Blots were normalized against β-actin (sc-47778, Santa Cruz Biotechnology; 44 kDa) at 1/1000 or Pgk1 (ab113687, Abcam; 44 kDa) antibodies incubated at

1/10000 in TBS-T for 1 h at room temperature. HRP-conjugated secondary antibodies used were anti-mouse (Amersham) at 1/6000 or anti-rabbit (1/10000) for 1 h at room temperature in TBS-T. ECL™ Prime Western Blotting Detection (GE Healthcare) was used to visualize antigen-antibody complexes, followed by exposure to appropriated films (Hyperfilm ECL, Amersham Biosciences). Normalizations were performed by dividing values of detected bands related to proteins of interest by values for β-actin or Pgk1. ImageJ software (National Institutes of Health, USA) was used to quantify film data.

Statistical analyses

Results were analyzed by one-way ANOVA followed by Tukey's post-hoc test using GraphPad Prism (GraphPad Software Inc., version 4.00, CA). Differences were considered statistically significant at a p-value of ≤ 0.05. All data are expressed as percent of control or absolute values ± standard deviation (SD).

Results

S. cerevisiae transformation

Cells strains were successfully transformed with A30P or A53T alpha-synuclein. Thus, 12 cell strains were used in this study as exemplified in Table 1. As expected, all cell strains showed an 18 kDa band for both alpha-synucleins (Figure 1). Interestingly, BY4741, ΔYpt53, and ΔAtg8 expressing A53T alpha-synuclein showed an additional band in the stacking gel for A53T alpha-synuclein, indicating protein aggregation. In ΔGem, the A53T band in the stacking gel was absent.

BY4741	Control cell line
BY4741 E.V. (empty vector)	Control cell line expressing E.V.
BY4741 A30P (pontual mutated alpha-synuclein A30P)	Control cell line expressing A30P alpha-synuclein
BY4741 A53T (pontual mutated alpha-synuclein A53T)	Control cell line expressing A53T alpha-synuclein
Δ Gem (homologous of MIRO1 knockout)	Control cell line of Δ Gem
Δ Gem E.V. (empty vector)	Cell line expressing E.V.
Δ Gem A30P (pontual mutated alpha-synuclein A30P)	Cell line expressing A30P alpha-synuclein
Δ Gem A53T (pontual mutated alpha-synuclein A53T)	Cell line expressing A53T alpha-synuclein
Δ Ypt53 (homologous of Rab5 knockout)	Control cell line of Δ Ypt53
Δ Ypt53 E.V. (empty vector)	Cell line expressing E.V.
Δ Ypt53 A30P (pontual mutated alpha-synuclein A30P)	Cell line expressing A30P alpha-synuclein
Δ Ypt53 A53T (pontual mutated alpha-synuclein A53T)	Cell line expressing A53T alpha-synuclein
Δ Atg8 (homologous of LC3 knockout)	Control cell line of Δ Atg8
Δ Atg8 E.V. (empty vector)	Cell line expressing E.V.
Δ Atg8 A30P (pontual mutated alpha-synuclein A30P)	Cell line expressing A30P alpha-synuclein
Δ Atg8 A53T (pontual mutated alpha-synuclein A53T)	Cell line expressing A53T alpha-synuclein

Table 1: Description of cell strains used in this study. BY4741 was used as the control cell line. ΔGem (homologous to Miro1), ΔYpt53 (homologous to Rab5), and ΔAtg8 (homologous to LC3) were cell lines used to study alpha-synuclein toxicity related to Miro1, Rab5 and LC3 genes. Cell lines expressed E.V. (empty vector), A30P alpha-synuclein, or A53T alpha-synuclein.

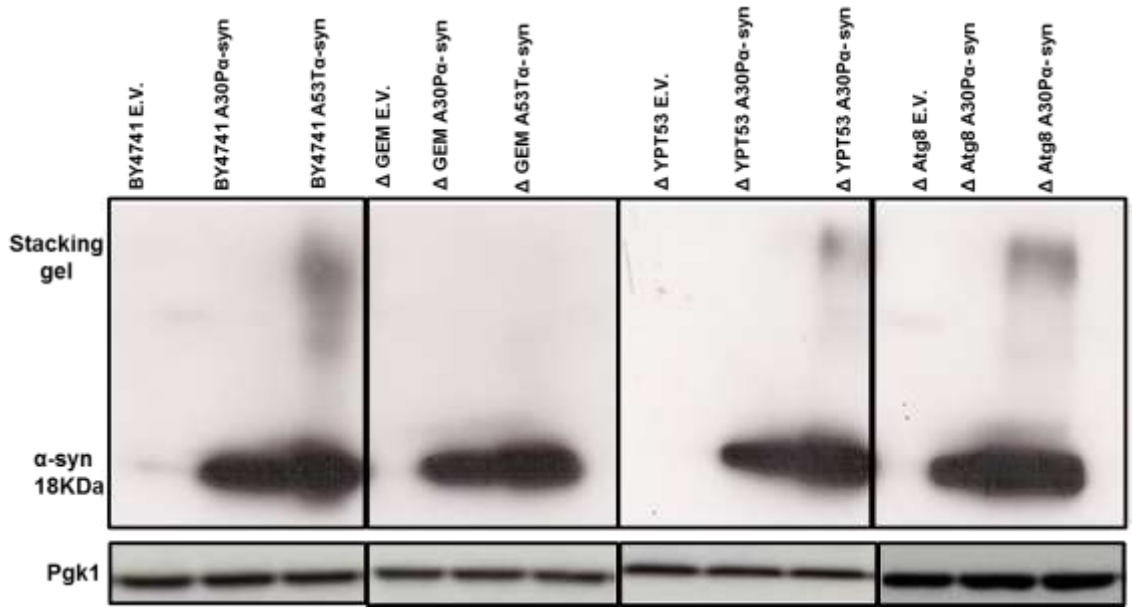

Figure 1: Western blotting for alpha-synuclein (18 kDa) and loading control Pgk1 (44 kDa). Expression of A30P and A53T alpha-synuclein in BY4741, ΔGem, ΔYpt53, and ΔAtg8 cultured in SD medium for 8 h. BY4741, ΔYpt53, and ΔAtg8 showed an additional band in the stacking gel for A53T alpha-synuclein, indicating protein aggregation.

Viability and H_2O_2 sensitivity assays

From now on the BY4741 and the deletions of Gem, YPT53 and Atg8 will be represented in the graphs as B., G., Y, and A., respectively.

The viability of cell strains was analyzed by a dilution series and growth curve in SD medium (fermentation medium; Figure 2), SG (intermediary; Figure 3) medium or SGE (respiratory) medium (Figure 4).

In the dilution series assay using SD medium, cells grew for 4 days. It was observed that deletions of Ypt53 and Atg8 genes did not change yeast growth. On the other hand, deletion of the Gem gene inhibited yeast growth. Alpha-synuclein expression changed the growth of the cell lines. A30P alpha-synuclein expression inhibited the growth of the BY4741 and ΔYpt53 lines relative to that in their respective lines expressing E.V. (Figure 2A and C). A53T expression inhibited growth of the BY4741 (Figure 2A), ΔYpt53 (Figure 2C), and ΔAtg8 (Figure 2D) lines relative to that in

their respective controls. Curiously, A30P and A53T alpha-synuclein expression ameliorated ΔGem line growth (Figure 2B).

In the growth curve assay, Gem, Ypt53, and Atg8 gene deletions significantly impaired normal growth of cells relative to that in BY4741 after 24 h of growth (Figure 2M). A30P and A53T alpha-synuclein impaired the growth of the BY4741 (E), ΔYPT53 (Figure 2G), and ΔAtg8 (Figure 2H) cells. After 24 h of growth, OD_{600} was measured again, revealing significantly decreased growth of the BY4741 (Figure 2I), ΔYPT53 (Figure 2K), and ΔAtg8 (Figure 2L) cells. Expression of A30P and A53T alpha-synuclein led to significantly higher ΔGem strain growth than that in the line expressing E.V. (Figure 2F and J).

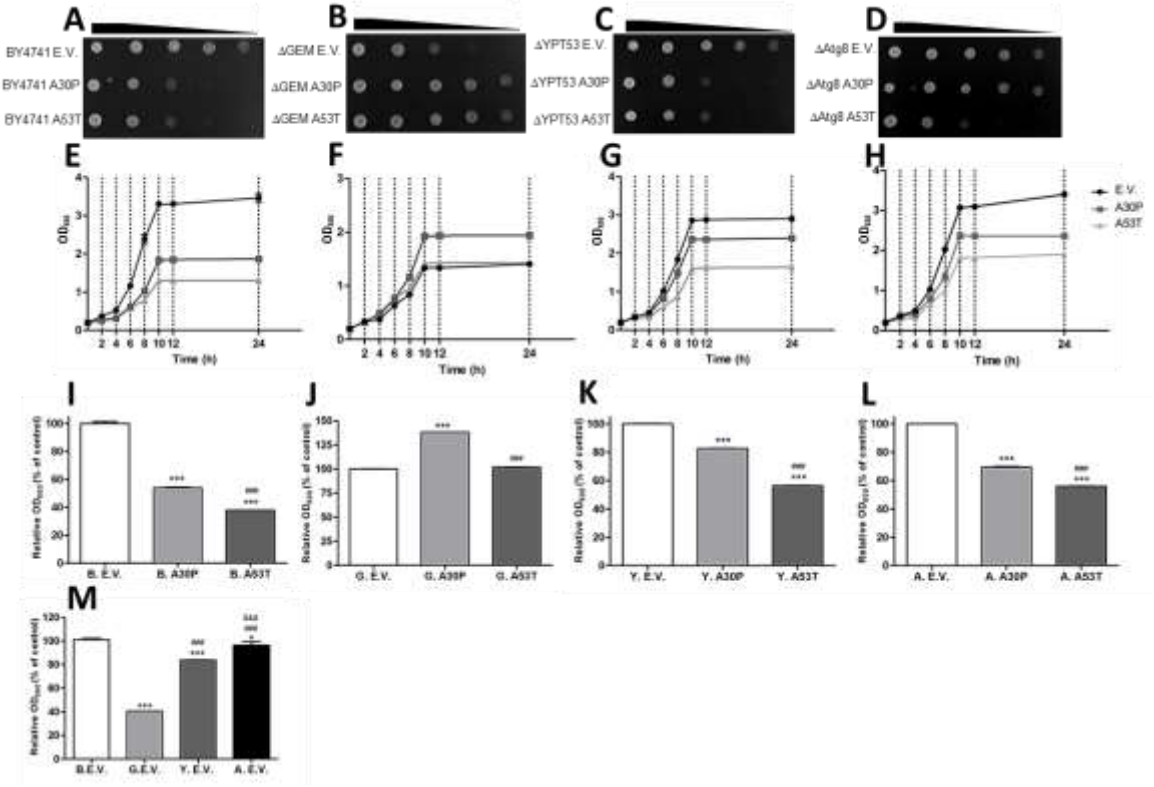

Figure 2: Viability of cell strains expressing A30P or A53T alpha-synuclein cultured in SD medium. A, B, C, and D: dilution series from a concentration of 1.0 OD diluted at 1/10, 1/100, 1/1000, or 1/10000 and cultured for 4 days. E, F, G, and H: growth curve from a concentration of 0.2 OD measured every 2 h. I, J, K, L and M: Quantification of growth curve at 24 h. Absence of Gem, Ypt53 and Atg8 genes impaired normal growth relative to that of BY4741 (B, F, G, H and M). A30P and A53T alpha-synuclein impaired normal growth of BY4741, ΔYpt53, and ΔAtg8 (A, C, D, E, G, H, I, K and L). A30P and A53T alpha-synuclein ameliorated ΔGem growth (B, F and J). The values of 3 independent experiments (n=3) are expressed as percent of control (E.V.) ± SD. One-way ANOVA followed by Tukey's post-hoc test were employed. *$p \leq 0.05$ compared with control. # $p \leq 0.05$ compared with ΔGem. & $p \leq 0.05$ compared with ΔYpt53.

In the viability assays using SG medium, cells grew for 4 days. All cell strains grew less than cells that grew in SD medium (Figure 3).

In the dilution series performed with SG medium, deletions in Gem and Ypt53 genes impaired normal growth of cells relative to that in BY4741 (Figure 3 A, B, C and D). Interestingly, A30P alpha-synuclein did not change ΔGem growth, whereas it ameliorated ΔYpt53 cell growth and inhibited ΔAtg8 cell growth relative to that in their respective controls. While A53T alpha-

synuclein inhibited BY4741 and ΔYpt53 growth (Figure 3 A and C), it did not change ΔAtg8 or ΔGem growth.

In the growth curve assay, deletion of Gem, Ypt53, and Atg8 significantly inhibited normal growth of cells relative to that of the BY4741 strain (M) after 24 h of growth. A30P and A53T alpha-synuclein did not change BY4741 growth (Figure 3E and I), whereas they significantly inhibited the growth of ΔYpt53 (Figure 3G and K) and ameliorated the growth of ΔGem. Contrasting the observations of the dilution series, A30P alpha-synuclein did not change ΔAtg8 growth, while A53T alpha-synuclein significantly inhibited growth of this cell strain (Figure 3H and L).

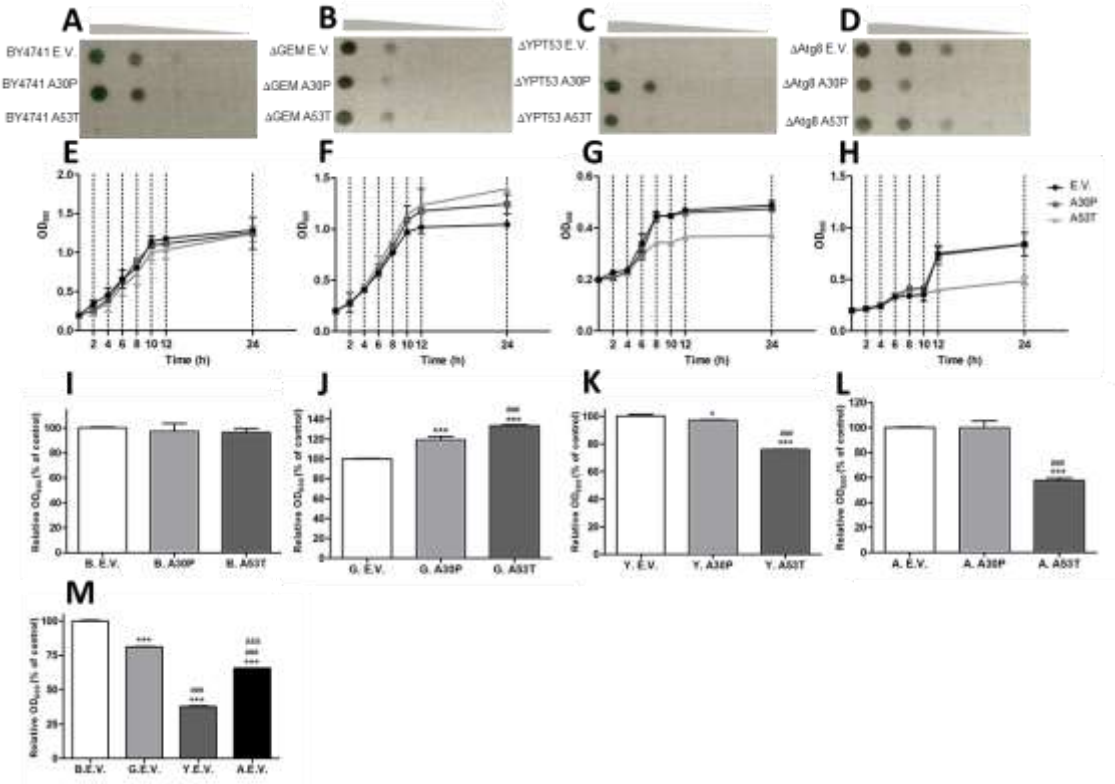

Figure 3: Viability of cell strains expressing A30P or A53T alpha-synuclein cultured in SG medium. A, B, C, and D: dilution series from a concentration of 1.0 OD diluted at 1/10, 1/100, 1/1000, or 1/10000 cultured for 4 days. E, F, G, and H: growth curve from a concentration at 0.2 OD measured every 2 h. I, J, K, L, and M: quantification of growth curve at 24 h. Absence of Gem, Ypt53, and Atg8 genes impaired normal growth relative to that of BY4741 cells (B, C, F, G, H and M). A53T alpha-synuclein impaired normal growth of BY4741, ΔYpt53, and ΔAtg8 (A, C, E, G, H, K and L). A30P and A53T alpha-synuclein ameliorated ΔGem growth (B, F, and J). The values of 3 independent experiments (n=3) are expressed as percent of control (E.V.) ± SD. One-way ANOVA followed by Tukey's post-hoc test were employed. *$p \leq 0.05$ compared with control. # $p \leq 0.05$ compared with ΔGem. & $p \leq 0.05$ compared with ΔYpt53.

In SGE medium, all cell strains grew in solid medium for more than a week. SGE medium inhibited the growth of all cell strains (Figure 4). BY4741 and ΔYpt53 strains expressing A53T alpha-synuclein had their growth completely inhibited (Figure 4A and C). However, A30P or A53T alpha-synuclein expression did not change ΔGem or ΔAtg8 growth (Figure 4B and D). Though the ΔAtg8 strain also showed inhibited growth in SGE medium, this line grew better than the other lines in this medium (Figure 4D).

It was not possible to perform growth curve assays because the cells did not grow at all in the liquid SGE medium. Even after 12 h of culture in liquid SGE medium, the OD_{600} did not change from 0.2.

