The role of alpha-synuclein on transcriptional deregulation in Parkinson's disease

Dissertation

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Hora

Sinto que hoje novamente embarco
Para as grandes aventuras,
Passam no ar palavras obscuras
E o meu desejo canta - por isso marco
Nos meus sentidos a imagem desta hora.

Sonoro e profundo
Aquele mundo
Que eu sonhara e perdera
Espera
O peso dos meus gestos.

E dormem mil gestos nos meus dedos.

(...)
As minhas mãos estão cheias
De expectativa e de segredos
Como os negros arvoredos
Que baloiçam na noite murmurando.

Ao longe por mim oiço chamando
A voz das coisas que eu sei amar.

E de novo caminho para o mar.

Sophia de Mello Breyner Andresen

Affirmation

I hereby declare that I have written this thesis entitled “The role of alpha-synuclein on transcriptional deregulation in Parkinson’s disease” independently and with no other sources and aids other than quoted. This thesis has not been submitted elsewhere for any academic degree.

Isabel Paiva de Castro

Göttingen, February 7th, 2018

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List of abbreviations

acH3 - Acetylated histone 3

AD - Alzheimer's Disease

AK - Adenylate kinase

ANOVA - Analysis of Variance

aSyn - Alpha-synuclein

ATM - ATM serine/threonine kinase

BDNF - Brain-derived neurotrophic factor

bFGF - Basic fibroblast growth factor

BiFC - Bimolecular fluorescence complementation

BRCA1 - DNA repair associated 1

BRCA2 - DNA repair associated 2

bSyn – beta-synculein

cAMP - Dibutyryl cAMP

CBA - Chicken/β-actin

ChIP - Chromatin immuneprecipitation

ChIP-seq - Chromatin immuneprecipitation followed by sequencing

COL4A1 - Collagen Type IV Alpha 1 Chain

COL4A2 - Collagen Type IV Alpha 2 Chain

COMT - Catechol-O-methyltransferase

CREB - cAMP response element binding protein

CREB – cAMP response element binding

D2 - Two days

D5 - Five days

D8 - Eight days

DAT - Dopamine transporter

DCFDA - 2',7'–dichlorofluorescein diacetate

DLB - Dementia with Lewy Bodies

DMEM/F12 - Advanced Dulbecco's modified Eagle's medium/F12

DNMT1 - DNA methyltransferase

DR5 - Death receptor 5

ER - Endoplasmic reticulum

ERAD - Endoplasmic-reticulum-associated protein degradation

FC - Fold-change

FOXM1 - Forkhead box M1

GDNF - Glial cell derived neurotrophic factor

GO - Gene ontology

H1 - Histone 1

H3 - Histone 3

HATs - Histone acetyltransferases

HDAC - Histone deacetylases

HDACi - Histone deacetylase inhibitors

HEK293 - Human embryonic kidney cells 293

IPA - Ingenuity Pathway Analysis

iPSC - Induced pluripotent stem cells

IRE1 - serine/threonine-protein kinase/endoribonuclease inositol-requiring enzyme 1

IRES - Internal ribosome entry site

L-DOPA - 3,4- dihyphroxy-L-phenylalanine

LBs - Lewy Bodies

LRRK2 - leucine rich repeat kinase 2

LUHMES - Lund Human Mesencephalic

MAP2 - Microtubule-associated protein 2

miRNAs – microRNAs

mitROS - Mitochondrial ROS

MPP+ - 1-methyl-4-phenylpyridinium

NaB - Sodium butyrate

NAC - Non-amyloid-beta component

NF-κB - factor nuclear kappa B

NGS - Normal goat serum

NGS - next generation sequencing

NLS - Nuclear localization system

p-H2AX - Phosphorylated H2AX

p-p53 - Phosphorylated p53

padj - p-adjusted value

PARK2 - Parkin

PBS - 1xPhosphate Buffer

PCA - Principle component analysis

PD - Parkinson's Disease

PERK - protein kinase R-like endoplasmic reticulum kinase

PGC1 - Peroxisome proliferator-activated receptor gamma coactivator 1

PKC - Protein kinase C

pS129 - Phosphorylated on serine 129

PTEN - Phosphatase and tensin homolog

PTMs - Post-translational modifications

qPCR - Real-time PCR

RAD18 - E3 ubiquitin protein ligase

REM - rapid eye movement

RNA-seq - RNA-sequencing

ROS - Reactive oxygen species

RT - Room temperature

S129 - Serine 129

SAHA - Suberoylanilide hydroxamic acid

SD - Standard deviation

SDS-PAGE - Sodium dodecyl sulfate polyacrylamide

SN - *substantia nigra*

SNARE - Soluble NSF attachment protein receptor

SNCA - Synuclein alpha

SNpc - *Substantia nigra pars compacta*

TBST - 1xTris Base Solution/0.05% Tween

TH - Tyrosine hydroxylase

TOP2A - Topoisomerase (DNA) II alpha

TUJ1 - Neuron-specific class III β-tubulin

UPR - unfolded protein response

WPRE - Woodchuck hepatitis virus posttranscriptional regulatory element

WT - Wild-type

XBP1 - X-box binding protein 1

Abstract

Parkinson's disease (PD) is a complex neurodegenerative disorder that manifests through a broad range of motor and non-motor symptoms. Alpha-synuclein (aSyn) is the major protein component of Lewy bodies and Lewy neurites, considered the pathological hallmarks in PD and other synucleinopathies. Some familial forms of PD can be caused by duplication, triplication, or missense mutations in the gene encoding for aSyn. However, the precise molecular mechanisms linking aSyn to the disease are still elusive. Although it has been shown that aSyn plays a role in transcriptional deregulation, the effect of specific aSyn mutants associated with familial forms of PD, such as the A30P mutant, remains unclear. This thesis compiles two studies that contain our major findings focused on the role of aSyn on transcriptional deregulation in PD.

In the first study, we aimed to investigate the impact of aSyn on transcriptional deregulation using Lund Human Mesencephalic (LUHMES) cells as a model, since they can be differentiated into dopaminergic neurons. To achieve this, we generated two cell lines expressing wild-type (WT) or mutant A30P aSyn and performed gene expression analysis using RNA-sequencing. We observed that both WT and A30P aSyn induced robust transcriptional deregulation, including changes in expression of DNA damage/repair genes. Interestingly, increased DNA damage and was only observed in WT aSyn dopaminergic neurons. Furthermore, WT aSyn affected mitochondrial ROS (miROS) handling, unlike A30P aSyn. In these cells, aSyn expression decreased expression of acetylated histone 3 (acH3) levels that were restored by treatment with sodium butyrate (NaB), a histone deacetylase inhibitor (HDACi). Interestingly, NaB was able to rescue the DNA damage induced by aSyn expression, possibly by upregulation of DNA repair genes observed upon the treatment. Moreover, treatment with NaB was shown to ameliorate miROS handling in WT aSyn cells.

In the second study, our main goal was to investigate the role of aSyn on transcriptional deregulation in transgenic mice models of PD. For this purpose, we used transgenic mice overexpressing human WT aSyn and A30P and conducted gene

expression studies. We observed that A30P aSyn promotes stronger transcriptional deregulation and increases DNA binding when compared to endogenous aSyn, consistently with the results obtained previously in LUHMES cells. Importantly, we identified several biological processes affected by A30P mutant aSyn, such as ER-associated pathways. Interestingly, COL4A2, a pro-apoptotic gene, was found to be upregulated in both A30P aSyn transgenic mice and in dopaminergic neurons expressing A30P aSyn. Finally, we observed that aSyn A30P alters Golgi morphology and increases endoplasmic reticulum (ER) stress in dopaminergic cells.

Our findings suggest that aSyn can impact on transcription, both in dopaminergic neurons and in transgenic mouse models of PD, and that A30P aSyn has a stronger effect than WT aSyn. These studies provide novel insight into the mechanism underlying aSyn-toxicity, including gene deregulation, histone modification, DNA damage, miROS handling and Golgi-ER systems. Ultimately, our studies open novel avenues for future therapeutic intervention in PD and other synucleinopathies.

Introduction

1. Parkinson's disease

Parkinson's disease (PD) is the second most common neurodegenerative disease, after Alzheimer's disease (AD), and the most common progressive motor disorder. This pathology affects around 1% of the worldwide population at the age of 60 and around 5% of people over 85 [1]. It is expectable that ageing associated diseases, such as PD, will dramatically increase in the future, taking into account the steadily increasing age of the world's population.

PD was first described in 1817 by James Parkinson in his "An essay on the Shaking Palsy" [2] and, 200 hundred years later, the molecular mechanisms of the disease remain unclear. Clinical diagnosis of PD still relies on the identification of motor features, such as resting tremor, rigidity, postural instability and bradykinesia [3, 4] (Figure 1). Currently it is known that not only motor symptoms are associated with the disease. Non-motor disabilities are known to precede or succeed motor symptoms, including neuropsychiatric disorder, such as depression, dementia, anxiety and rapid eye movement (REM) sleep disorder [5]. Also, gastrointestinal disturbances, anemia, hyposmia or anosmia and cardiac sympathetic denervation are common symptoms of PD patients [5] (Figure 1).

The first pathological insights on PD came in 1912, when Friedrich Heinrich Lewy described the presence of protein inclusions in *post mortem* brain tissue of patients who had suffered from shaking palsy, defining the term Lewy bodies (LBs) [6]. These depositions are now considered the major hallmark of PD. Some years later, the *substantia nigra* (SN) was found to be particularly affected in PD brains [7]. A progressive loss of dopaminergic neurons in the *substantia nigra pars compacta* (SNpc) was identified in PD brains (Figure 1). These neurons project to the striatum, the region of the brain responsible for motor coordination. The loss of dopaminergic neurons, which are the main

source of dopamine, results in impairment of coordination and movement, since dopamine is the neurotransmitter that stimulates motor neurons [8] (Figure 1). In 2003, a correlation between LB pathology and the temporal sequence of PD symptomatology was established [9]. In the dual-hit hypothesis, pathology is proposed to start from the periphery (nose and gut) and spreads through the brain [9]. More recently, it was reported that PD pathology can spread from damaged to healthy neurons, since it was found the presence of LBs in neurons grafted into the brains of PD patients [10, 11]. Consistently, new evidence suggested that aSyn can exhibit prion-like behavior [12].