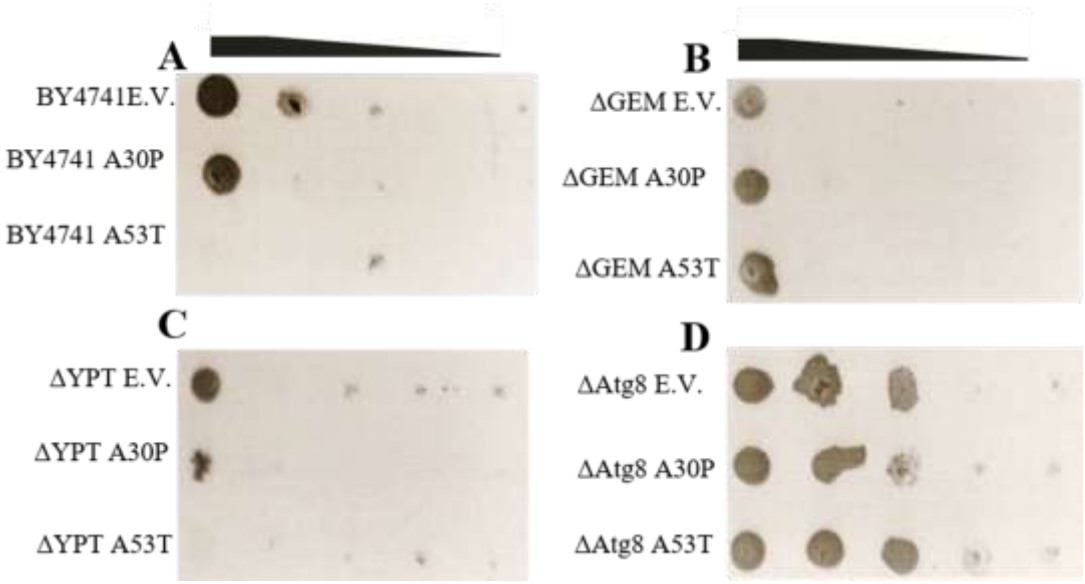

Figure 4: Viability of cell strains expressing A30P or A53T alpha-synuclein cultured in SGE. A, B, C, and D: dilution series from a concentration of 1.0 OD diluted at 1/10, 1/100, 1/1000, or 1/10000 and cultured for 8 days. All cell strains showed difficulties growing in SGE medium. The ΔAtg8 strain grew better than other strains.

Taken together, the results suggest that Gem, Ypt53 and Atg8 gene deletions led to lower viability of cells than that of the BY4741 strain in SD and SG medium. BY4741, ΔYpt53, and ΔAtg8 strains were more susceptible to toxicity of A30P and A53T alpha-synuclein in SD medium. In SG medium, only the ΔYpt53 strain was affected by both mutated alpha-synuclein proteins, while ΔAtg8 was only affected by A53T alpha-synuclein, and BY4741 strains were not affected by the mutated proteins at all.

Next, BY4741 cells were exposed to H_2O_2 for 10 days. Cells were cultured in SD medium, and a dilution series was done to analyze viability/sensitivity of cells to H_2O_2. ΔGem, ΔYpt53, and ΔAtg8 did not grow with exposure to H_2O_2 at 0.5 mM or smaller concentrations.

A sensitivity assay showed that in the absence of H_2O_2 or in the exposure of H_2O_2 at 0.5 mM, A30P and A53T alpha-synuclein decreased BY4741 viability (Figure 5A and B). However, 1 mM H_2O_2 decreased the viability of all cell strains (Figure 5C, D, E, and F). Furthermore, BY4741 cells expressing A53T alpha-synuclein and exposed to 2 mM or 3 mM H_2O_2 showed lower viability than cells expressing A30P alpha-synuclein. These data suggest that A53T alpha-synuclein is more toxic to cells than A30P alpha-synuclein.

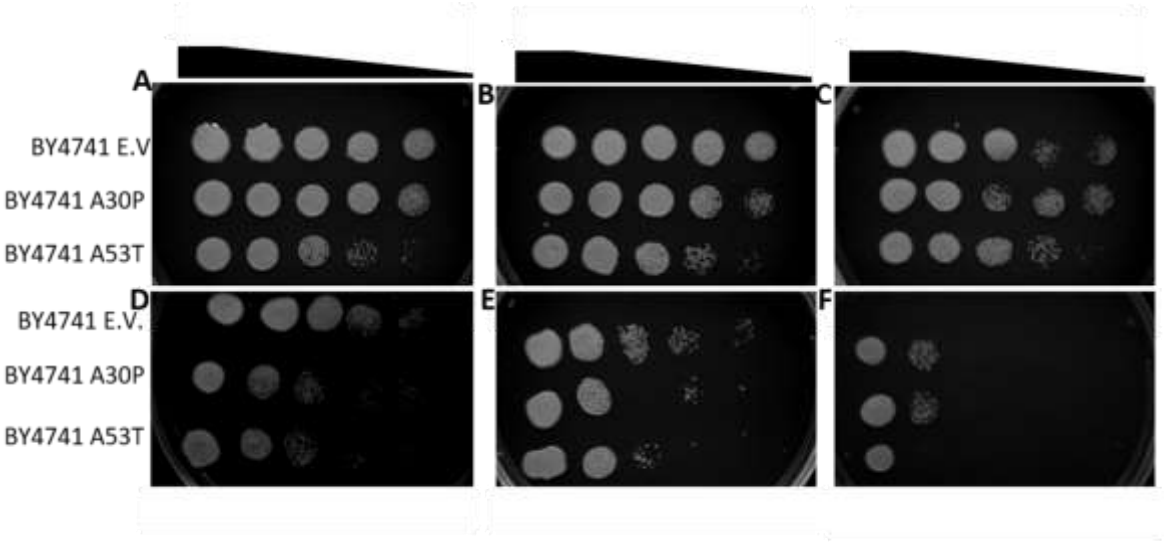

Figure 5: Sensitivity assay of BY4741 cells expressing A30P or A53T alpha-synuclein cultured in SD medium. Cell strains were exposed to H_2O_2 at 0 mM (A), 0.5 mM (B), 1.0 mM (C), 1.5 mM (D), 2.0 mM (E), or 3 mM (F) for 8 days. Yeast cells were plated in a dilution series at 1/10, 1/100, 1/1000, or 1/10000 from 1.0 OD A53T alpha-synuclein impaired yeast growth. H_2O_2 at 1.0 mM impaired normal growth of all cell strains.

H_2O_2 production

After 10 min of incubation with Amplex Red™ in samples containing isolated mitochondria and cultured in SG medium, deletion of Gem and Atg8 genes led to higher H_2O_2 levels that those in

the BY4741 and ΔYpt53 strains (Figure 6E). BY4741 and ΔYpt53 cells expressing A53T alpha-synuclein showed higher levels of H_2O_2 than those observed in BY4741 cells expressing E.V. or A30P alpha-synuclein (Figure 6A) and ΔYpt53 cells expressing E.V. (Figure 6C). On the contrary, ΔGem cells expressing A53T alpha-synuclein showed lower levels of H_2O_2 than did strains expressing E.V. or A30P alpha-synuclein (Figure 6B). ΔYpt53 cells expressing A30P alpha-synuclein showed higher levels of H_2O_2 than did cells expressing E.V. or A53T alpha-synuclein (Figure 6C). On the other hand, no changes in levels of H_2O_2 were observed in ΔAtg8 cells (Figure 6D).

These results strongly suggest that the absence of Gem protected mitochondrial dysfunction caused by A53T alpha-synuclein. Alternatively, absence of Ypt53 left mitochondria more susceptible to alpha-synuclein toxicity than ΔYpt53 cells expressing E.V.

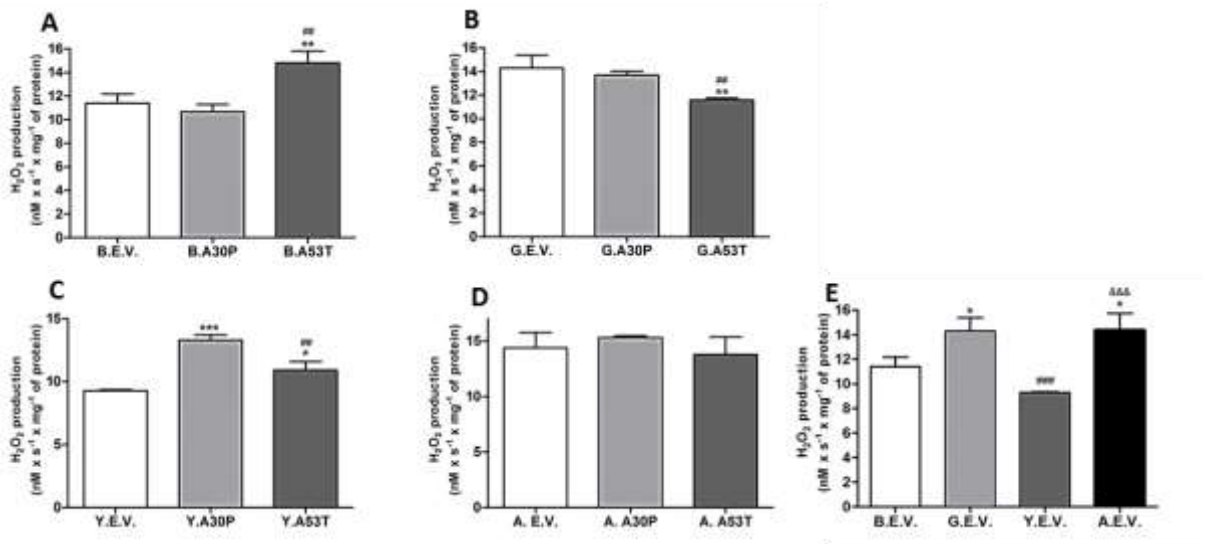

Figure 6: Mitochondrial H_2O_2 production using Amplex Red™. Inoculums were incubated in SG medium for 8 h. ΔGem E.V. and ΔAtg8 E.V. showed higher levels of H_2O_2, whereas ΔYpt53 showed lower levels of H_2O_2 than BY4741 cells expressing E.V. (E). BY4741 and ΔYpt53 expressing A53T α-synuclein showed higher levels of H_2O_2 than their respective controls (A, C). ΔYpt53 cells expressing A30P alpha-synuclein showed the highest levels of H_2O_2 relative to that in ΔAtg8 cells expressing E.V and ΔAtg8 expressing A53T alpha-synuclein (C). ΔGem cells expressing A53T alpha-synuclein showed lower levels of H_2O_2 than cells expressing E.V. or A30P alpha-synuclein. The values of 3 independent experiments (n=3) are expressed as absolute value ± SD. One-way ANOVA followed by Tukey's post-hoc test were employed. *$p \leq 0.05$ compared with control. # $p \leq 0.05$ compared with ΔGem. & $p \leq 0.05$ compared with ΔYpt53.

GSH/GSSG ratio measurements in cell strains cultured in SGE liquid medium

Increased levels of H_2O_2 changes the redox state of cells and could lead to imbalances between reduced forms of glutathione (GSH) and oxidized forms of glutathione (GSSG). In order to investigate the balance of both glutathione forms, the GSH/GSSG ratio was analyzed after growing of inoculum in SGE medium.

The GSH/GSSG ratio was significantly lower in ΔGem (E.V.) and ΔYpt53 (E.V.) strains than in the BY4741 (E.V.) strain, indicating higher levels of GSSG in these strains (Figure 7E). Interestingly, the ΔAtg8 line show the same GSH/GSSG ratio as BY4741, indicating that deletion of this gene did not affect the balance between the forms of glutathione. In addition, A30P and A53T expression decreased the GSH/GSSG ratio in BY4741 (Figure 7A), ΔGem (Figure 7B), ΔYpt53 (Figure 7C), and ΔAtg8 (Figure D), indicating that the cellular environment was more oxidative in the presence of mutated forms of alpha-synuclein.

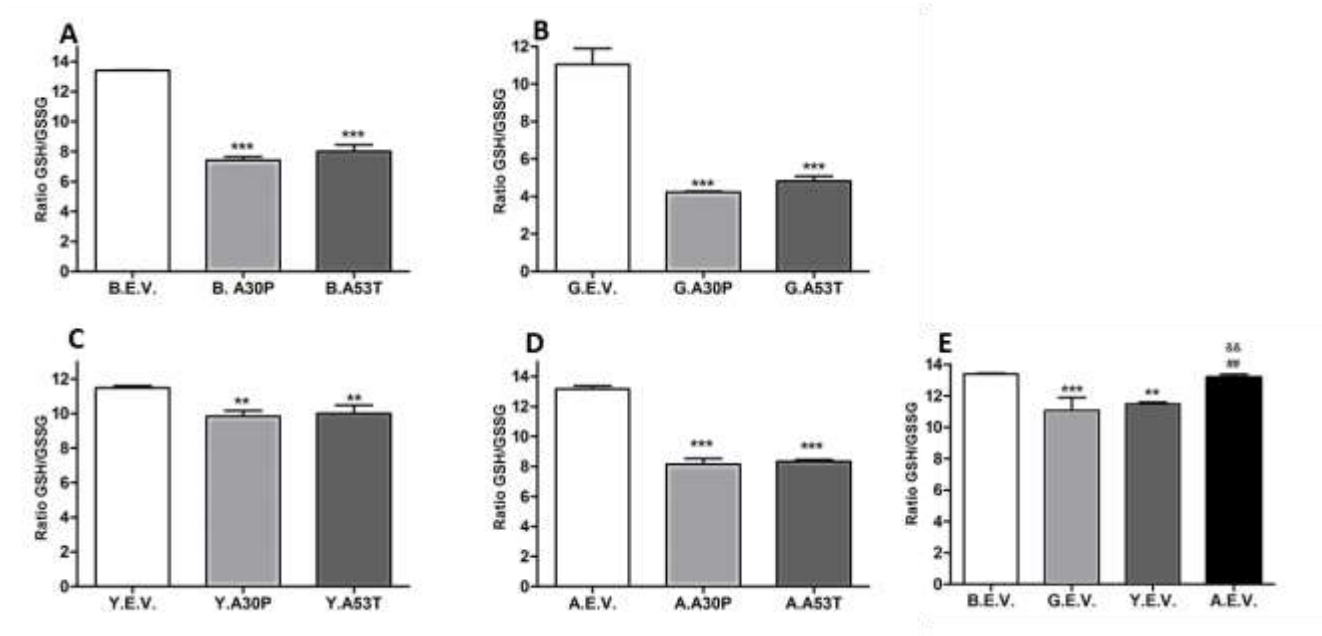

Figure 7: Quantification of GSH/GSSG ratio using HPLC-ECD. Inoculums were incubated in SGE medium for 8 h. ΔGem E.V., ΔYpt53 E.V. and ΔAtg8 E.V. showed lower GSH/GSSG than that in BY4741 E.V. (E). BY4741 (A), ΔGem (B), ΔYpt53 (C), and ΔAtg8 (D) expressing A30P or A53T alpha-synuclein showed lower GSH/GSSG ratios than their respective controls. The values of 3 independent experiments (n=3) are expressed as absolute number ± SD. One-way ANOVA followed by Tukey's post-hoc test were employed. *$p \leq 0.05$ compared with control. # $p \leq 0.05$ compared with ΔGem. & $p \leq 0.05$ compared with ΔYpt53.

UPR and endoplasmic reticulum stress analysis by evaluation of ^{S}Hac1/^{U}Hac1 ratio and Pdi and CHOP protein expression

Increased *Hac1* gene expression and alternative splicing of Hac1 is a well established parameter to evaluate UPR activation and ER stress. After incubation of strains in SD medium, levels of ^{U}Hac1 and ^{S}Hac1 were analyzed using RT-PCR (Figure 8A). ΔGem and ΔYpt53 lines showed higher ^{S}Hac1/^{U}Hac1 ratios than the BY4741 and ΔAtg8 strains (Figure 8F). ΔGem and ΔYpt53 strains expressing A30P or A53T alpha-synuclein showed lower ^{S}Hac1/^{U}Hac1 ratios than the ΔGem E.V. strain (Figure 8C) and ΔYpt53 E.V. strain (Figure 8D), respectively. Furthermore, the ΔYpt53 and BY4741 cells expressing A53T alpha-synuclein showed lower ^{S}Hac1/^{U}Hac1 ratios than ΔYPT53 expressing E.V. or A30P alpha-synuclein (Figure 8D) and BY4741 expressing E.V. or A30P alpha-synuclein, respectively (Figure 8A). Curiously, ΔAtg8 cells expressing A30P alpha-synuclein showed higher ^{S}Hac1/^{U}Hac1 ratios than ΔAtg8 cells expressing E.V. (Figure 8E).

These results indicate that, in SD medium, deletion of Gem Ypt53 and Atg8 genes activated UPR and ER stress. Curiously, all cell strains expressing E.V. showed an ^{S}Hac1 band and UPR activation. A30P and A53T alpha-synuclein did not activate the UPR in all cell strains significantly more than in their respective controls, except ΔAtg8 cells expressing A30P alpha-synuclein. Despite the ΔAtg8 strain expressing A53T alpha-synuclein showing a higher ^{S}Hac1/^{U}Hac1 ratio than its control (ΔAtg8 E.V.), the increase was not statistically significant. Therefore, only expression of A30P alpha-synuclein in the ΔAtg8 strain activated the UPR response and ER stress.

Representative bands of ^{U}Hac1 and ^{S}Hac1 were analyzed after incubation of cells in SGE medium (Figure 8G). Comparing all the cell strains expressing E.V., only the ΔGem strain showed a higher ^{S}Hac1/^{U}Hac1 ratio than the BY4741 E.V. and ΔYpt53 E.V. strains. Interestingly, ΔYpt53 E.V. showed the lowest ^{S}Hac1/^{U}Hac1 ratio relative to those of the BY4741, ΔGem, and ΔAtg8 strains. There were no significant differences in the ^{S}Hac1/^{U}Hac1 ratios between BY4741 and ΔAtg8 cells (Figure 8L).