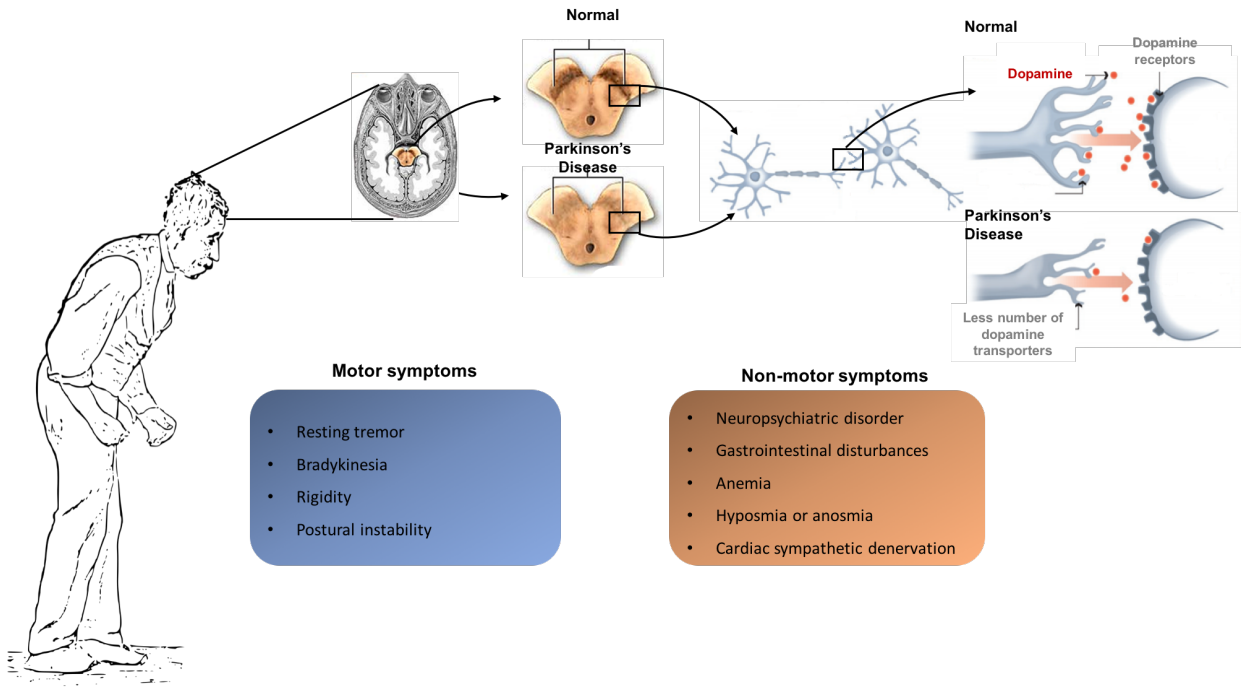

Figure 1. Pathophysiology of PD. PD patients show a progressive loss of dopaminergic neurons in the SNpc and dysfunctions on dopamine release affects coordination and movements. In addition to the motor symptoms, non-motor symptoms, including anxiety, depression and dementia (neuropsychiatric disorders) are also linked to PD [3, 4].

The loss of dopaminergic neurons in the SNpc is an attractive target for PD therapeutic approaches, but all efforts thus far have proven unsuccessful. Nevertheless, certain motor features can be improved by replacing dopamine by using L-DOPA (3,4-dihyphroxy-L-phenylalanine), a dopamine precursor [13]. Other available therapeutics include dopamine receptor agonists monoamine oxidase B inhibitors, anticholinergic medications, and catechol-O-methyl transferase inhibitors [14]. Deep brain stimulation can also be used as a treatment in case patients cannot be sufficiently controlled with medication [13]. However, these treatments cause many adverse effects on PD patients. Thus, investing in new therapeutic strategies for this pathology is still crucial. Furthermore, the scientific community has not been successful identifying reliable biomarkers to diagnose PD at pre-clinical stages. Further efforts need to be done in order to find an accurate diagnosis and prognosis of the disease.

1.1 Parkinson's disease etiology

The etiology of PD is complex and multifactorial with genetic and environmental factors playing a crucial role in the disease onset [15]. Most PD cases are sporadic (90%) and aging is the major known risk factor in these cases [16]. Furthermore, exposure to several environmental factors, such as 1-methyl-4-phenyl-1,2,5,6-tetrahydropyridine (MPTP), rotenone, or paraquat, promotes similar degeneration effects to the ones observed in sporadic PD cases [17]. Additionally, exposure to iron, smoking habits and caffeine intake, have been associated with the disease [18]. Although PD symptoms are thought to affect mostly the brain, the disease also affects non-neuronal tissues. Currently, the hypothesis that PD may start in the gastrointestinal track is becoming stronger. Some studies showed consistent changes in the gut microbiome composition, in both early and advanced PD [19]. Therefore, it is being suggested that these modifications on the gut microbiome represent a risk factor for PD [20]. Moreover, genome-wide association studies identified several polymorphisms in non-coding regions of the SNCA locus, representing a genetic risk factor for the sporadic form of PD [21-23].

Significant advances concerning the genetic mechanisms underlying PD pathogeneses have been made.

Only a small percentage (10%) of PD cases are associated to a familiar genetic origin by the presence of alterations in various genes [24, 25]. The first mutation associated wtih PD was identified in 1997 in the *SNCA* gene, encoding for aSyn [26]. Currently, it is known that missense mutations, as well as duplications/triplications of the *SNCA* gene are associated with autosomal dominant cases of PD [27, 28]. On the other hand, mutations in DJ1, PTEN-induced putative kinase (PINK1) and ATP13A2, among others genes, are associated with autosomal-recessive forms of PD (Table 1).

Thus far, many genetic alterations associated with PD were found in several chromosomal regions and numerous proteins were identified in LBs of PD brains. However, aSyn is thought to be a central player in PD [29, 30].

Table 1. Genes implicated in PD.

Locus	Gene	Description	Reference
PARK 1/4	*SNCA*	Presynaptic/nuclear protein	[26]
PARK2	*Parkin*	Ubiquitin ligase	[31]
PARK3	SPR (?)		[32]
PARK5	*UCH-L1*	Ubiquitin protease	[33]
PARK6	*PINK1*	Mitochondrial protein kinase	[34]
PARK7	*DJ-1*	Multifunctional protein	[35]
PARK8	LRRK2	Leucine-rich repeat kinase 2	[32]
PARK9	ATP13A2	Lysosomal ATPase	[36]
PARK10	(?)		[37]
PARK11	GIGYF2		[38]
PARK12	(?)		[39]
PARK13	Omi/HTRA2	Serine Protease	[40]
PARK14	PLA2G6	Phospholipase	[41]
PARK15	FBXO7	F-box protein	[42]
PARK16	(?)		[21]
PARK17	VPS35	Retromer Complex	[43]
PARK18	EIF4G1	Translation Initiation Factor	[44]
PARK19	DNAJC6	DNAJ/HSP40 homolog, Subfamily C, Member 6	[45]
PARK20	SYNJ1	Synaptojanin 1	[46]
PARK21	DNAJC13	DNAJ/Hsp40 Homolog, Subfamily C, Member 13	[47]
PARK22	CHCHD2	Coiled-coil-helix-coiled-coil-helix domain containing 2	[48]
PARK23	VPS13C	Vacuolar protein sorting 13 homolog C	[49]

1.2 Molecular mechanisms underlying Parkinson's disease

Several molecular mechanisms have been associated to PD pathophysiology. These include, among others, the accumulation of misfolded protein aggregates, impaired function of protein degradation machinery, mitochondrial damage, oxidative stress, ER stress, or neuroinflammmation [50]. However, it is not well understood which mechanisms can trigger neurotoxicity and that might be in the origin of the neurodegeneration process.

Another important mechanism that appears to be common to several neurodegenerative diseases is transcriptional deregulation. A previous study from our group identified transcriptional changes in peripheral cells of PD patients [51]. Gene expression alterations in blood of rapid progression PD patients were mostly involved in immune response, nucleic acids metabolic process and mitochondria [51]. Recent meta-analysis using SN post-mortem tissue of PD patients observed deregulation of genes related to general key cellular functions, such as protein degradation, mitochondrial energy metabolism, synaptic function, as well as survival mechanisms, including immune system processes and response to stimulus [52]. Furthermore, epigenetic changes associated with the expression of microRNAs (miRNAs) were also linked to PD [53-55]. miRNAs are small, non-coding RNAs (length, 19-24 nucleotides) that bind target messenger RNAs (mRNAs) leading to their degradation or repression of translation [56]. This process of RNA silencing is thought to influence several mechanisms underlying neurodegeneration process [53-55]. The transcriptomic knowledge of PD is still in its early stage and a better understanding of the molecular mechanisms that underlie gene expression and epigenetic changes will contribute to great advances in PD field.

DNA damage and impairment of DNA repair, have also been associated with PD [57]. It was shown that DNA strand breaks occur in the SNpc in MPTP mouse model and this was associated with poly (ADP-ribose) polymerase (PARP) activation [58]. Furthermore, recent reports described that specific defects in DNA repair, such as flawed nucleotide excision repair (NER) capacity, affect the dopaminergic system and can constitute an age-related risk factor for the disease [57]. Although many studies indicate

a connection between increased oxidative stress and nuclear DNA damage [59-61], it is not clear which event occurs first.

Mitochondrial dysfunction is also an important aspect in PD. Several PD-linked mutations affect genes with specific functions in mitochondrial dynamics. Among those, mutations in DJ-1, Parkin, PINK1, vacuolar protein sorting-associated protein 35 (VPS35) and LRRK2 highlight the possibility of mitochondrial dysfunction as a major cause of neuronal toxicity in PD [62-64]. Additionally, inhibitors of the mitochondrial transport chain, such as MPTP, paraquat or rotenone, leads to dopaminergic neuronal dysfunction, supporting the strong involvement of mitochondrial dysfunction in PD [65-68].

Accumulating evidence shows that disruption in the secretory pathway function is a significant contributor to PD, since this leads to the accumulation of aggregated proteins and loss of dopaminergic neurons [69]. Using neuronal cells derived from PD patients, it was shown that ER stress is a relevant molecular signature of the pathology, leading to the accumulation of ER-Associated Degradation (ERAD) protein in neurons [70]. Familial mutations of PD revealed a closer relationship with the secretory pathway. Mutations in ATP13A2, a lysosomal protein, lead to a rare type of early onset parkinsonism and promotes retention of its protein at the ER, enhancing ER stress and inducing cell death [63]. Changes in LRKK2 are the most common genetic alteration in PD patients [71]. It is known that LRRK2 is partially located to the ER in PD dopaminergic neurons [72] and lack of its homologue in *C.elegans* leads to high susceptibility to develop ER stress and toxicity [73]. In response to ER stress, a complex signaling transduction pathway, the unfolded protein response (UPR), is activated [74]. Many studies indicate that UPR activation exerts a rate-limiting role in neurodegeneration [75]. In the neurons of PD patients, the UPR is activated and its modulation protects or enhances disease progression. It was shown that low UPR expression protects neurons from aSyn-induced toxicity, apparently by stimulating chaperones and secretory pathways [76, 77]. Furthermore, previous studies indicated that activation of IRE1/XBP1, a particular UPR branch, is essential for PD neuronal survival, and that this may be selectively relevant for SN neurons [77]. Additionally, changes in PERK/eIF2α pathway, another branch of UPR and crucial in protein synthesis shutdown, was previously related to neurodegeneration

[78]. This suggests that shutdown of protein translation process is a fundamental mechanism for neuronal dysfunction. Nevertheless, the majority of the studies associating ER stress and PD were done *in vitro*. Therefore, *in vivo* confirmation is necessary to identify the relevant components of this pathway in order to identify therapeutic targets for PD.

2. aSyn is a central player in Parkinson's disease

Proteins of the synuclein family have been implicated in various diseases. This family was first described and isolated from the electric organ of the Torpedo ray and its expression was found in both synapses and nuclei. Thus, the name of this family of proteins was entitled synuclein due to its cellular localization (SYNapse, NUCLEus, and protEIN) [79].

The synuclein family comprises three members: aSyn, beta-synuclein (bSyn) and gamma-synuclein [80]. Although all these proteins have been studied in the context of some diseases, aSyn emerged as a major player in PD. In fact, aSyn is the main component of LBs which are present in a variety of disorders called synucleinophaties [81]. These disorders include PD, Dementia with LB (DLB), Pure Autonomic Failure and Multiple Systems Atrophy [82, 83].

aSyn is composed of 140 amino acids and it is defined as an intrinsically disordered protein. Structurally, it can be divided into 3 regions: the N-terminal, the non-amyloid-beta component (NAC) and the C-terminal domain (Figure 2). The N-terminal region comprises residues 1-60 and contains seven conserved KTKEGV repeat followed by a variable short hydrophilic tail [84]. It is unfolded in solution and the amphipathic structure formed by the repeats allows lipid binding. Thus, aSyn interacts vesicular structures and it is mostly attached to membranes [85, 86]. The central NAC domain (amino acids 61-95) is highly hydrophobic and prone to aggregation, while the hydrophilic C-terminal domain (amino acids 96-140) is in general unstructured and contains mostly

charged amino acids, such as glutamate and aspartate. The C-terminal domain is able to minimize the aggregation propensity, since the lack of this domain increases aSyn fibrilization [87] (Figure 2).

The possible existence of an aSyn tetrameric conformation has been discussed in recent years [88-90]. Although the existence of this conformation of aSyn is still controversial, new studies found that aSyn displays tetrameric conformation under physiological conditions [88-90]. The shifting between monomeric to tetrameric states, resultant from mutations within the N-terminal domain, was shown to increase cellular toxicity [91].