A30P and A53T expression led to lower ^{S}Hac1/^{U}Hac1 ratio in BY4741 (Figure 8H) and ΔYpt53 (Figure 8J) cells than in their respective controls (E.V.). ΔGem cells expressing A53T alpha-synuclein also showed decreased ^{S}Hac1/^{U}Hac1 ratios, whereas A30P expression increased the ^{S}Hac1/^{U}Hac1 ratio (Figure 8 I). On the other hand, the ^{S}Hac1/^{U}Hac1 ratio in ΔAtg8 cells expressing mutated alpha-synuclein did not differ from that of ΔAtg8 E.V. (Figure 8K).

Analysis of UPR activation (and consequently ER stress) through evaluation of the ^{S}Hac1/^{U}Hac1 ratio showed different results among the strains depending on the type of medium used. In SD medium, the UPR was activated in all Δ strains relative to its activity in BY4741; on the other hand, only the ΔGem strain showed UPR activation relative to its activity in \BY4741 (and other strains) after incubation in SGE medium. In general, alpha-synuclein expression decreased the ^{S}Hac1/^{U}Hac1 ratio except in ΔAtg8 cells cultured in SD medium and ΔGem cells cultured in SGE medium expressing A30P alpha-synuclein, which increased the ^{S}Hac1/^{U}Hac1 ratio, indicating these cells strains were more susceptible to UPR activation than were the other cell strains expressing alpha-synuclein or E.V.

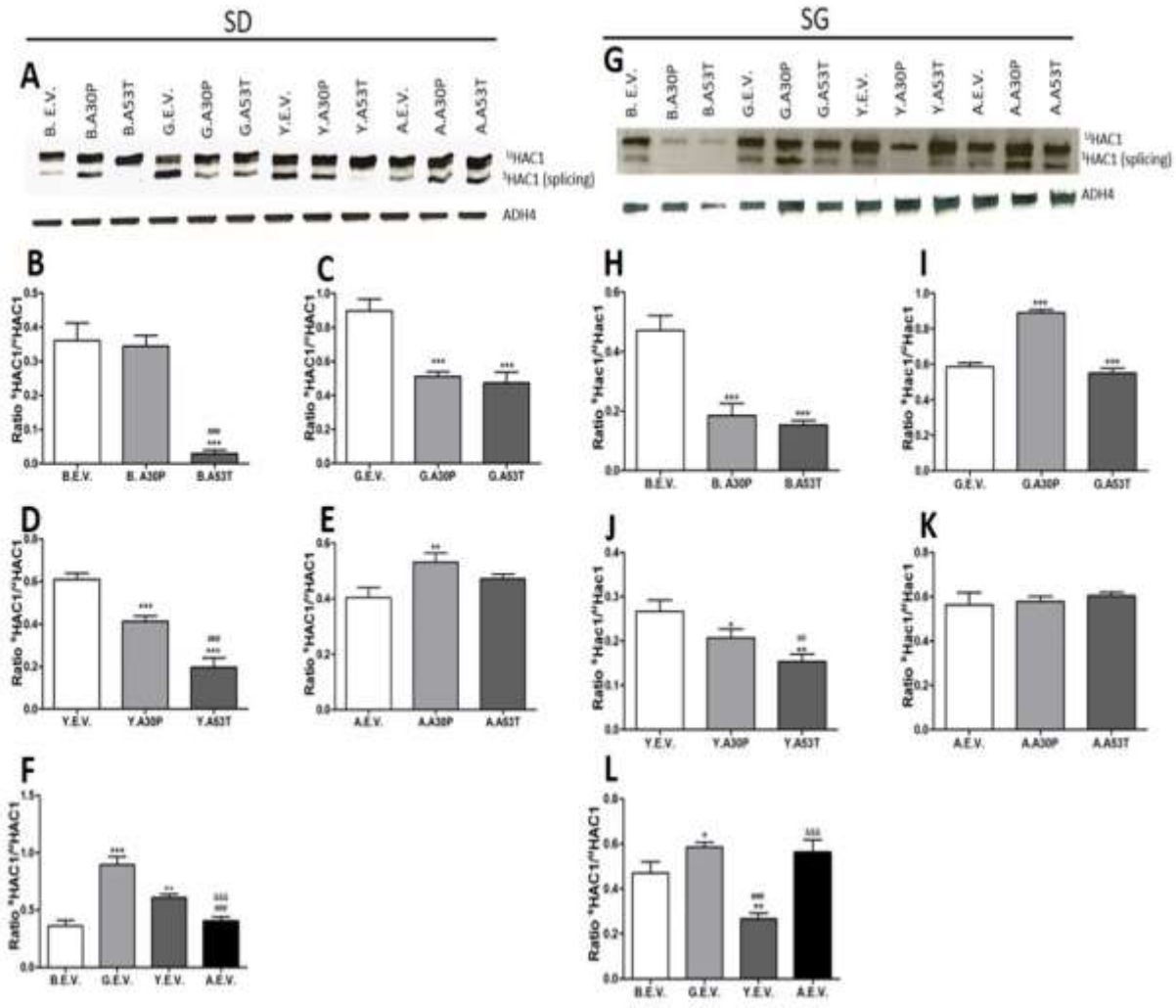

Figure 8: mRNA expression and ratio of ^{U}HAC1 (650 bp) and ^{S}HAC1 (splicing 450 bp) and loading control ADH4 (1369 bp) of cells cultured in SD medium (A) or SGE medium (G). In SD medium, all cell lines showed a spliced *Hac1* indicating ER stress except BY4741 cells expressing A53T alpha-synuclein. ΔGem and ΔYpt53 showed higher ^{S}HAC1/^{U}HAC1 ratios than BY4741 cells (F). BY4741 cells expressing A53T alpha-synuclein did not show *Hac1* splicing (B). ΔGem (C) and ΔYpt53 lines (D) expressing A30P or A53T alpha-synuclein showed lower ^{S}HAC1/^{U}HAC1 ratios than their respective strains expressing E.V. The ΔAtg8 line expressing A30P showed higher ^{S}HAC1/^{U}HAC1 ratios (E). All cell lines cultured in SGE medium showed a spliced *Hac1* except BY4741 expressing A30P or A53T alpha-synuclein and ΔYPT53 expressing A30P alpha-synuclein (G). Compared to BY4741 cells, the ΔGem strain showed a higher ^{S}HAC1/^{U}HAC1 ratio, whereas ΔYpt53 showed a lower ^{S}HAC1/^{U}HAC1 ratio, and ΔAtg8 did not show any differences in the ^{S}HAC1/^{U}HAC1 ratio (L). BY4741 E.V. showed a high ^{S}HAC1/^{U}HAC1 ratio (H). ΔYpt53 expressing A30P or A53T alpha-synuclein showed lower ^{S}HAC1/^{U}HAC1 ratios than strains expressing E.V. (J). ΔGem cells expressing A30P alpha-synuclein showed higher ^{S}HAC1/^{U}HAC1 ratios than the E.V. strain (I). ΔAtg8 did not show differences in the ^{S}HAC1/^{U}HAC1 ratio among the strains (K). The values of 3 independent experiments (n=3) are expressed as absolute number ± SD. One-way ANOVA followed by Tukey's post-hoc test were employed. *$p \leq 0.05$ compared with control. # $p \leq 0.05$ compared with ΔGem. & $p \leq 0.05$ compared with ΔYpt53.

Pdi1 plays a crucial role in protein folding. Specifically, in the presence of mutated alpha-synuclein, Pdi1 expression increases and removes misfolded proteins from the ER, preventing ER stress. We analyzed Pdi1 protein expression among our cell strains cultured in SD medium expressing alpha-synuclein and their respective controls. Representative blots of Pdi1 expression in the presence or absence of alpha-synuclein are shown for BY4741 (Figure 9A1), ΔGem (Figure 9B1), ΔYpt53 (Figure 9C1), and ΔAtg8 (Figure 9D1). Lower levels of protein were found in the ΔGem, ΔYpt53, and ΔAtg8 strains than in the BY4741 strain (Figure 9E). Expression of A30P alpha-synuclein in the BY4741 and ΔGem strain decreased Pdi1 expression (Figure 9A and B, respectively). Expression of A53T alpha-synuclein in the ΔGem strain also decreased protein levels (Figure 9B). These results indicate that protein folding in the ER may be impaired in these strains with decreased Pdi1 expression. In contrast, BY4741 cells expressing A53T alpha-synuclein showed increased levels of Pdi1 (Figure 9A), as did ΔAtg8 cells expressing both A53T alpha-synuclein and A30P alpha-synuclein (Figure 9D), indicating increased removal of misfolded proteins from the ER, a protective response mechanism against ER stress. Alternatively, expression of both mutated alpha-synucleins did not change Pdi1 levels in the ΔYpt53 strain (Figure 9C).

These findings revealed that, compared with BY4741, deletions in *Gem*, *Ypt53* and *Atg8* genes significantly decreased Pdi1 expression. Furthermore, deletions in such genes combined with mutated alpha-synuclein changed Pdi1 expression in different ways among the strains.

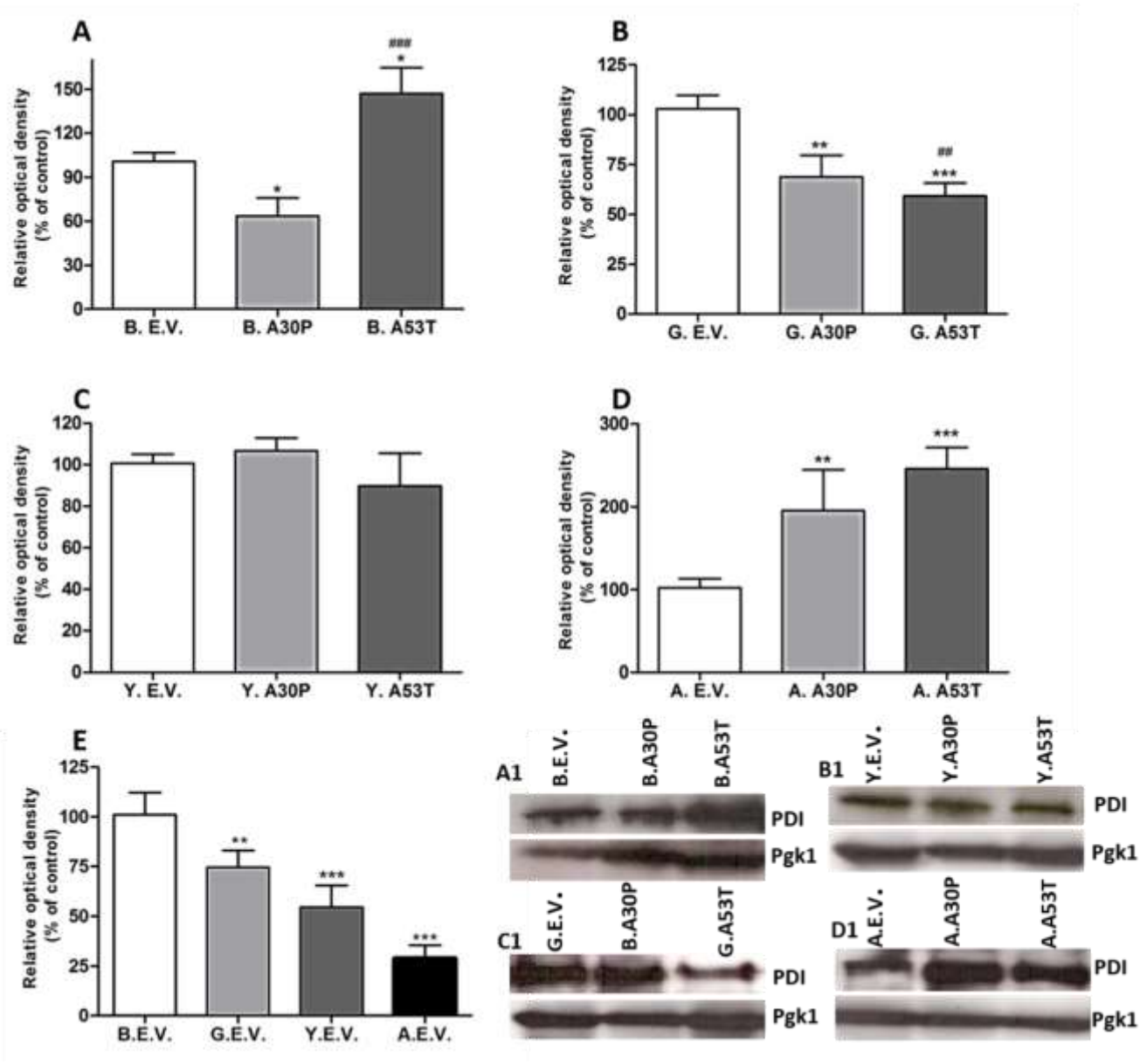

Figure 9: Representative blots of Pdi (anti-PDI, 55 kDa) and loading control Pgk1 (44 kDa) expression in BY4741 (A1), ΔGem (B1), ΔYpt53 (C1), and ΔAtg8 (D1) strains. Cell strains were cultured in SD medium. Compared with BY4741, all cell strains showed reduced levels of Pdi expression (E). BY4741 cells expressing A30P alpha-synuclein showed decreased levels of Pdi, whereas cells expressing A53T alpha-synuclein showed higher levels of Pdi than lines expressing E.V. (A). ΔGem cells expressing A30P or A53T alpha-synuclein showed lower levels of Pdi than cells expressing E.V. (B). ΔYpt53 did not change Pdi levels among lines expressing E.V. or alpha-synuclein (C). ΔAtg8 cells expressing A30P or A53T alpha-synuclein showed higher levels of Pdi than E.V. cells (D). The values of 3 independent experiments (n=3) are expressed as percent of control (E.V.) ± SD. One-way ANOVA followed by Tukey's post-test were employed. *$p \leq 0.05$ compared with control. # $p \leq 0.05$ compared with ΔGem. & $p \leq 0.05$ compared with ΔYpt53.

ER stress and UPR activation can modulate CHOP expression, increasing protein levels leading to cell death. Western blots for CHOP expression among the cell lines (Figure 10A) showed that deletion of GEM, YPT53, and Atg8 genes led to higher levels of CHOP than those in the BY4741 line (Figure 10F). BY4741, ΔGEM, and ΔYPT53 cells expressing A53T alpha-synuclein showed higher levels of CHOP than lines expressing E.V. These analyses strongly suggest that apoptosis pathways were activated in these lines. In contrast, A30P expression led to lower CHOP levels in ΔGEM, ΔYPT53, and ΔAtg8 than in their respective controls, indicating apoptosis activation by ER stress was repressed in these lines.

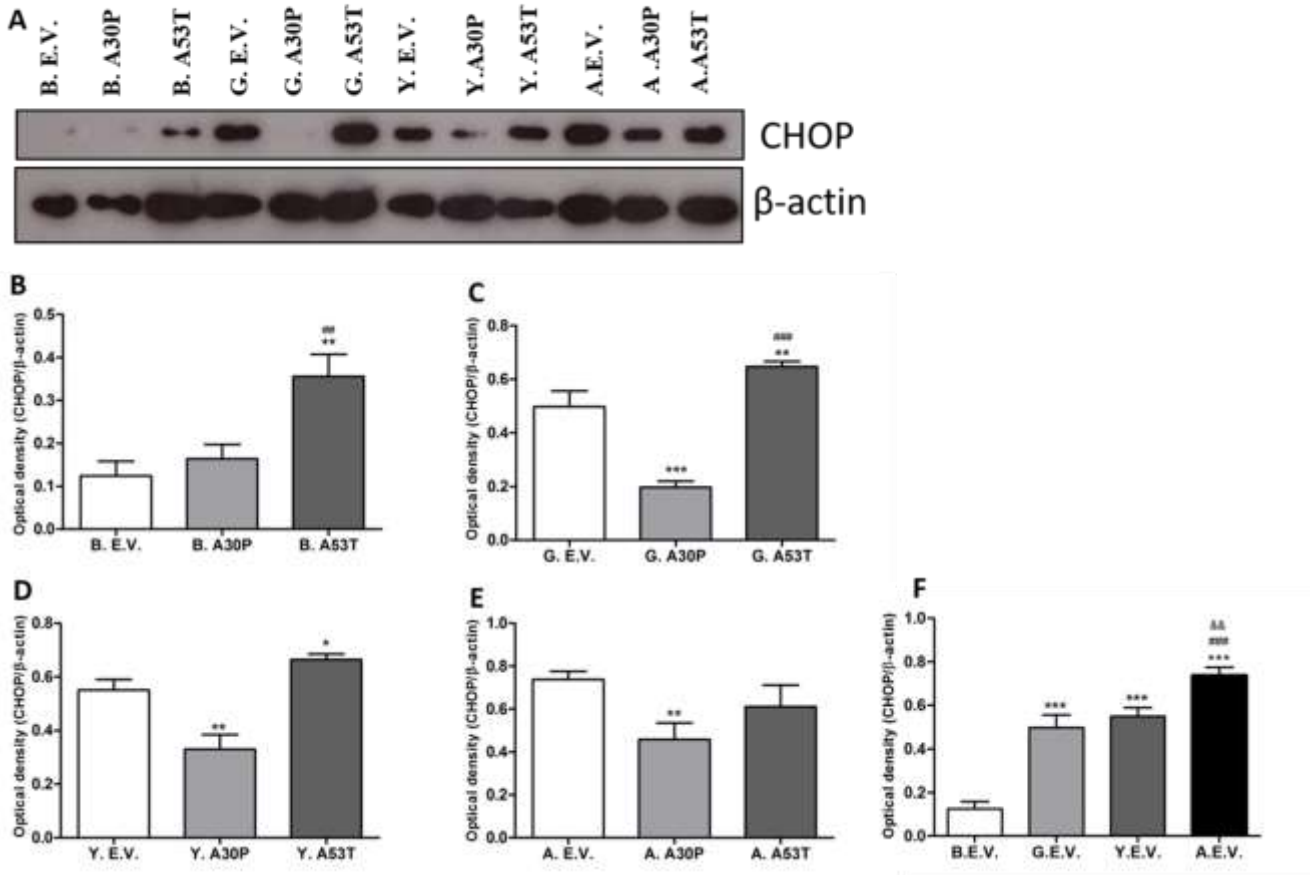

Figure 10: Representative blotting of CHOP expression among cell lines cultured in SD medium (A). Compared with BY4741, all cell lines showed significant increases in CHOP levels (F). The BY4741 line expressing A53T alpha-synuclein showed higher levels of CHOP than cells expressing E.V. or A30P alpha-synuclein (B). The ΔGEM line expressing A30P alpha-synuclein showed decreased levels of CHOP, whereas cells expressing A53T alpha-synuclein showed higher levels of CHOP than cells expressing E.V. (C). The ΔYPT53 line expressing A30P alpha-synuclein showed decreased levels of CHOP, whereas cells expressing A53T alpha-synuclein showed increased levels of CHOP (D). The ΔAtg8 line expressing A30P alpha-synuclein showed lower levels of CHOP than cells expressing E.V. (E). The values of 3 independent experiments (n=3) are expressed as absolute number ± SD. One-way ANOVA followed by Tukey's post-test were employed. *$p \leq 0.05$ compared with control. # $p \leq 0.05$ compared with ΔGEM. & $p \leq 0.05$ compared with ΔYPT53.