The aggregation of aSyn into LBs is believed to play an important role in PD pathogenesis. Nevertheless, this process is not fully understood. In pathological conditions, misfolded aSyn monomers can interact with each other, forming unstable dimers and oligomers that can aggregate in amyloid fibrils [81]. However, the question of which aSyn species represent the major toxicity in the cells is still debatable in the field. Some studies showed that the toxic species are the aSyn oligomers [92-94] while others pointed towards the aSyn aggregates as the major player in toxicity [95, 96]. The most accepted hypothesis is that the prefibrillar oligomers are the toxic species of aSyn and this might lead to disease progression [97]. Furthermore, in vitro studies showed that inclusion formation may act as a protective mechanism reducing the toxicity caused by oligomeric species of aSyn [98-100]. Recently, it was shown that aSyn might be constantly shifting between monomeric and a functional oligomeric state, which emphasizes the need to discriminate between aSyn functional oligomers and toxic oligomers [90, 101, 102].

2.1 Mutations in the *SNCA* gene

Missense mutations and multiplications in *SNCA*, encoding gene of aSyn, cause rare cases of autosomal dominant early-onset of PD. Several mutations were implicated

in the pathology (A53T [26], A30P [103], E46K [104], H50Q [105], G51D [106], A53E [107]). Moreover, duplications and triplications of the *SNCA* locus are also associated with disease onset, and those cases are more frequent than those carrying point mutations [81]. Studies in cellular and animal models showed that overexpression of wild-type (WT) aSyn and the presence of *SNCA* mutations induced toxicity [85, 108-110]. Several reports indicated that mutations in *SNCA* gene influence the kinetics of aSyn aggregation due to their different propensities rates of oligomer and fibrillary inclusions formation [37, 111, 112]. Thus, it is of great importance to deeply investigate familial mutations of aSyn in order to better understand the mechanisms underlying the disease.

The first aSyn mutation associated with PD was the A53T mutation. It was identified in an Italian kindred and, later, in three Greek families with autosomal dominant inheritance of PD [26, 113]. The substitution of an alanine for a tyrosine at position 53 is enough to disrupt the alpha-helix and increase the beta-sheet conformation, enhancing the aggregation propensity [114]. This mutant aSyn also shows increased membrane binding propensity and affects mitochondria homeostasis [111, 115-117].

Later, another aSyn mutation was identified in a German family. The substitution of an alanine for a proline at position 30 (A30P) was described as a rare autosomal dominant trait [103]. An interesting characteristic of this mutation is the reduced affinity for membrane and vesicle binding. This effect leads to decrease fibrillation kinetics, promoting the accumulation of protofibrillar and oligomeric structures in the cell [112, 118, 119]. Furthermore, overexpression of A30P aSyn was shown to affect tyrosine hydroxylase (TH) synthesis, impairing neurite and axonal regeneration in dopaminergic neurons [120].

Other mutations in the *SNCA* gene linked to familial forms of PD were later identified. The E46K mutation was detected in a Spanish family with autosomal dominant parkinsonism [104, 121]. This mutation shows a higher rate of fibril formation comparing to the WT aSyn [121]. It was also reported that E46K mutation increased the percentage of cells with inclusions in human neuroglioma cells [122]. Additionally, transgenic mice expressing E46K aSyn develop severe motor impairment and show phosphorylated S129 aSyn-positive inclusions [123].

More recently, two other aSyn mutations were characterized, the H50Q and G15D mutation [105, 106, 124]. The H50Q mutation was associated to a late-onset idiopathic PD and it increased aggregation and amyloid formation of aSyn. This effect was associated with an enhancement of aSyn secretion and cellular toxicity [125, 126].

The G15D mutation was found in a French family with parkinsonian-pyramidal syndrome and was associated with an early disease onset, showing rapid progression [106]. *In vitro*, this mutation showed reduced aggregation propensity, impaired membrane binding and increased mitochondrial fragmentation [127].

The most recent aSyn familial mutation, the A53E mutation, was identified in a Finish patient with atypical PD at the age of 36. The patient showed accumulation of aSyn inclusions in the striatum and severe cortical pathology [107]. Also, this mutation was shown to decrease aSyn aggregation and increase oligomers accumulation [128, 129]. A study reported that A53E aSyn mutation has also lower membrane binding affinity compared to the WT aSyn [128]. An effect on mitochondrial dysfunction and Golgi fragmentation was also implicated for this mutation [129, 130] .

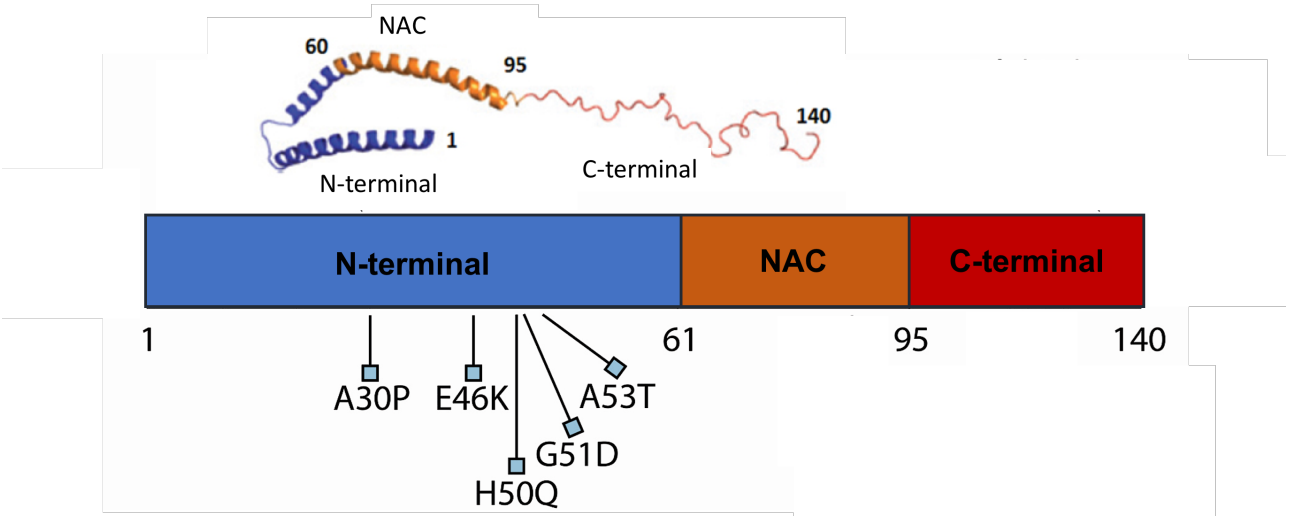

Figure 2. Structure of human aSyn. Schematic representation of the 3 different domains of the human aSyn: N-terminal, NAC and C-terminal. aSyn mutations associated to familial forms of PD are located in the N-terminal (adapted from Gallegos *et al.*, 2015 [131]).

2.2 Putative functions of aSyn

The neurotoxicity of aSyn, due to either increased levels of expression or to the effect of mutations (as described above), appears to be associated with a wide-range of pathways and cellular mechanisms (Figure 3). Due to the high abundance and accumulation of aSyn in the pre-synaptic compartment [132, 133], one emerging consensus is that aSyn is involved with the maintenance of the pre-synaptic vesicle pool and participates in the neurotransmitter release process [134]. Several studies supported this hypothesis showing that aSyn facilitates the interaction between synaptic vesicles and participates in the regulation of their assembly [101, 102]. aSyn is able to interact with synaptic SNARES enhancing vesicle fusion [101]. Moreover, aSyn knockout mice display alterations in synaptic vesicles dynamics and lower levels of striatal dopamine [135, 136].

Another important aspect of aSyn-induced toxicity is its association with mitochondrial dysfunction (Figure 3). Although the localization of aSyn in the mitochondria is still controversial [137], studies have shown that aSyn has a mitochondrial targeting sequence at its N-terminus and that it is able to disrupt mitochondrial protein import mechanisms [138]. Recent data indicated that aSyn can bind to the mitochondrial outer membrane and that can be imported to the mitochondria [139, 140]. Studies in a *C.elegans* model showed that overexpression of aSyn leads to disruption in mitochondrial fusion, resulting in mitochondrial fragmentation [141]. Moreover, the prefibrillar oligomeric form of aSyn was shown to promote complex I dysfunction, using isolated mitochondria [142] and A53T aSyn was capable to inhibit function of the complex I in dopaminergic neurons of transgenic mice [143]. While aSyn overexpressing mice are more prone to MPTP neurotoxicity and show severe mitochondrial alterations [144], the aSyn knockout mice show resistance to the toxin [145].

aSyn can also affect ER and Golgi systems [129, 146] (Figure 3). It was described that expression of aSyn can interfere with ER-to-Golgi transport process [147, 148]. Additionally, it was also observed that expression of aSyn increases ER stress and activates UPR [149]. It is not clear, however, how aSyn is able to promote ER stress. One

possible mechanism would be the inhibition of ER-to-Golgi transport and/or dysfunction of the secretory pathway, resulting in overload of the ER [150, 151]. Another possible mechanism would be the aggregation of aSyn with chaperones into the ER lumen, leading to ER stress [152] . Additionally, aSyn can also trigger ER stress by inhibition of ER associated degradation (ERAD), leading to the accumulation of unfolded proteins. Interestingly, Homocystein-induced ER protein (Herp) plays an essential role in ERAD and it is overexpressed in PD and present in LB of PD patients [153].

Dysfunctions in Golgi homeostasis are also observed in several neurodegenerative diseases, including PD [154, 155] (Figure 3). Particularly, alterations in Golgi morphology are commonly associated to these diseases [156]. Previous studies reported that expression of aSyn led to Golgi fragmentation in neurons. Also, aSyn prefibrillar aggregates were associated with disruption of Golgi [156]. Other studies, using yeast as a model, showed that aSyn accumulation leads to mislocalization of Golgi markers and secretory vesicles aggregation [157]. Although several mechanisms have been proposed, further investigations need to be done in order to understand how aSyn leads to changes in Golgi morphology.