Discussion

Alpha-synuclein accumulation and aggregation leads to mitochondrial dysfunction, ER stress, aberrant autophagy, and consequently high levels of ROS. A30P or A53T point mutations in the alpha-synuclein gene can exacerbate alpha-synuclein toxicity mechanisms (Mullin and Schapira, 2013; Cali et al., 2011).

Mechanisms for alpha-synuclein toxicity may involve its interaction with GEM, Rab5, and Atg8 proteins. GEM protein (Miro in mammals) is a protein bound to the outer mitochondrial membrane. In the presence of accumulated alpha-synuclein, this protein is involved in dysregulation of autophagy, mitophagy, and communication between mitochondria and the ER, worsening autophagy dysfunction and ROS production (Frederick et al., 2004; Devine et al., 2016; Wang et al., 2011; Grimm, 2012). YPT53 (Rab5 in mammals) participates in the endocytic pathway and formation and trafficking of primary endosomes. It has been shown that alpha-synuclein interacts with Rab5 and may lead to abnormal vesicle distribution, consequently impairing the first step of autophagy. Atg8 (LC3 in mammals) is the main component of autophagy responsible for the formation of autophagosomes and fusion of vesicles in lysosomes. Specially mutated forms of alpha-synuclein impair autophagy, blocking their own degradation and leading to accumulated lysosomal vesicles. In this condition, lysosomes suffer alterations and play a role in the activation of apoptosis. Further, LC3 drives dysfunctional mitochondria to mitophagy, but in the long-term, the protein can activate excessive mitophagy leading to mitochondrial loss and cell death (Bourdenx et al., 2014; Chu et al., 2014).

In this study, we investigated alpha-synuclein toxicity related to GEM, YPT53 and Atg8 genes in a simple model using *S. cerevisiae* expressing A30P or A53T alpha-synuclein. We found all lines expressed both alpha-synucleins; BY4741, ΔYPT53, and Atg8 cells showed a second band for A53T alpha-synuclein in stacking gels, indicating the protein was aggregated in these lines. Other studies showed that mutated alpha-synuclein is prone to aggregation, and A53T alpha-synuclein oligomerizes and form aggregates faster and more easily than A30P alpha-synuclein

(Ostrerova-Golts et al., 2000; Stefanovic et al., 2015). Surprisingly, the ΔGEM line did not show an A53T band in stacking gels, suggesting GEM deletion prevented A53T alpha-synuclein aggregation.

Absence of GEM, YPT53, and Atg8 hampered cell growth in SD and SG medium relative to that in the control BY4741 line. As cited above, these genes play important roles that when disrupted consequently impair cell growth. A30P and A53T alpha-synuclein hamper growth of BY4741 cells in the absence or presence of different concentrations of H_2O_2, ΔYPT53, and ΔAtg8 in SD medium. It is known that A30P or A53T alpha-synuclein in different ways lead to mitochondrial damage and ATP depletion (Mullin and Schapira, 2013). Since cell division requires high ATP production, alpha-synuclein expression can first impair cell division. In contrast, growth of the ΔGEM line was not affected by A53T alpha-synuclein expression, whereas A30P alpha-synuclein ameliorated cell growth relative to that in the ΔGEM-expressing E.V. or A53T alpha-synuclein, suggesting absence of the GEM gene protects cells against alpha-synuclein damage.

Cells grown in SG medium showed another pattern of growth. In general, cells grew less in SG medium than in SD medium. *S. cerevisiae* is known to activate genes that promote growth in the presence of glucose, justifying yeast's preference for glucose (Williams et al., 2015; Zeng et al., 2016). Expression of A30P or A53T alpha-synuclein did not affect the BY4741 line, while A30P impaired ΔYPT53 growth, and A53T alpha-synuclein expression impaired growth of both the ΔYPT53 and ΔAtg8 lines. Alternatively, the ΔGEM line expressing A30P or A53T alpha-synuclein showed better growth than the line expressing E.V., indicating alpha-synuclein expression ameliorated ΔGEM growth. The changed patterns of growth observed in the BY4741, ΔGEM expressing A30P or A53T alpha-synuclein, and ΔAtg8 expressing A30P alpha-synuclein lines can be related to mitochondrial activity. In SD medium, cells received glucose, and mitochondrial respiration was repressed; however, in SG medium, cells could both ferment or activate

mitochondrial respiration, increasing the number of mitochondria in the cells. These observations suggest mitochondrial activity ameliorated yeast growth in the presence of mutated alpha-synuclein.

In SGE medium, yeasts are unable to utilize anaerobic respiration (are not able to ferment) and can only use mitochondrial respiration to obtain energy. In this medium, cells need to increase the amount/number of mitochondria to supply energy levels. In general, all cell lines showed difficulty growing. BY4741 and ΔYPT53 expressing A53T alpha-synuclein did not grow, whereas ΔGEM and ΔAtg8 grew in the presence of the mutated proteins, revealing that compared to other lines, A53T alpha-synuclein was less toxic for ΔGEM and ΔAtg8 when they grew in SGE medium. Interestingly, the ΔAtg8 line grew better than other lines, revealing that mitochondrial activity improved cell growth in this line. It is known that alpha-synuclein disturbs autophagy and over the long term overload the lysosome, leading to accumulation of vesicles and cell death (Freeman et al., 2013). Arduíno and colleagues (2013) have shown that mitochondrial dysfunction plays an important role in aberrant autophagy, leading to accumulation of vesicles and consequent autophagy dysfunction. These findings suggest that functional mitochondria can prevent aberrant autophagy. Using SGE medium, mitochondria are functional and may ameliorate damages caused by absence of the Atg8 gene.

Mitochondrial dynamics are crucial to keep the quality of the organelle. This process involves GEM, which is responsible for coordinating the correct localization and signaling to renew the organelle (Frederick et al., 2004; Devine et al., 2016), and Atg8 to remove nonfunctional mitochondria through macroautophagy (Hamacher-Brady and Brady, 2016; Chen et al., 2015). Further, it was already shown that impairment in mitochondrial quality control generates excessive ROS (Hamacher-Brady and Brady, 2016). In our model, isolated mitochondria from the ΔGEM and ΔAtg8 lines showed higher H_2O_2 levels than the BY4741 and ΔYPT53 lines when grown in SG medium. These data reveal that the absence of these genes was enough to increase H_2O_2 produced by mitochondria, indicating they are crucial to maintain mitochondrial function.

Mitochondrial dysfunction caused by overexpression of alpha-synuclein or expression of mutated alpha-synuclein also increases ROS levels, such as H_2O_2 (Nakamura, 2013). A53T expression increased H_2O_2 levels in BY4741 and ΔYPT53 cells. However, comparing levels of H_2O_2 in these 2 cell lines, we observed that H_2O_2 produced by mitochondria in BY4741 cells expressing A53T alpha-synuclein was higher than H_2O_2 produced by the ΔYPT53 line, indicating that the absence of the YPT53 gene ameliorated mitochondrial dysfunction caused by A53T alpha-synuclein. In fact, it has been shown that A53T alpha-synuclein damaged mitochondria more intensively by interacting with the mitochondrial membrane, leading to increased ROS levels.

On the other hand, A30P alpha-synuclein did not interact with the mitochondrial membrane and its toxicity is more related to proteasome inhibition (Smith et al., 2005). A30P alpha-synuclein did not change mitochondrial production of H_2O_2 in most lines except in ΔYPT53 cells, which showed the highest levels of H_2O_2 relative to those in ΔYPT53 expressing E.V. or A53T alpha-synuclein. These data indicate that alpha-synuclein, especially A30P alpha-synuclein toxicity involving mitochondrial dysfunction, is related to deficits in the formation and trafficking of primary endosomes. In earlier stages of neurodegeneration, alpha-synuclein is endocytosed by YPT53 to be degraded in lysosomes. However, accumulated alpha-synuclein disturbs degradation processes, disturbing primary endosomes and lysosome dynamics, leading to alpha-synuclein aggregation (Sung et al., 2001; Sancenon et al., 2012). In addition, overexpression of alpha-synuclein in yeast leads to increased YPT53 expression in order to remove excessive protein accumulation. Moreover, expression of alpha-synuclein in the long term blocks autophagy, including mitophagy, and YPT53 can drive mitochondria to degradation independent of mitophagy signals (Sancenon et al., 2012; Hamacher-Brady et al., 2014). Thus, deleting the YPT53 gene led to inhibition of the protective response of cells to remove mutated alpha-synuclein, promoting excessive alpha-synuclein accumulation that consequently damaged mitochondria. Beyond that, damaged mitochondria could not be degraded via autophagy leading to increased levels of H_2O_2.

However, both alpha-synucleins expression did not change H_2O_2 levels in the ΔAtg8 line, suggesting that autophagy inhibition in the presence of mutated alpha-synuclein did not increase mitochondrial dysfunction. Moreover, a comparison of the ΔYPT53 and ΔAtg8 lines indicates that damage to the first steps of degradation processes in the endocytic pathway and primary endosomes formation/trafficking and their accumulation can play an important role in alpha-synuclein aggregation and in mitochondrial dysfunction caused by alpha-synuclein toxicity.

Interestingly, the ΔGEM line expressing A53T alpha-synuclein showed the same levels of H_2O_2 as the BY4741 line expressing E.V., whereas, compared to ΔGEM E.V., cells expressing A30P alpha-synuclein did not show differing levels of H_2O_2. Once more, these results suggest that the absence of GEM attenuated alpha-synuclein toxicity, especially with the mutated form A53T alpha-synuclein. In early stages of PD, A53T alpha-synuclein induces aberrant mitochondrial fission, producing many fragmented mitochondria that need to be degraded by mitophagy. In mammals, it is reported that proteins related to fusion and fission of the mitochondrial target Miro (GEM) protein signal mitophagy.

In yeast, GEM participates of mitochondrial dynamics, but the protein is not required for the mitochondrial fission process (Frederick et al., 2004). However, it was already shown that in mammalian cells and yeast, A53T alpha-synuclein perturbs mitochondrial fusion, fission, and mitophagy (Chen et al., 2015; Sampaio-Marques, et al., 2012). By impairing mitophagy, A53T alpha-synuclein blocks removal of dysfunctional mitochondria contributing to increased H_2O_2 levels (Chen et al., 2015). Interestingly, Lui and colleagues (Devine et al., 2016) demonstrated in mammalian cells that knockdown of Miro induced mitophagy, removing dysfunctional mitochondria. Taken together, these findings suggest that absence of GEM plays a role in the maintenance of healthy mitochondria in the presence of A53T alpha-synuclein, and GEM is involved in mitochondrial dysfunction caused by A53T alpha-synuclein.

One of the routes involved in H_2O_2 elimination requires Glutathione peroxidase which oxidizes GSH to GSSG. Therefore, analysis of the ratio between GSH/GSSG is considered a good parameter to evaluate the redox state of cells. All cell lines were cultured in SGE medium, and the GSH/GSSG ratio was evaluated. Surprisingly, we found no differences in the GSH/GSSG ratio between the BY4741 and ΔAtg8 lines even though H_2O_2 levels were increased in ΔAtg8 line. Even so, the ΔYPT53 line, which did not show different levels of H_2O_2 from those of BY4741, showed reduced GSH/GSSG ratio. Together, these results indicate an oxidative environment in the absence of both YPT53 (which consequently impairs autophagy initiation) and Atg8 (which blocks autophagy). However, only absence of Atg8 led to mitochondrial dysfunction and increased H_2O_2. On the other hand, as expected, compared to BY4741, the ΔGEM line showed a reduced GSH/GSSG ratio, indicating an oxidative environment due to the high levels of H_2O_2.

All cell lines expressing either alpha-synuclein showed a lower GSH/GSSG ratio than that in their respective controls. These data indicate that both alpha-synucleins led to an oxidized environment without necessarily damaging mitochondria. This imbalance in the redox state of cells can be caused by accumulated misfolded proteins in the ER. Overexpression of alpha-synuclein or mutated alpha-synuclein can cause ER stress, leading to oxidative stress and consequently decreased GSH/GSSG levels (Smith et al., 2005). Moreover, mitochondria and ER communicate and transfer calcium and other signals to maintain homeostasis through a membrane protein complex (Raturi and Simmen, 2013).

Alpha-synuclein has been shown to interfere in cross-talk between the mitochondria and ER, leading to an imbalance of calcium transference (Krols et al., 2016). In cases of ER stress, calcium leaks from ER are recaptured by mitochondria, increasing mitochondrial metabolism and consequently increasing H_2O_2. Increased H_2O_2 levels also lead to release of calcium from ER, creating a vicious cycle (Zeeshan et al., 2016).

Mitochondrial dysfunction leads to ATP depletion and excessive H_2O_2 levels in the ER, oxidizing GSH in GSSG and altering the redox state of cells. This chronic oxidized state leads to cell death. This situation can be worsened by the absence of GEM, YPT53, and Atg8. GEM modulates the contact between mitochondria and ER, and it is already known that the absence of GEM disrupts this communication, leading to excessive autophagy (Grimm, 2012). Furthermore, autophagy also plays a role in regulation of mitochondria and ER contacts; thus, absence of YPT53 or Atg8 can also be involved in redox imbalance caused by miscommunication between mitochondria and ER (Krols et al., 2016).

Mutated alpha-synuclein induces accumulation of unfolded and misfolded proteins in ER, leading to ER stress. In order to alleviate ER stress, the UPR is activated, leading to increased expression of the transcription factor Hac1, which is spliced (Mizuno et al., 2015). Thus, in order to evaluate ER stress, we analyze the ratio of spliced Hac1 (^{S}Hac1) and Hac1 mRNA (^{U}Hac1). We found Hac1 splicing indicated UPR activated in all cell lines cultured in SD medium except in BY4741 cells expressing A53T alpha-synuclein.

Evaluation of the ^{S}Hac1/^{U}Hac1 ratio revealed that the ΔGEM and ΔYPT53 lines cultured in SD medium had an increased ^{S}Hac1/^{U}Hac1 ratio, indicating UPR activation as expected. Conversely, the ratio of ^{S}Hac1/^{U}Hac1 in the ΔAtg8 line did differ from that in BY4741. Taken together, these results indicate that the absence of GEM and YPT53 stress both mitochondria and ER. However, absence of Atg8 and the consequent autophagy blocking causes selective damage in mitochondria. Curiously, A30P alpha-synuclein expression in the BY4741 line did not lead to differing ^{S}Hac1/^{U}Hac1 ratios from those in BY4741 expressing E.V., and A53T alpha-synuclein, which is more toxic than A30P alpha-synuclein, showed a decreased ^{S}Hac1/^{U}Hac1 ratio, indicating A53T alpha-synuclein blocked UPR in BY4741 cells. Despite alpha-synuclein activating the UPR, in the long term or with A53T alpha-synuclein overexpression, the UPR can be blocked to activate apoptosis signaling (Colla et al., 2012; Credle et al., 2015). Interesting, ΔGEM and ΔYPT53 lines

expressing A30P or A53T alpha-synuclein showed decreased ^{S}Hac1/^{U}Hac1 ratios, indicating alpha-synuclein expression combined with deletion of these genes repressed the UPR response relative to that in their respective controls. On the other hand, ΔAtg8 cells expressing A30P alpha-synuclein showed an increased ^{S}Hac1/^{U}Hac1 ratio, indicating greater UPR activation in these cells than in cells expressing E.V. or A53T alpha-synuclein.

Evaluation of ^{S}Hac1/^{U}Hac1 ratios in cells cultured in SGE medium showed the presence of spliced Hac1 in most cell lines except in BY4741, ΔYPT53 lines expressing A30P alpha-synuclein, and BY4741 cells expressing A53T alpha-synuclein, indicating UPR was blocked in these cell lines. Comparisons among the lines revealed increased, decreased, and unchanged ^{S}Hac1/^{U}Hac1 ratios in ΔGEM, ΔYPT53, and Atg8, respectively, from those in BY4741. Expression of both alpha-synucleins decreased ^{S}Hac1/^{U}Hac1 ratio in BY4741 and ΔYPT53 lines, suggesting both alpha-synucleins were repressing the UPR response in these lines relative to that in their respective controls. Interestingly, in SD medium, A30P alpha-synuclein expression did not repress the UPR response, indicating that mitochondrial activity may play a role in activation or repression of UPR in the presence of A30P alpha-synuclein. On the other hand, ΔGEM cells expressing A30P alpha-synuclein showed an increased ^{S}Hac1/^{U}Hac1 ratio, suggesting a more activated UPR relative to that in cells expressing E.V. or ΔGEM cultured in SD medium. These data suggest that A30P alpha-synuclein overactivated the UPR in the absence of GEM combined with mitochondrial activity. Both alpha-synucleins' expression did not change the ^{S}Hac1/^{U}Hac1 ratio in the ΔAtg8 line, indicating that mitochondrial activity and absence of Atg8 repress UPR activation by A30P alpha-synuclein in SD medium. Taken together, these results strongly suggest, once more, that alpha-synuclein toxicity is dependent on mitochondrial activation.

ER stress can impair folding machinery of ER. Activated UPR and Hac1 induce expression of chaperones, such as PDI, in order to enhance ER capacity to fold and secrete misfolded proteins to alleviate ER stress. We found lower PDI levels in all Δ cell lines cultured in SD medium than in

BY4741, indicating deletion of GEM, YPT53 and Atg8 decreased ER folding machinery capacity. Beyond that, as expected, BY4741 cells showed decreased levels of PDI in the presence of A30P alpha-synuclein. In contrast, expression of A53T alpha-synuclein was accompanied by increased PDI levels, corroborating with ER stress.

According to previous results, both alpha-synucleins decreased PDI levels in the ΔGEM line, indicating absence of this gene impairs signaling to enhance folding and secretion of misfolded proteins in ER. This mechanism could be involved in the impairment of A53T protein aggregation only observed in this line. Nonetheless, increased expression of PDI is related to protective responses against ER stress, redox imbalance, and formation of alpha-synuclein aggregates.

Chronically increased PDI levels lead to increased levels of ROS, consequently favoring oligomerization and aggregation of alpha-synuclein. Moreover, it was reported that inhibition of PDI also could prevent ER stress and alpha-synuclein aggregation (Mizuno et al., 2016; Zeeshan et al., 2015; Lehtonen et al., 2016). In the ΔYPT53 line, alpha-synuclein expression did not change PDI levels, whereas both alpha-synucleins increased PDI levels in the ΔAtg8 line, indicating autophagy impairment; mutated alpha-synuclein expression activated ER folding and secretion of protein machinery.

The paradox of adaptive responses against ER stress is that when they do not work, they can activate apoptosis signaling, such as the transcription factor CHOP (Yang et al., 2016). Therefore, we evaluated CHOP levels in cells cultured in SD medium. We found higher CHOP expression in all Δ lines than in BY4741, which did not show any band for CHOP, indicating that deletion of these genes was enough to signalize cell death.

Interestingly, expression of A30P alpha-synuclein did not activate CHOP, whereas A53T alpha-synuclein increased CHOP levels, suggesting that even though Hac1 was not spliced in BY4741 cells expressing A53T, ER was stressed and activated apoptosis, as demonstrated by high levels of PDI and CHOP, respectively.

Surprisingly, ΔGEM cells expressing A30P did not show CHOP expression, revealing A30P alpha-synuclein combined with deletion of GEM completely repressed CHOP expression.

Interestingly, A30P alpha-synuclein expression also decreased CHOP levels in the ΔYPT53 and Atg8 lines, indicating that A30P alpha-synuclein repressed CHOP expression at different levels in all Δ lines. These data suggest that these genes may play a role in apoptosis signaling caused by A30P expression. On the other hand, ΔGEM and ΔYPT53 cells expressing A53T alpha-synuclein showed higher levels of CHOP than in their respective controls. These cell lines did not show increased ER stress markers in this study, suggesting that in the absence of these genes, A53T alpha-synuclein directly activates apoptosis pathways without activating protective responses. Alternatively, ΔAtg8 was the only line where A53T alpha-synuclein expression did not increase CHOP levels, suggesting deletion of Atg8 can repress apoptosis signaling through CHOP and prevent cell death.

Finally, we observed that A30P alpha-synuclein toxicity was dependent on mitochondrial and autophagy dysfunction since mitochondrial levels of H_2O_2 only changed in the ΔYPT53 line, revealing that impairment in the initial steps of autophagy and the endocytic pathway can damage mitochondria in the presence of this mutation. Moreover, the spliced band of Hac1, indicating UPR activation, only appeared in all cell lines cultivated in SD medium, whereas in cells cultivated in SGE medium (which have functional mitochondria), only ΔGEM and ΔAtg8 showed spliced Hac1. Since deletion of GEM and Atg8 promotes mitochondrial and autophagy dysfunction, respectively these data strongly suggest UPR is activated only when mitochondria and autophagy is compromised. Furthermore, PDI levels were increased only in the ΔAtg8 line, suggesting that impairment of autophagy may trigger ER stress.

It is known that A53T alpha-synuclein is prone to forming aggregates faster and more easily than A30P alpha-synuclein. However, we observed that absence of ΔGEM could impede aggregation formation and preserve mitochondrial function, whereas absence of GEM, YPT53, and

Atg8 could repress UPR activation. Additionally, deletion of GEM and YPT53 prevented increases of PDI, whereas deletion of Atg8 prevented ER apoptosis signaling. Taken together, these results suggest that A53T alpha-synuclein toxicity involving damage to mitochondrial and apoptosis signaling caused by ER stress are dependent on the GEM and Atg8 genes, respectively.

Nevertheless, more studies are needed to clarify how these genes are involved in alpha-synuclein toxicity.

Conclusion

In our study, we observed that:

A30P alpha-synuclein toxicity was dependent on mitochondrial and autophagy dysfunction.

A53T alpha-synuclein was more toxic than A30P alpha-synuclein.

A53T alpha-synuclein toxicity involving damage to mitochondrial and apoptosis signaling caused by ER stress was dependent on GEM and Atg8, respectively.

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Part III

3.3. Decreased mitochondrial mobility and increased number of lysosome vesicles in human dopaminergic neurons derived from SNCA^3 patients

Abstract

Insoluble alpha-synuclein protein aggregates and oxidative stress caused by mitochondrial dysfunction are the main characteristics of Parkinson's disease (PD). Mutations in the alpha-synuclein gene (SNCA) constitute the primary risk for PD. Triplications in the alpha-synuclein gene (SNCA^3) have been reported to cause overexpression of the protein and lead to early-onset PD. Studies using several models of PD have revealed that alpha-synuclein toxicity involves mitochondrial and autophagy dysfunction. Overexpression of alpha-synuclein causes disturbed intracellular trafficking, harming mitochondrial and autophagy dynamics. Induced pluripotent stem cells (hiPSC) derived from dopaminergic neurons allow investigations into the phenotypes and cellular mechanisms that lead to neurodegeneration. Further, this cell model maintains the genetic background of the patient the cells were derived from. In this study, using dopaminergic neurons derived from hiPSCs, we reported that overexpression of alpha-synuclein decreased mitochondrial trafficking, and accumulated lysosome vesicles. In a sensitivity assay, SNCA^3 neurons were demonstrated to be more susceptible to rotenone toxicity, which alters intracellular mitochondrial distribution, impairing retrograde transport of the organelle.

Introduction

Alpha-synuclein is a small pre-synaptic protein and a major constituent of Lewy bodies, which are insoluble clusters characteristic of Parkinson's disease (PD) (Scharre et al., 2016). The etiology of PD is unknown in about 75% of cases; however, evidence shows that PD is a consequence of both environmental factors and genetic susceptibility (Biernacka et al., 2016; Marques et al., 2011). Mutations in the alpha-synuclein gene (SNCA) have been suggested as the

main risk for PD (Nalls et al., 2014). Triplicated copies of the gene ($SNCA^3$) cause overexpression of protein and are known to be associated with autosomal dominant PD (Olgiati et al., 2015). It has been reported that overexpression of alpha-synuclein favors oligomer formation, which damages mitochondria and increases oxidative stress (Byers et al., 2011). Indeed, oligomers may be more cytotoxic than protein aggregates and interact with membranes of vesicles and organelles, such as mitochondria, leading to increased reactive oxygen species (ROS) (Chemerovski-Glikman et al., 2016; Kalia et al., 2013; Cremades et al., 2012). However, the mechanisms involved in mitochondrial dysfunction caused by overexpressed alpha-synuclein are not well understood. Maintenance of mitochondrial function is dependent on equilibrated mitochondrial trafficking (Sheng, 2014). In our previous studies, we revealed that overexpressed alpha-synuclein impaired mitochondrial trafficking and changed motor protein expression (Melo et al., 2013; Chaves et al., 2013). Furthermore, high levels of alpha-synuclein promote hyperphosphorylation of tau protein, causing microtubule disassembly and disturb in intracellular trafficking (Roy and Jackson, 2014).

Alterations in intracellular trafficking also affect autophagy dynamics. Arduíno and colleagues (2013) demonstrated that mitochondrial dysfunction causes microtubule disassembly, impairing lysosome vesicle trafficking and harming autophagy dynamics. Autophagy dysfunction has been implicated in the pathology of PD. Initially, it was proposed that the proteasome degraded alpha-synuclein. However, recent studies showed that alpha-synuclein is preferentially degraded via lysosomes, especially with protein overexpression (Aflaki et al., 2016; Mak et al., 2010).

Several studies have investigated alpha-synuclein toxicity involving autophagy dysfunction. Alpha-synuclein impaired autophagy to interact with proteins related to vesicle trafficking, leading to accumulation of vesicles or autophagy disruption. Additionally, alpha-synuclein increased lysosome activity and, in the presence of aggregates, chronically led to rupture of the organelle, worsening mitochondrial dysfunction (Freeman et al., 2013; Sancenon et al., 2012). Interestingly, experiments using human induced pluripotent stem cells (hiPSC) derived from neurons of patients

with PD showed preserved mitochondrial function and prevented lysosome vesicles accumulation (Su and Qi, 2013). However, mechanisms underlying mitochondrial and autophagy dysfunction are still unclear.

hiPSC-derived neurons allow investigations of cellular mechanisms involved in degeneration in a model that preserves genetic background of patients. Flier and colleagues (2014) demonstrated altered mitochondrial metabolism in human neural precursor cells carrying alpha-synuclein gene triplications, suggesting that neurons also could have affected mitochondrial metabolism in the presence of this mutation. In this study, we generated neurons from iPSCs from patients with $SNCA^3$ to investigate mitochondrial trafficking and autophagy dysfunction in the presence of overexpressed alpha-synuclein. In addition, we tested dopaminergic neuron sensitivity and observed mitochondrial localization after exposure to rotenone, a natural pesticide that blocks complex I of the electron transport chain and triggers neurodegeneration in PD (Jalewa et al., 2016; Ferrante et al., 1997).

MATERIAL AND METHODS

Generation and characterization of human induced pluripotent stem cells (hiPSC) from fibroblasts of patients diagnosed with familial PD

Fibroblasts from epidermis of elderly normal females or patients with familial PD expressing the triplicated alpha-synuclein gene ($SNCA^3$) were purchased from the Coriell Institute (New Jersey). To generate iPSCs, fibroblasts were reprogrammed with episomal vectors containing human genes (Takashi and Yamanaka, 2006) following manufacturer instructions. Briefly, fibroblasts were plated (1×10^6 cells per 35 mm^2 well) and cultured in DMEM media supplemented with 15% of FBS (fetal bovine serum). The next day, cells reached about 75% of confluence and were transfected with L-MYC and Lin28 (Addgene #27080) at a concentration of 0.85 µg/ml, Sox-2 and KLF4 (Addgene #270778) at a concentration of 1.3 µg/ml, and OCT3/4 (Addgene #27077

and #27076) at a concentration of 0.85 µg/ml using nucleofector (Amaxa™ Basic Nucleofector™ Kit for Mammalian Fibroblasts). The electric parameter used was the U-023 program.

About 5 days after transfection, colonies were observed and passaged onto geltrex- coated dishes (35 mm^2) and cultured in E8 media (Essential 8™, Invitrogen). Media were changed every 2 days. For expansion of lines, colonies were cultured for another 30 days and passaged onto dishes coated with VTN (Vitronectin, Invitrogen). E8 media were changed every day. After about 10 passages, colonies were characterized by immunocytochemistry (fluorescence) for pluripotency markers and for 3 germ lineages of embryoid bodies.

To generate embryoid bodies (EBs), cells were plated in non-adherent dishes without coating for 1 week. Colonies were maintained in E8 media for 2 days. E8 was replaced with a medium for spontaneous differentiation composed of: 75% DMEM, 20% FBS hyclone, 1% non-essential amino acids, 1% glutamax, 1% HEPES, and 0.1% β-mercaptoethanol. After 2 days, EBs formed were cultivated for 5 more days. EBs were plated in dishes coated with gelatin for 8 days. EBs were fixed and stained for ectoderm, endoderm and mesoderm markers.

Dopaminergic neuronal differentiation and characterization

Differentiation was based on dual SMAD-inhibition protocols described by Kriks (2011). Briefly, at day 1, E8 media was replaced with normal media: DMEM supplemented with 15% KSR (Knockout replacement medium, Invitrogen), 0.1 mM non-essential amino acids, 2 mM L-glutamine, 10 µM β-mercaptoethanol, plus 100 nM LDN193189 (Stemgent) and 10 µM SB432542 (Tocris). The next day, normal media was replaced with 100 ng/ml SHH C25II (R&D), 100 ng/ml FGF8 (R&D) and 2 µM purmorphamine. At day 4, normal media was replaced, and CHIR at 3 µM and 25% N2 were added to the media. At day 6, 50% N2 was added, and SB432542 was removed from the normal media. At day 8, 75% N2 was added to the normal media containing only LDN193189 and CHIR. At day 10, 100% N2 was added to the normal media. At day 11, cells were cultured in the neurobasal media supplemented with CHIR (until day 13), B27, L-glutamine, BDNF

at 20 ng/ml, GDNF at 20 ng/ml, ascorbic acid at 0.2 mM, TGFb3 at 1 ng/ml, cAMP at 0.5 mM, and DAPT at 10 μM. At day 20, neurons were dissociated using Accutase (Millipore) and plated at 3 × 10^5 cells/cm^2 in pretreated dishes with geltrex. Neurons were cultured for another 40 or 70 days until they achieved maturity.

At 60 and 90 days of differentiation, neurons were stained for the following markers: neuronal marker βIII-tubulin (Tuj1, anti-rabbit), dopaminergic marker TH (tyrosine hydroxylase, anti-mouse) and alpha-synuclein.

Mobility of viable mitochondria

Neurons were plated on coverslips coated with geltrex, and mitochondria were labeled in orange using a fluorescent probe MitoTracker® Orange CMTMRos (Invitrogen), which only binds to mitochondria with intact membrane potentials. The probe was dissolved in culture medium at 75 nM for 30 min at 37 °C. This procedure does not affect the mitochondrial membrane potential (Pendergraas et al., 2004). MitoTracker was dissolved in DMSO at 1 mM following the manufacturer's instructions. This solution was stored at -20 ºC. After staining, neurons were rinsed three times with dPBS in the dark, and mitochondrial trafficking and the total number of mitochondria per frame was immediately evaluated using confocal microscopy with a 554–576 nm wavelength and 63× objective, as previously described.

Lysosome vesicles

Acidic vesicles were labeled in red with a fluorescent probe, LisoTracker™ Red DND-99 (Invitrogen) diluted in the media at 75 nM. Neurons were incubated with the probe for 10 min at 37 °C, rinsed three times, and immediately visualized using confocal microscopy and a 63× objective.

Treatment with rotenone

Rotenone was diluted in DMSO (stock solution 1 mM) and added in the culture media at 50 nM or 100 nM. Neurons were exposed to rotenone or DMSO (as the control) at 0.01% for 48 h followed by fixation with 4% PFA and staining for dopaminergic neurons, alpha-synuclein, and

mitochondria. The morphology of dopaminergic neurons and mitochondria and localization of mitochondria were analyzed using confocal microscopy with a 63× objective.

Immunocytochemistry

Cells were rinsed with PBS and fixed with 4% PFA for 20 min at room temperature. Permeabilization and nonspecific labeling was blocked with PBS containing 0.2% Triton and 4% bovine serum albumin (BSA, Sigma) for 30 min at room temperature.

hiPSCs were stained for the following pluripotent markers: rabbit anti-OCT-4 (1/300, Abcam, ab137427), mouse anti-SOX-2 (1/300, Abcam, ab171380), mouse anti-Tra1-60 (1/500, Abcam, ab16288), and mouse anti-Tra1-81 (1/500, Abcam, ab16289).

Embryoid bodies were prepared for immunocytochemistry as cited above. The primary antibodies employed for identification of embryonic three germ layer were ectoderm marker (neuronal marker) rabbit anti-βIII-tubulin (Tuj1, 1/500, Abcam, ab18207), endoderm marker rabbit anti-GATA-4 (1/200, Abcam, ab61767), and mesoderm marker rabbit anti Desmin (1/400, Abcam, ab8592).

Neurons were rinsed carefully with warm dPBS and prepared for immunocytochemistry as cited above. The primary antibodies employed were neuronal marker rabbit anti βIII-tubulin (Tuj1, 1/1000), dopaminergic marker mouse anti-TH (1/500, Millipore, MAB318), rabbit anti-alpha-synuclein (1/500, Abcam, ab138501), and mitochondrial marker rabbit anti-TOM20 (1/1000, sc-11415).