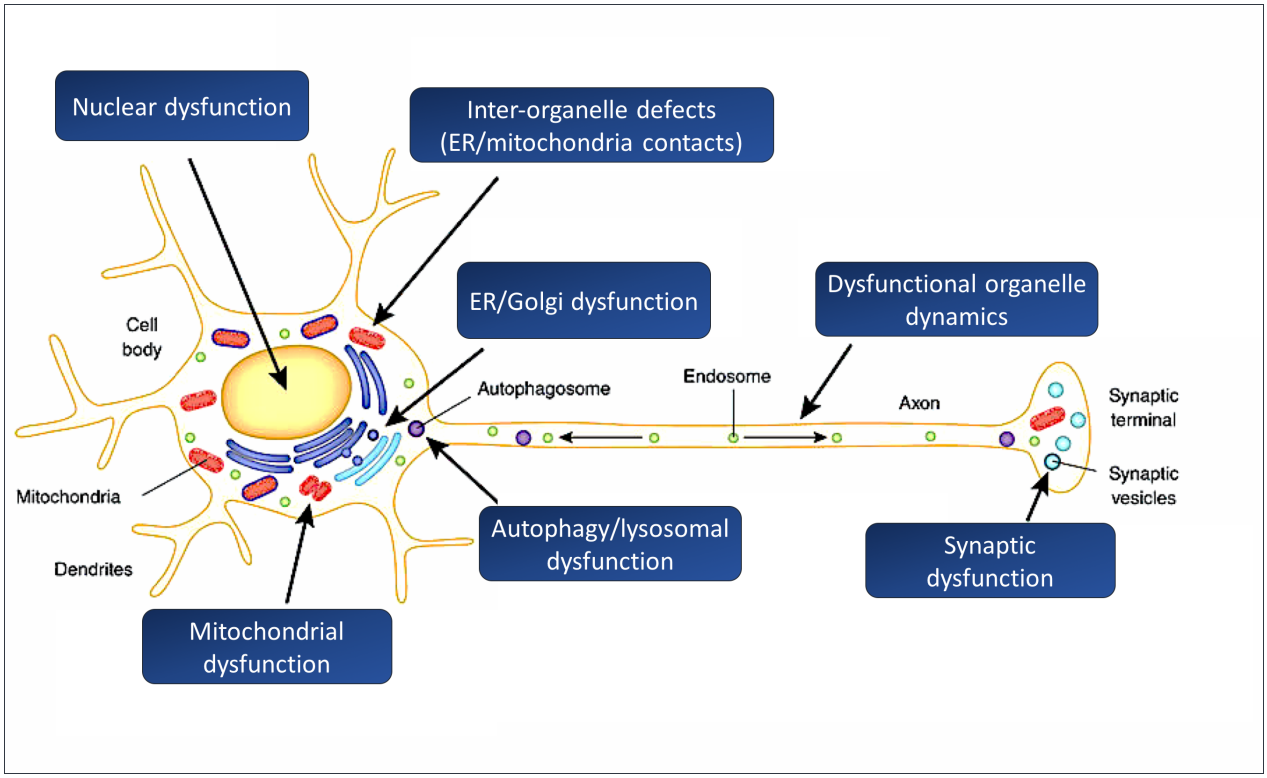

Figure 3. Pathways associated with aSyn-induced toxicity. Many molecular mechanisms have been implicated in aSyn-induced neurotoxicity, including synaptic, nuclear and mitochondrial dysfunction. aSyn was also associated to dysfunction in organelle dynamics that affects the autophagy and lysosomal pathway. ER/Golgi dysfunctions, such as ER stress and Golgi fragmentation were previously implicated with aSyn-induced toxicity. aSyn was also shown to have a role in the nucleus by binding to histones and DNA. (adapted from *Wong et al*., 2017 [158])

2.2.1 aSyn and transcriptional deregulation

The putative occurrence of aSyn in the nucleus raises the possibility of a role in transcription deregulation [159, 160] (Figure 4). However, it is not clear which are the genes and biological processes affected by aSyn expression and its precise role in the nucleus. It is also not defined whether the interactions of aSyn in the nucleus can trigger apoptosis or if, on the other hand, can lead to neuroprotection.

Accumulation of aSyn was found to downregulate numerous genes upstream of cyclic adenosine monophosphate (cAMP) response element binding protein (CREB),

including glycogen synthase kinase 3b (GSK3b), protein kinase C (PKC) and nuclear factor kappa B (NF-κB) [161, 162]. It is known that downregulation of CREB downregulates brain-derived neurotrophic factor (BDNF) [163] and Bcl-2 [162] leading to decreased levels of TH [164]. Interestingly, nuclear aSyn downregulates Nurr1, A nuclear receptor implicated in dopamine biosynthesis and dopaminergic neuron survival. Decreased levels of Nurr1 have been associated with PD and its ablation in adult mice led to behavioral characteristics of parkinsonism during aging [165]. Recently, it was indicated that activation of Nurr1 can provide neuroprotection which can be a potential target for monotherapy in PD [166].

A relevant question in the field is whether aSyn is able to bind directly to DNA and, consequently, alter gene regulation (Figure 4). Several studies have shown that aSyn can change DNA conformation and stability by binding directly to transcriptionally active zones, histone-free and single copy DNA [167-169]. Curiously, aSyn was also shown to cause DNA nicking leading to DNA damage [170]. Additionally, DNA can itself regulate aSyn folding. While binding of aSyn to double-stranded DNA was associated with its aggregation [171], binding of aSyn to supercoilded plasmid and single-strand circular DNA resulted in alpha-helical folding, preventing aggregation of aSyn [172]. It was also reported that the presence of nuclear factors increases aSyn fibrilization [173]. Interestingly, other neurodegenerative related proteins, such as Tau, exhibits also DNA binding properties [174]. It was also reported that aSyn shows stronger DNA-binding in PD brains compared to the controls and also in transgenic mice upon oxidative stress [175]. However, it remains unclear whether mutants of aSyn affect differently the DNA binding process.

It is also proposed that aSyn may interact with several promoter regions regulating the expression of their genes. One of these promoter regions is the PGC1 alpha gene, which is known to be a major mitochondrial transcription factor. Interestingly, PGC1 alpha is found downregulated in brains of PD patients [175].

Besides the nuclear localization of aSyn, regulation of gene expression can also be affected by its cytoplasmic localization, as aSyn is able to retain some transcription factors and their regulatory kinases in the cytoplasm [81]. As an example, aSyn can

sequestrate DNA methyltransferase 1 (Dnmt1), leading to DNA hypomethylation [176]. Consistently, it is known that decreased levels of Dnmt1 and DNA hypomethylation are found in PD and DLB brains [176]. Furthermore, the transcription factor Elk-1 was found phosphorylated within aSyn cytoplasmatic inclusions in glia [177].

Although several studies support the toxic effect of aSyn accumulation in transcriptional deregulation, other reports suggest its neuroprotective role in gene regulation. Nanomolar concentrations of aSyn were shown to exert a neuroprotective effect by activating prosurvival signalling pathways, such as PI13K/Akt and Bcl-2 family. However, higher amounts of aSyn led to cell toxicity. The question of whether aSyn can be neuroprotective or, in contrast, causes toxicity, appears to be related to the amount of protein within the neurons [178].

Although some studies reported that both WT and mutant aSyn affect gene expression in a similar manner [175, 179], other studies suggested that some genes are only affected by WT or mutant aSyn [180]. TH and GTP cyclohydrolase, important dopamine homeostasis regulators, were affected only by WT aSyn expression, unlike mutant aSyn [180]. Thus, the question whether the mutants of aSyn have a different impact in gene regulation compared with the WT aSyn is still not answered.

2.2.2 aSyn and histones

It was previously shown that nuclear aSyn can interact with histones and, consequently, decrease the number of free histones that are available to bind to DNA [173, 178, 181]. The interaction between aSyn and histones was proven when aSyn was found co-localized with acetylated histone 3 (acH3) in nucleus of nigral neurons of paraquat-treated mice [173]. It was also observed that aSyn is able to bind to histone 1 creating a tight 2:1 complex, *in vitro*, leading to fibrilization of aSyn [173]. Later studies reported that aSyn binds to histone 3 *in vitro* and in *Drosophila* [182]. Although some reports showed that some aSyn mutations increase aSyn nuclear localization [122, 182,

183], the effect of these mutations on the conformational state of histones was not investigated.