Primary antibodies were incubated overnight at 4 °C, whereas secondary antibodies were incubated with fluorescent anti-mouse (1/120, Jackson) or anti-rabbit (1/120, Jackson) secondary antibodies for 1 h at room temperature protected from light.

To stain nuclei, Hoechst was incubated at 1/1000 for 10 min protected from light.

Statistical Analysis

Mitochondrial mobility was analyzed using Student's *t* test. Experiments were repeated three times (n=3). A p value ≤ 0.05 was considered statistically significant. Data are expressed as absolute values or percentage of control ± SD (standard deviation). The statistical software employed was GraphPad Prism (GraphPad Software Inc., version 4.00, CA).

Results

Characterization of hiPSCs

Fibroblasts from controls and patients with the autosomal dominant form of PD expressing 3 copies of the alpha-synuclein gene ($SNCA^3$) were successfully reprogrammed into iPSCs. Cells from controls (Figure 1A) and from patients (Figure 1B) showed stem cell morphology and stained positively for the pluripotent markers OCT-4, SOX-2, Tra1-60, and Tra1-81 as shown in Figure 1.

Next, embryoid bodies were generated and three germ layers were detected by staining ectoderm, endoderm, and mesoderm in control hiPSCs (Figure 2A) and $SNCA^3$ hiPSCs (Figure 2B).

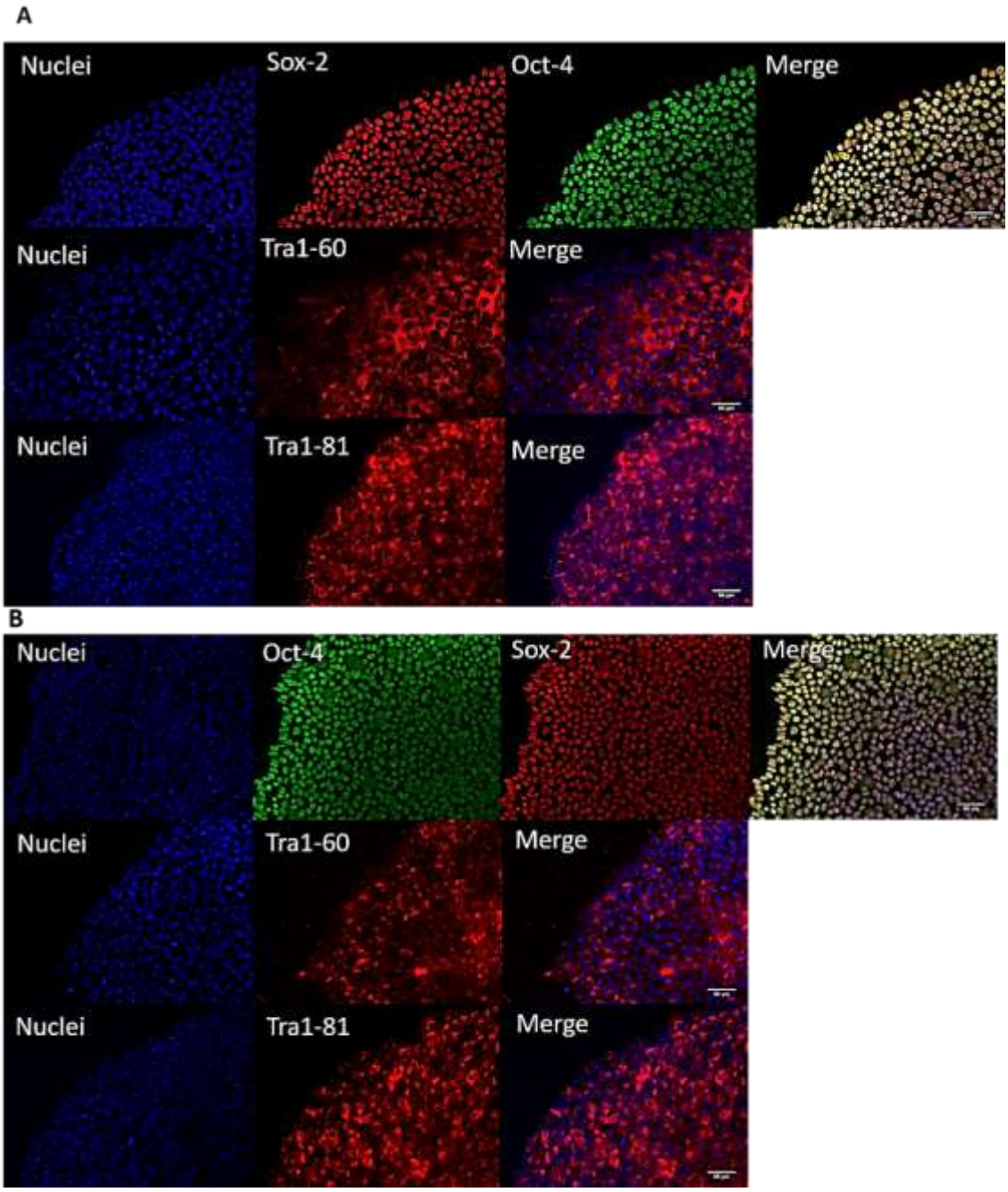

Figure 1: Characterization of hiPSCs by immunofluorescence. Photomicrographs illustrating immunoreactivity of pluripotent markers SOX-2, OCT-4, Tra1-60, and Tra1-81 (overlapping stains in merge) in control (A) and patient cells carrying $SNCA^3$ (B). Hoechst stained nuclei in blue. Confocal images used a 63× objective (scale of 50µm).

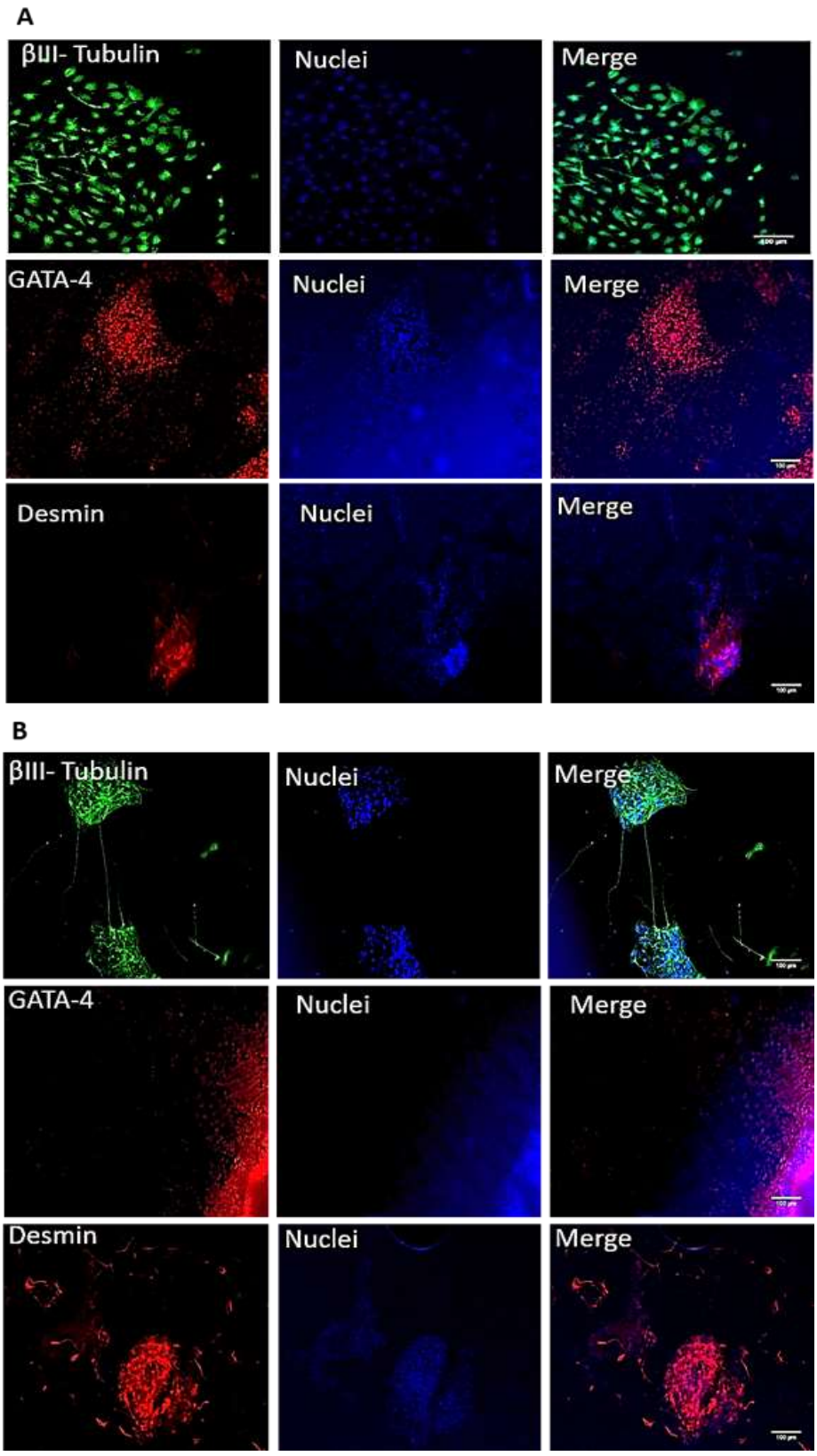

Figure 2: Characterization of embryoid bodies by immunofluorescence. Photomicrographs illustrating immunoreactivity of the germ layers ectoderm (BIII-tubulin), endoderm (GATA-4), and mesoderm (Desmin) (overlapping stains in merge) in control (A) and patient cells carrying $SNCA^3$ (B). Hoechst stained nuclei in blue. Confocal images were captured using 63× objective (scale of 50µm).

Taken together, these results showed both fibroblasts from control and $SNCA^3$ patients were correctly reprogrammed into hiPSCs with the same efficiency, showing stem cell morphology and primary pluripotent markers. In addition, control and $SNCA^3$ hiPSCs forms EBs and were positive for staining of the 3 germ layers, confirming pluripotency of the hiPSCs generated.

Subsequently, hiPSCs were differentiated into dopaminergic neurons. After 60 and 90 days of differentiation, cells were positive for a neuronal marker (Tuj1) and dopaminergic marker (TH) (Figure 3A and B, respectively). Kriks' protocol showed a differentiation efficiency of 80% for dopaminergic neurons; nevertheless, we observed about 10%-20% dopaminergic neurons after 60 or 90 days of differentiation.

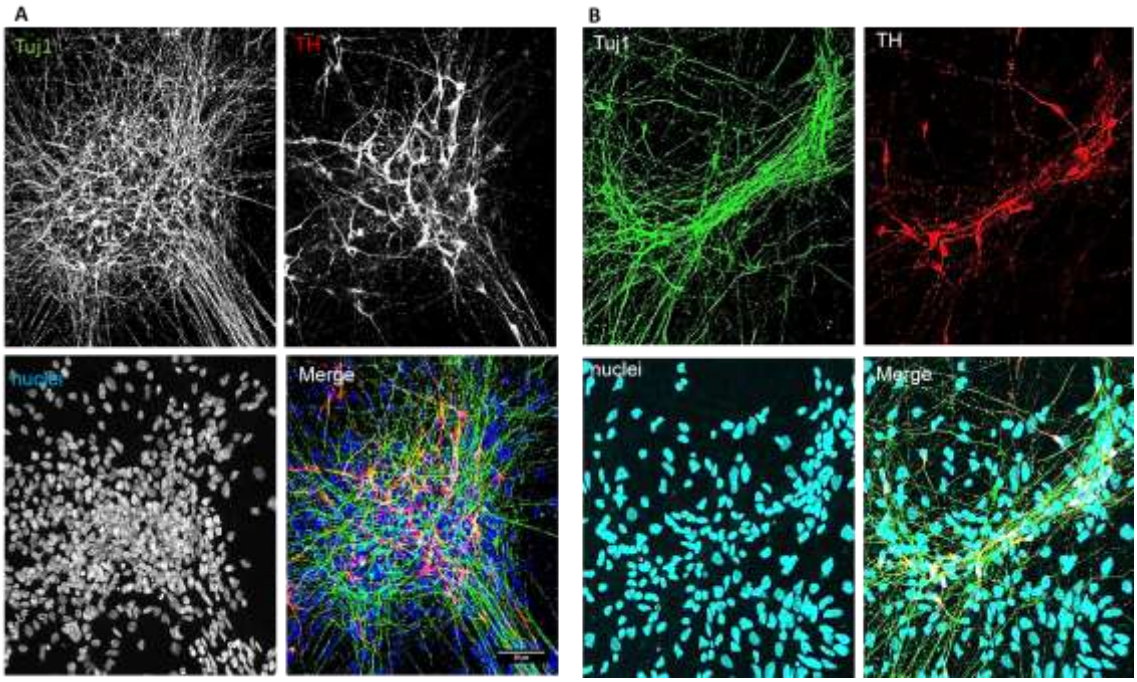

Figure 3: Characterization of dopaminergic neurons derived from hiPSCs by immunofluorescence after 60 days (A) and 90 days (B) of differentiation. Photomicrographs illustrating immunoreactivity of neurons in green (Tuj1) and dopaminergic neurons in red (TH) (overlapping stains in merge). Hoechst stained nuclei in blue. Confocal images were captured using a 63× objective.

Overexpressed alpha-synuclein is a phenotype of $SNCA^3$ neurons. Therefore, neurons were stained for alpha-synuclein after 60 days and 90 days of differentiation. After 60 days of differentiation, $SNCA^3$ neurons did not show alpha-synuclein staining. However, after 90 days of differentiation, homogeneous alpha-synuclein staining was observed, suggesting the protein was not aggregated (Figure 4). These data suggest that 90 days of differentiation was necessary to observe a PD phenotype.

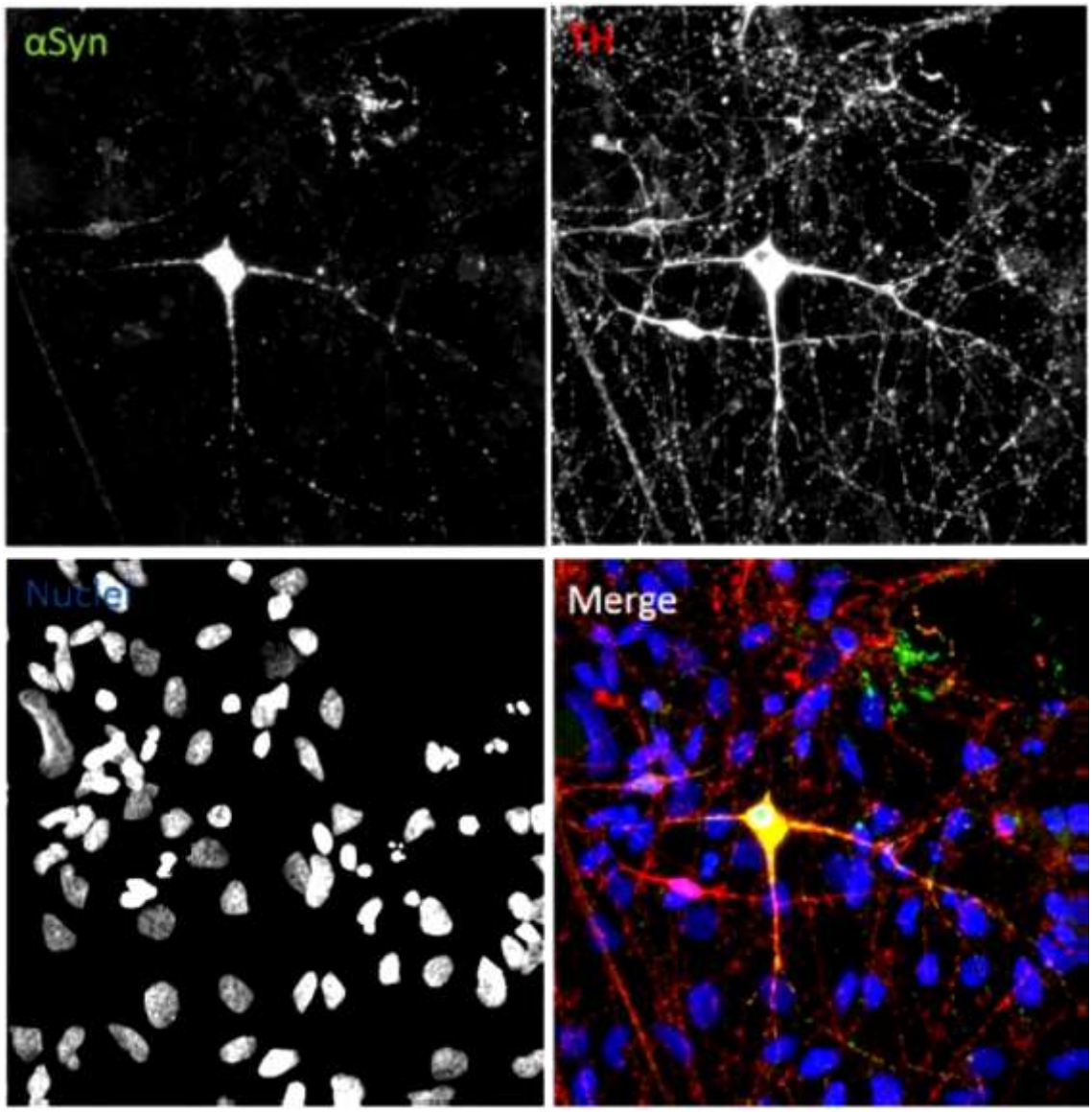

Figure 4: Immunofluorescence of alpha-synuclein and dopaminergic neurons after 90 days of differentiation of hiPSC into dopaminergic neurons. Photomicrographs illustrating alpha-synuclein expression in green (α-syn) and dopaminergic neurons in red (TH) (overlapping stains in merge). Hoechst stained nuclei in blue. Confocal images were captured using a 63× objective.

Several studies have demonstrated that mitochondrial dysfunction, such as differences in mitochondrial morphology and decreased membrane potential, occur in the presence of overexpressed alpha-synuclein, including in models using neurons derived from hiPSCs (Su and Qi, 2013; Yakhine-Diop et al., 2014; Sanchez et al., 2012). The mechanisms that lead to mitochondrial dysfunction are still unclear, but it has been suggested that alpha-synuclein disrupts mitochondrial

trafficking, causing mitochondrial dysfunction in PD (Ottolini et al., 2016). In order to investigate mitochondrial alterations in our model, we quantified mitochondrial trafficking and compared mitochondrial morphology and membrane potential using MitoTracker Orange (Figure 5).

After 60 days of differentiation, we observed no changes in mitochondrial morphology in control cells compared with $SNCA^3$ neurons (Figure 5A and B, respectively). Mitochondrial trafficking and the amount of mitochondrias with intact mitochondrial membrane potential also did not differ from that in $SNCA^3$ neurons (Figure 5C and D, respectively). After 90 days of differentiation, we did not observe differences in mitochondrial morphology between control and $SNCA^3$ neurons (Figure 5E and F). However, mitochondrial trafficking and the amount of mitochondrias with intact mitochondrial membrane potential were lower in $SNCA^3$ neurons than in control neurons (Figure 5G and H, respectively). Taken together, these results showed disrupted mitochondrial trafficking in the presence of alpha-synuclein overexpression.

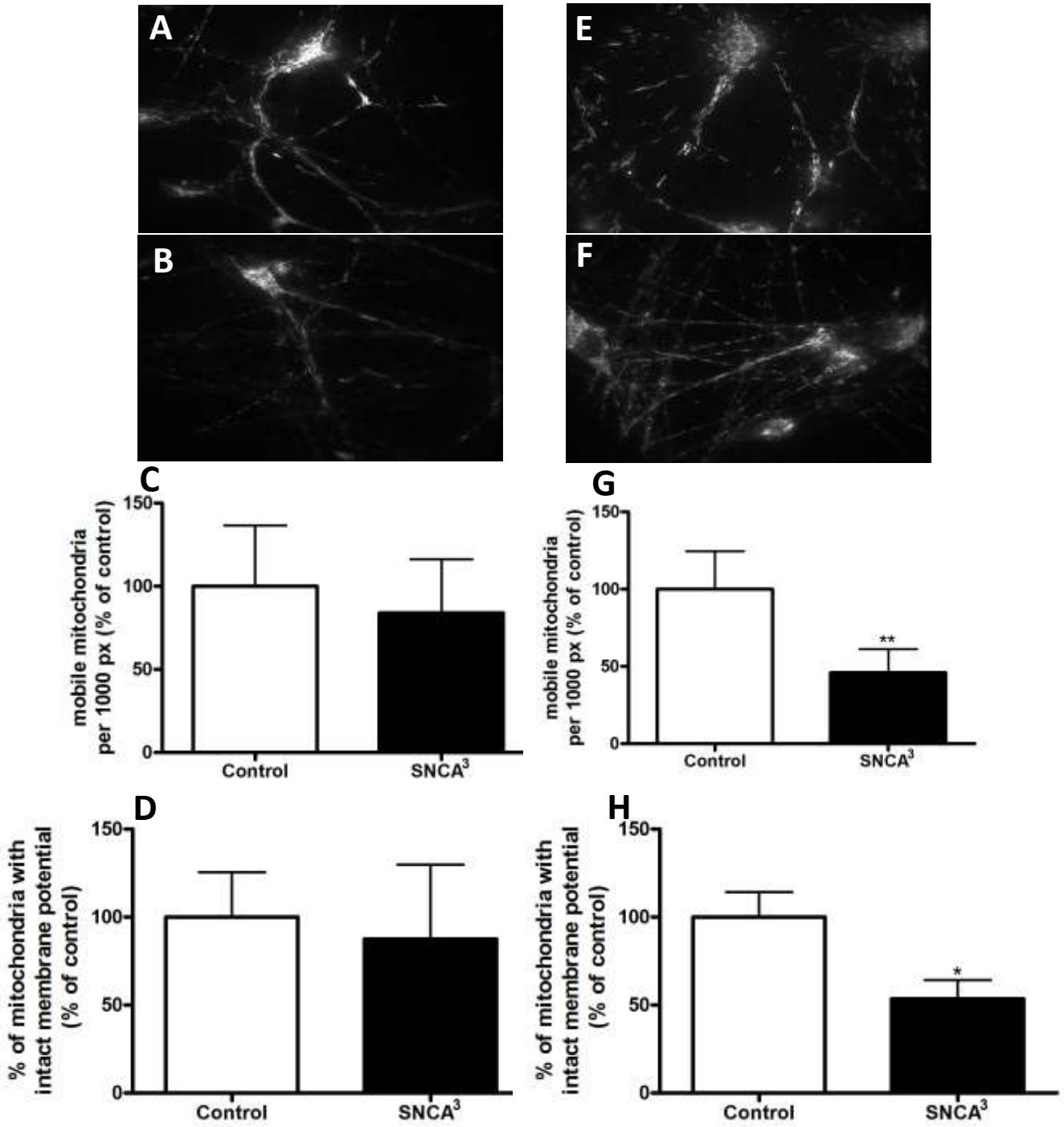

Figure 5: Mitochondrial trafficking and mitochondrial membrane potential in neurons incubated with MitoTracker Orange. Photomicrographs show mitochondria labeled after 60 days of differentiation in control neurons (A) and $SNCA^3$ neurons (B) or after 90 days of differentiation in control neurons (E) and $SNCA^3$ neurons (F). Mitochondria trafficking did not change after 60 days of differentiation (C). After 90 days of differentiation, mitochondrial trafficking was lower in neurons expressing $SNCA^3$ than in controls (G). The percentage of moving mitochondria with intact membrane potentials did not change after 60 days of differentiation (D). After 90 days of differentiation, neurons expressing $SNCA^3$ showed a decreased percentage of moving mitochondria with intact membrane potentials (H). Confocal images used a 63× objective. Student's *t* test was employed. n=6 cultures,*$p \leq 0.05$ compared with control. Experiments were repeated 3 times.

It has been reported that autophagy dysfunction in PD is caused by overexpression of alpha-synuclein, which can inhibit autophagy mechanisms as well as disrupt vesicles trafficking and the endocytic pathway. Further, aggregates of alpha-synuclein can directly impair lysosome function, causing lysosome rupture followed by mitochondrial dysfunction (Winslow and Rubinsztein, 2011; Sancenon et al., 2012; Freeman et al., 2013; Lin et al., 2015). Therefore, to investigate whether autophagy was impaired, we stained acidic vesicles using LysoTracker in neurons (Figure 6). After 90 days of differentiation, $SNCA^3$ neurons (Figure 6B) showed a significantly higher number of vesicles (Figure 6C) than was observed in control neurons (Figure 6A). These data indicated that overexpression of alpha-synuclein led to accumulation of lysosome, which strongly suggests that autophagy was impaired in these neurons.

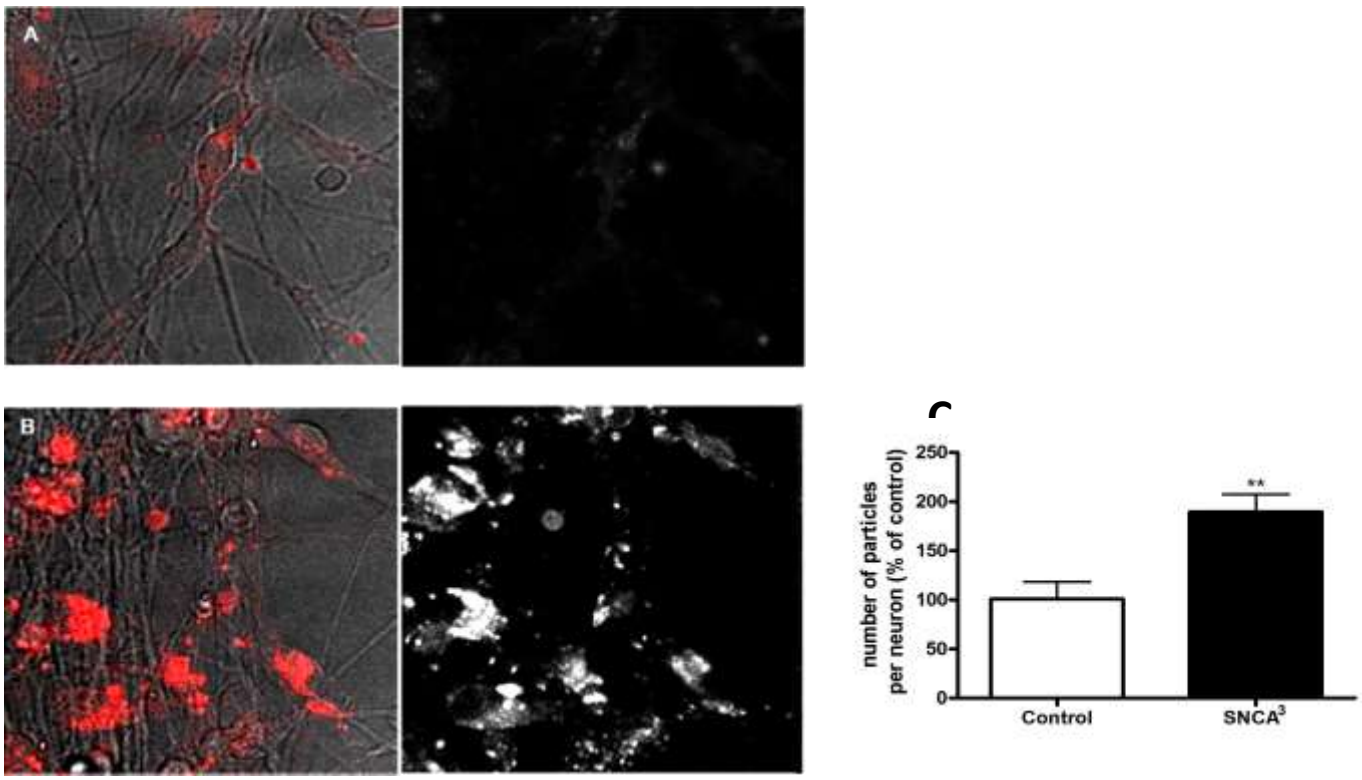

Figure 6: Lysosomes stained using LysoTracker in control neurons (A) and neurons expressing $SNCA^3$ (B) after 90 days of differentiation. Lysosome accumulation was greater in neurons expressing $SNCA^3$ than in the controls (B). Quantification of the number of particles stained red showed significantly more stained particles in neurons expressing $SNCA^3$ (C). Confocal images were captured using a 63× objective. Data expressed as percentage of control ± SD. Student's *t* test was employed. n=6 cultures,*$p \leq 0.05$ compared with control. Experiments were repeated 3 times.

The main risk factor for PD is age. It seems that the neurodegenerative process is a combination of age-related changes and accumulated alpha-synuclein. Protein aggregate formation, as well as injuries caused by alpha-synuclein toxicity, seems to be exacerbated by natural changes in aged neurons, such as oxidative stress caused by increased ROS. hiPSC-derived neurons are not subject to all changes involved in aging. Specifically, increased ROS is characteristic of both aged neurons and neurons from patients with PD (Scharre et al., 2016; Ross et al., 2015). Therefore, an addition stressor is required to mimic the alterations that occur during normal aging, including dopaminergic sensitivity.

To verify the sensitivity of neurons to ROS in the presence of alpha-synuclein, control neurons and $SNCA^3$ neurons were treated with low doses of rotenone at 50 nM or 100 nM for 48 h.

First, we analyzed control neurons morphology and expression of alpha-synuclein (Figure 7). The morphology of dopaminergic control neurons morphology after treatment with 50 nM of rotenone (Figure 7B) or 100 nM of rotenone (Figure 7C) did not differ from that of the control neurons exposed to DMSO (Figure 7A). However, control neurons exposed to 50 nM of rotenone showed more intense alpha-synuclein staining (Figure 7B) than control neurons treated with DMSO. Further, neurons treated with 100 nM rotenone showed higher alpha-synuclein labeling (Figure 7C) than that observed in neurons treated with 50 nM rotenone. On the other hand, compared to cells treated with DMSO, $SNCA^3$ neurons treated with 50 nM rotenone showed decreased number and length of neurites in more than 50% of neurons and decreased nucleus size, indicating cell death (Figure 7E). More than 50% of $SNCA^3$ neurons exposed to 100 nM rotenone showed severe shortening of neurites and also smaller nuclei, suggesting cell death (Figure 7F). Intensity of alpha-synuclein staining did not change in $SNCA^3$ neurons among the treatments. These findings strongly suggest that rotenone increased alpha-synuclein expression in control neurons and activated apoptosis in $SNCA^3$ neurons, demonstrating that these neurons were more sensitive to increased ROS caused by rotenone than control neurons.

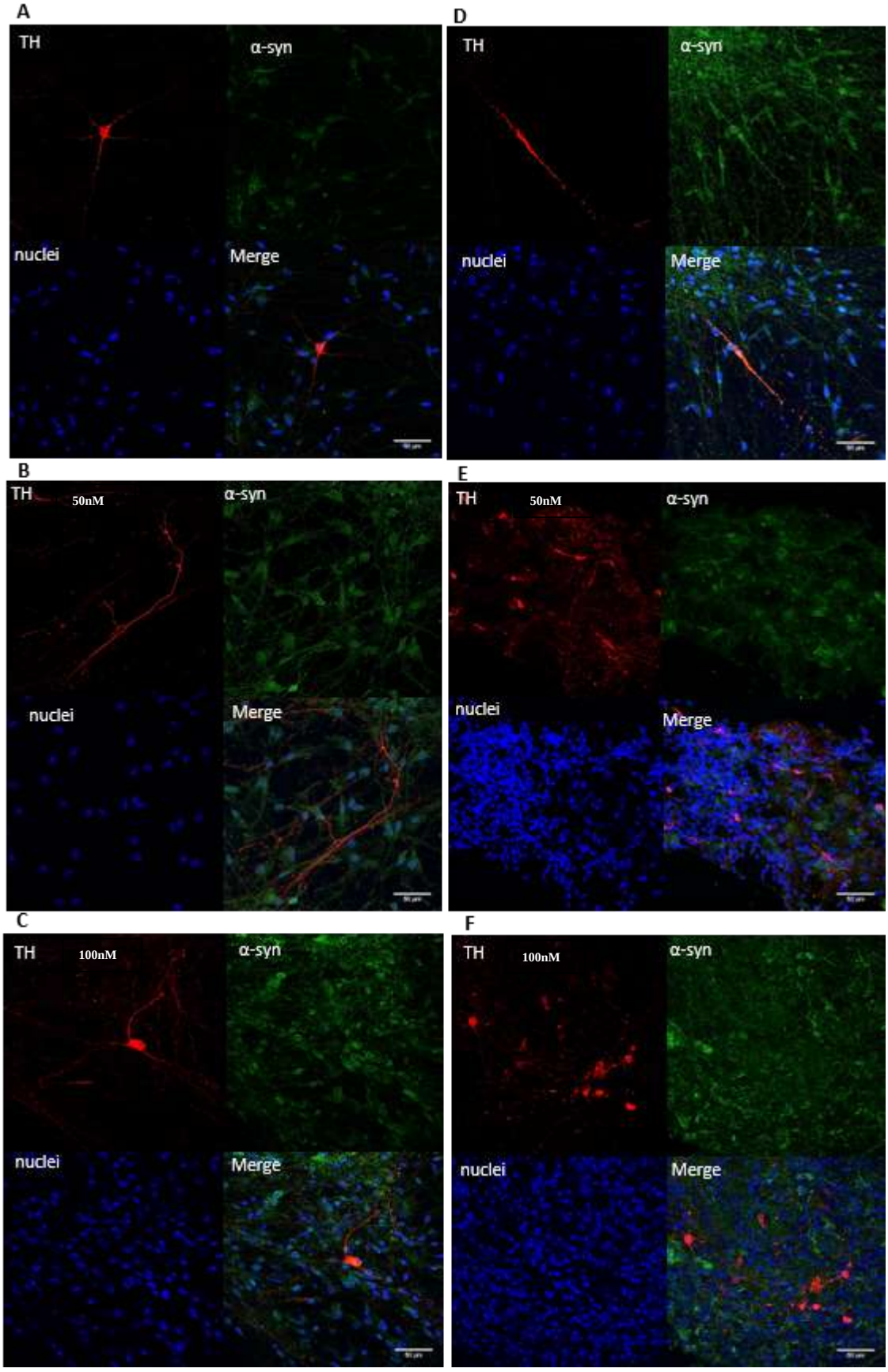

Figure 7: Photomicrographs showing immunoreactivity of dopaminergic neurons in red (TH) and alpha-synuclein in green (α-syn), (overlapping stains in merge) at 90 days of differentiation after treatment with 50 nM or 100 nM rotenone for 48 h. Hoechst stained nuclei in blue. Control and SNCA^{3} neurons treated with DMSO for 48 h (A and D, respectively). Control and SNCA^{3} neurons

treated with rotenone at 50 nM (B and E, respectively). Control and $SNCA^3$ neurons treated with 100 nM rotenone (C and F). Confocal images were captured using a 63× objective (scale of 50µm).

Rotenone targets mitochondria, blocking complex I of the respiratory chain and causing mitochondrial dysfunction. Furthermore, in our previous studies, we showed that rotenone disturbs motor proteins and mitochondrial trafficking (Chaves et al., 2010; Melo et al., 2013; Chaves et al., 2013).

In order to investigate mitochondrial localization, we stained mitochondria (TOM20) in dopaminergic neurons and analyzed mitochondrial localization in neurons with preserved neurites after rotenone exposure (Figure 8). We found that control neurons exposed to 50 nM (Figure 8B) or 100 nM (Figure 8C) rotenone had concentrated mitochondria in cell body and homogeneous mitochondrial distribution in neurites similar to DMSO control neurons (Figure 8A). In contrast, compared to $SNCA^3$ neurons exposed to DMSO (Figure 8D), $SNCA^3$ neurons exposed to 100 nM rotenone did not show mitochondria concentrated in cell body (Figure 8F), suggesting that retrograde trafficking was impaired in these neurons. All dopaminergic neurons exposed to 50 nM rotenone showed severe shortening of neurites, making it impossible to analyze mitochondrial distribution in these branches (Figure 8E).

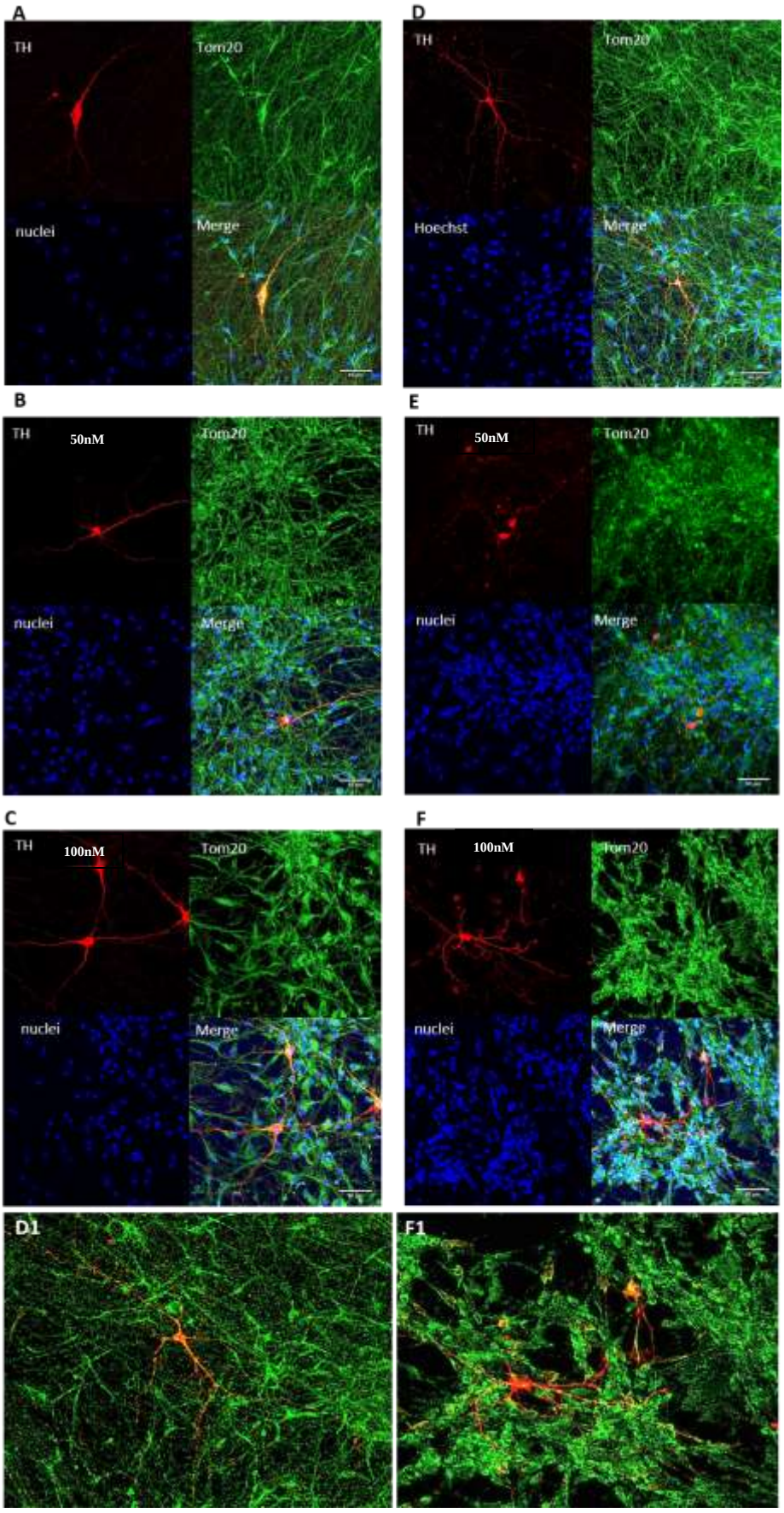

Figure 8: Photomicrographs showing immunoreactivity of dopaminergic neurons in red (TH) and mitochondria in green (TOM20), (overlapping stains in merge) at 90 days of differentiation after treatment with rotenone at 50 nM or 100 nM for 48 h. Hoechst stained nuclei in blue. Control and SNCA^3 neurons treated with DMSO for 48 h (A and D, respectively). Control and SNCA^3 neurons

treated with rotenone at 50 nM (B and E, respectively). Control and $SNCA^3$ neurons treated with 100 nM of rotenone (C and F) Amplified images of $SNCA^3$ neurons treated with DMSO (D1) and with rotenone (F1). Arrow indicates absence of mitochondria in the cell body (F1). Confocal images were captured using a 63× objective (scale of 50μm).

Discussion

In our study, we successfully generated hiPSCs from fibroblasts derived from a control subject and PD patient with triplicated copies of the alpha-synuclein gene. Both lines (control and patient) showed stem cell morphology, expressed pluripotent markers, and spontaneously differentiated into 3 germ layers. These findings indicated that the presence of the $SNCA^3$ gene did not interfere with fibroblast reprogramming or cell pluripotency. However, further differentiation generated only 10–20% dopaminergic neurons. Kriks protocol (2011) has been widely used to generate dopaminergic neurons. Nevertheless, some recent studies have shown low efficiency in the generation of dopaminergic neurons using this protocol. It has been reported that adaptions in the protocol are needed, as well as purification in order to get a culture containing 80% dopaminergic neurons (Nishimura et al., 2015; Woodard et al., 2014; Grealish et al., 2014; Schöndorf et al., 2014; Miller et al., 2013).

To characterize the PD phenotype, we cultured the neurons for 90 days. $SNCA^3$ neurons showed stronger homogeneous staining than control neurons, suggesting alpha-synuclein was overexpressed, although the protein was not aggregated. It has been reported that alpha-synuclein impairs intracellular trafficking of vesicles and organelles, such as mitochondria, in several models (Mazzulli et al., 2016; Ottolini et al., 2016). Moreover, overexpressed alpha-synuclein has been reported to cause mitochondrial and autophagy dysfunction during PD development (Bose and Beal, 2016; Ottoline et al., 2016). Mechanisms behind alpha-synuclein toxicity involving altered mitochondrial morphology, membrane potential, and abnormal autophagy are still unclear. It has been reported that overexpressed alpha-synuclein favors oligomer formation, which seems to be

more toxic than aggregates. Oligomer toxicity relies on a high affinity of oligomers with membranes, including mitochondrial membranes, causing destabilization of the mitochondrial membrane potential and consequently mitochondrial dysfunction (Marmolino et al., 2016; Chen et al., 2015; Luth et al., 2014). We did not observe any changes in mitochondrial dynamics after 60 days of differentiation, confirming that the $SNCA^3$ neuronal phenotype is observed only after this period of time. Further, mitochondrial morphology did not change in either control or $SNCA^3$ neurons. However, after 90 days of differentiation, we observed decreased mitochondrial trafficking and membrane potentials in $SNCA^3$ neurons concomitant with overexpression of alpha-synuclein. These results strongly suggest that at 90 days of differentiation, overexpressed alpha-synuclein formed oligomers that damaged mitochondria. This is the first study showing mitochondrial trafficking impairment and alterations in the mitochondrial membrane potential as the first steps that lead to mitochondrial dysfunction in human $SNCA^3$ neurons. In addition, our results strongly suggest that these changes precede formation of protein aggregates and changes in mitochondrial morphology.

Alpha-synuclein can also cause autophagy dysfunction. Additionally, mitochondrial dysfunction also can impair microtubule trafficking, leading to inefficient autophagy (Arduino et al., 2013). In our study, we observed accumulated lysosome vesicles in $SNCA^3$ neurons, indicating that overexpressed alpha-synuclein was impairing autophagy in these neurons. Once alpha-synuclein is overexpressed, lysosomes increase their activity possibly to degrade excessive alpha-synuclein. However, in the long term, lysosomes are not efficient in degrading alpha-synuclein, and vesicles containing protein to be degraded start accumulating and disturbing autophagy dynamics. Moreover, alpha-synuclein overexpression can directly inhibit autophagy by impairing proteins related to vesicle trafficking, leading to accumulation of vesicles, favoring protein aggregation, and also damaging mitochondria (Mazzulli et al., 2016; Sancenon et al., 2012; Winslow and Rubinsztein, 2011).

We did not observe indications of protein aggregates in $SNCA^3$ neurons. However, alpha-synuclein protein aggregation and its toxicity seems to be a consequence of natural aging changes in cells, such as increased ROS, and a predisposition toward developing PD (Scharre et al., 2016). Interestingly, other studies also did not show alpha-synuclein aggregation naturally, and sensitivity of dopaminergic neurons expressing alpha-synuclein to oxidative stress, the main hallmark of PD, has been investigated through exposure to neurotoxins such as rotenone (Peng et al., 2013; Ryan et al., 2013). Though, it has been demonstrated that, human dopaminergic neurons derived from $SNCA^3$ patients expressed double amount of alpha-synuclein. In addition, these neurons were susceptible to oxidative stress (Byers et al., 2011).

Our previous results showed rotenone increase alpha-synuclein expression in primary culture of substantia nigra (Chaves et al, 2010). Analysis of rotenone exposure revealed that rotenone could increase alpha-synuclein expression in control neurons without affecting dopaminergic neuron morphology. In contrast, $SNCA^3$ neurons were more sensitive to rotenone, showing loss of neurites and cell death. In addition, retrograde mitochondrial trafficking was impaired, suggesting that mitochondrial biogenesis that preferentially occurs in the cell body was impaired. Since mitochondria are crucial organelles in controlling ROS levels and apoptosis, biogenesis impairment can lead to increased oxidative stress and consequently cell death. However, new experiments are needed to clarify the mechanisms behind mitochondrial and autophagy dysfunction in our model using hiPSC-derived dopaminergic neurons.

Conclusion

Overexpressed alpha-synuclein disrupts mitochondrial trafficking and impairs autophagy. Further, overexpressed protein makes neurons more sensitive to neurotoxins such as rotenone.

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4. Discussion

4. Discussion

In this study we have investigated mechanisms related to mitochondria dysfunction caused by overexpression of different types of alpha-synuclein. We have also investigated autophagy and endoplasmic reticulum stress, which have been shown to be a consequence and/or worsened mitochondria dysfunction (Krols et al., 2016; Arduino et al., 2013; Su and Qi, 2013, Grimm, 2012).

Recent reports have shown alpha-synuclein also leads to aberrant autophagy and increased ROS worsening mitochondria dysfunction (Redmann et al., 2016; Giordano et al., 2013). Specially mutated alpha-synuclein, or overexpression of WT protein overloads lysosome and endoplasmic reticulum. Consequently, degradation in general is impaired in the cell, including degradation of alpha-synuclein and mitochondria, favoring protein aggregates formation and mitochondria dysfunction (Sarkar et al., 2016; Freeman et al., 2013; Winslow and Rubinsztein, 2011). Moreover, overloaded endoplasmic reticulum causes organelle stress, increased oxidative stress also worsening mitochondria dysfunction (Sarkar et al., 2016; se Oliveira et al., 2015; Grimm, 2012). Together, these events create a vicious cycle leading to cell death. However, recent studies focused on unveil mechanisms involved in all these processes, demonstrated alpha-synuclein firstly targets mitochondria, causing mitochondria dysfunction, that lead to stress of organelles such as lysosome and endoplasmic reticulum (Krols et al., 2016; Chen et al., 2015; Arduino et al., 2013; Grimm, 2012). Therefore, the main focus to understand alpha-synuclein toxicity has been to understand mitochondria dysfunction in PD.

It is known mitochondria function is dependent of mitochondria dynamics, which consists basically in equilibrated anterograde and retrograde trafficking and anchoring mitochondria in sites with high demand of energy (Cieri et al., 2016; Sheng, 2014). Anterograde transport is crucial to maintenance of synapses, whereas, retrograde transport is crucial to renew mitochondria, since mitochondria biogenesis occurs preferentially at soma (Sheng et al., 2014). In our previous studies

we have observed alterations in motor proteins expression and mitochondria trafficking in the presence of overexpression of alpha-synuclein, before mitochondria membrane potential alterations (Melo et al., 2013; Chaves et al., 2013). Further, other studies also showed that impaired mitochondria trafficking is involved in earlier PD pathogenesis (Bose and Beal, 2016). Based on this study, we analyzed impact of overexpression of different types of alpha-synuclein in mitochondria trafficking and proteins related to trafficking of mitochondria and lysosome vesicles.

For this purpose, we have used 3 different cellular models expressing human alpha-synuclein: human SH-SY5Y cells (neuroblastoma) derived neurons, *S. cerevisiae* and hiPSC-derived dopaminergic neurons from $SNCA^3$ patients. On the contrary of our first studies, SH-SY5Y neurons allowed investigations of mitochondria trafficking direction, providing the link among impairment on direction of trafficking and changes observed in mitochondria and autophagy flux. In addition, we have used NAP at a concentration that restores mitochondria trafficking and observed recovering of mitochondria and autophagy dysfunction, revealing that disturbed trafficking can be the key to cascade of cellular alterations in PD. hiPSC dopaminergic neurons overexpressing alpha-synuclein also showed disrupted trafficking, indicating changed trafficking play a role in mitochondria dysfunction in human neurons derived from $SNCA^3$ patients.

Our previous studies already showed disturbed motor and adaptors proteins expression such as KIF1B, KIF5A, Miro, dynein and dynactin accompanied by disturbed mitochondria trafficking (Melo et al., 2013; Chaves et al., 2013). In addition, several studies have shown that especially Miro plays an important role in all processes involved in mitochondria dynamics (Devine et al., 2016). Moreover, it has been demonstrated that alpha-synuclein can interact with and/or change dynamics of proteins related to trafficking and autophagy such as Rab5 and LC3 (Koch et al., 2015; Sancenon et a., 2012; Dalfó et al., 2004). However, the consequences of these interactions or changes between alpha-synuclein and proteins related to trafficking, mitochondria and autophagy function are poorly understood.

Absence of proteins/genes is used to clarify their role in cellular events that contribute to neurodegeneration. However, viability of polarized cells like neurons, are totally dependent of intracellular trafficking. Therefore, we have used yeast model to investigate deeper alpha-synuclein toxicity related to disturbed mitochondria and lysosome vesicles trafficking. We have found that A30P alpha-synuclein toxicity differs of A53T alpha-synuclein, which formed aggregates. As expected, cells expressing A53T alpha-synuclein showed earlier changes in mitochondria, autophagy, endoplasmic reticulum and apoptosis signalization which could be prevented by absence of GEM (Miro in mammalian), YPT53 (Rab5 in mammalian) and Atg8 (LC3 in mammalian). These data indicate, understanding of mechanisms which lead to alpha-synuclein aggregation and toxicity involve specific signalization of genes related to intracellular trafficking.

To underline intracellular trafficking disruption or target genes involved in alpha-synuclein dysfunction, we investigated toxicity of overexpression of WT alpha-synuclein, or expression of A30P or A53T alpha-synuclein and also exposure to rotenone. Although most cases of PD are sporadic, only about 10% of cases have defined genetic causes (Kalinderi et al., 2016). However, despite mutations in alpha-synuclein gene and also neurotoxins that damage mitochondria could cause specific cell injuries, they are widely used in cellular models to study PD pathology in order to optimize time and conditions to observe PD phenotype.

In this study, we observed several changes characteristics of PD most in neurons expressing A53T alpha-synuclein. Since it is known WT, A30P and A53T alpha-synuclein lead to PD and they oligomerizes faster than others, likely with longer SH-SY5Y culture or addition of neurotoxin, we would observe mitochondria trafficking changed and other changes that were observed in cells expressing A53T alpha-synuclein. Other studies also performed chronical exposure to neurotoxins to observed PD phenotype (Koch et al., 2015; Melo et al., 2013). The same principle is applied for hiPSC neurons. It has been demonstrated that hiPSC neurons expressing A53T alpha-synuclein showed PD phenotype related to mitochondria dysfunction after 35 days of culture, whereas we

needed to wait 90 days to observe PD phenotype (Ryan et al., 2013). New investigations are needed to find out how to accelerate PD phenotype in these neurons and optimize hiPSC work in our study.

Together, analysis in our models revealed that mitochondria dysfunction caused by alpha-synuclein toxicity involves disruption in intracellular trafficking, preferentially first in mitochondria retrograde trafficking. Besides that, alpha-synuclein toxicity and apoptosis are dependent of proteins related to intracellular trafficking of mitochondria and autophagy, demonstrating understanding of impairment of intracellular trafficking could be a key to unveil PD cellular pathogenesis.

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5. Conclusions

5. Conclusions

Based on our data, we suggest that alpha-synuclein impairs mitochondria trafficking leading to mitochondria, autophagy and endoplasmic reticulum dysfunction, dependent of Miro1, Rab5 and LC3. However, the stabilization of microtubules using NAP recovers mitochondria and autophagy dysfunction. Therefore, we propose that disrupted intracellular trafficking plays a crucial role in PD pathology.