Furthermore, expression of aSyn has been linked to histone posttranslational modifications (PTMs), such as acetylation, phosphorylation and ubiquitination. Histone PTMs are known to affect gene expression and are also associated to DNA repair/damage and replication processes [184].

2.2.2.1 aSyn and histone acetylation

The interaction between aSyn and histones PTMs is not fully understood. However, the few available studies refer mostly to histone acetylation – as we have recently reviewed [185]. In the acetylation process, acetyl groups are transferred by histone acetyltransferases (HATs) or removed by histone deacetylases (HDACs) from lysine residues of histones. Acetylation of histones activates transcription by making chromatin more loosely packed while histone deacetylation induces transcription repression [186].

In physiological conditions, HAT and HDAC protein levels in neurons, maintain the balanced state in order to regulate gene expression. However, the acetylation homeostasis is found perturbed in neurodegenerative process [187]. A recent study demonstrated that histone acetylation is disease-dependently altered in PD, probably due to the effects of dopaminergic neurodegeneration and microglia infiltration [188]. Interestingly, decreased levels of acH3 at lysine residue 9 were observed in primary cortex of PD patients [189]. Furthermore, expression of WT aSyn and mutants A30P and A53T aSyn were shown to increase H3 hypoacetylation [182]. Decreased levels of acH3 were also associated with increased oxidative stress in mammalian cells [190]. Consistently, in aSyn transgenic mice, p300 HAT was found downregulated and this was related to lower levels of acH3 and transcriptional repression [191]. In cell cultures and brains of PD patients, p300 HAT was sequestered in aSyn-immunopositive aggregates

[192]. On the other hand, aSyn was shown to induce protection against hydroxyurea, a DNA synthesis inhibitor, by increasing the levels of acH3 in yeast [178].

These evidences showing a significant link between acH3 and neurotoxicity, supports modulation of acH3 levels as a promising therapeutic candidate for PD. Interestingly, treatment with histone deacetylase inhibitors (HDACi) rescued aSyn-induced toxicity by decreasing acH3 [182]. HDACi are currently used in therapeutic strategies for many diseases, such as cancer [193]. Furthermore, some immune and neurological disorders were shown to improve upon treatment with some HDACi [59]. Potential benefit of using this therapeutic strategy in neurodegenerative disorders have been studied. Interestingly, the effect of HDACi appear to be dependent on the cell type [59, 60]. In cancer cells, HDACi treatment is known to induce apoptosis by promoting DNA damage and increasing oxidative stress, an effect that can be associated to impairment of DNA repair process and transcription repression [59, 194]. Nevertheless, the use of HDACi in neuronal cells seems to be neuroprotective against oxidative stress [59, 60]. Treatment with valproic acid, a HDACi class I and IIa, induced neuroprotection in a rotenone PD model [195]. Additionally, treatment with sodium butyrate (NaB) and suberoylanilide hydroxamic acid (SAHA) promoted neuroprotection in aSyn models [59, 194]. Lately, HDACi have gained increasing attention as a promising alternative treatment for neurodegenerative diseases [196-198], however the possible mechanisms underlying its effect and its associated side effects need to be further investigated.

2.2.3 aSyn and miRNAs

In some neurodegenerative disease, including PD, dysregulation of non-coding RNAs, such as miRNAs, have been reported [54, 55, 199, 200] (Figure 4). Particularly, several miRNAs have been suggested as potential aSyn modulators. The mRNA of SNCA has a highly conserved 3'UTR that is twice longer than its coding sequence [201]. This fact suggests that the 3'UTR plays a role in maintaining the mRNA stability and regulating the protein translation. It was also reported that this region contains sequences

that can be target by some miRNAs, such as miR-7 and miR-153, which are known to decrease the protein levels of aSyn.

miR-7 expression was found decreased in MPTP treated mice. This fact can be associated with increased levels of aSyn and, consequently, its accumulation in the neurons [202]. Furthermore, miR-7 was shown to exert a protective role in MPP^+ treated cortical neurons by maintaining mTOR signaling pathway active [203], which is known to promote aSyn clearance by increasing autophagy [204].

Moreover, miR-153 targets aSyn 3'UTR and downregulates its expression. Interestingly, in a PD patient it was found a variation on the 3'UTR (C464A) in the binding site of miR-153 [205].

Other miRNAs, such as miR-34b and miR-34c are also known to target aSyn 3'-UTR. These miRNAs are found downregulated in PD patients and their inhibition lead to increased aSyn levels and aggregation [206, 207].

Furthermore, miR-214 also downregulates aSyn levels and its expression was found decreased in MPTP-treated mice and in cells treated with MPP^+ [208].

An effect of aSyn expression in modulating miRNAs expression has also been reported. It was shown that let-7 and miR-64/65 were downregulated in *C.elegans* models overexpressing human A53T aSyn, suggesting a role for these miRNAs in PD pathogenesis [55]. Using *Drosophila* expressing A30P aSyn, five miRNAs , dme-miR-13b-3p, dme-miR-932-5p, dme-miR-1008-5p, dme-miR-133-3p dme-miR-137-3p, were found upregulated [209]. Among those, miR-133, miR-13b and miR-137 are brain enriched and highly conserved from *Drosophila* to humans. Furthermore, some miRNAs were found deregulated in early-symptomatic A30P aSyn transgenic mice: miR-495, miR-10a, miR-10b, miR-212 and miR-132. One of them, miR-132 has been defined as a key regulator of neurite outgrowth [210]. These findings suggest a different role of aSyn mutations in miRNAs modulation, that might explain the distinct toxicity effects associated to their expression.

In the past few years, miRNAs research field has demonstrated strong relevance and significant impact in PD pathogenesis. Currently, there is accumulating evidence that miRNAs may be used as potential biomarkers or therapeutic targets in neurodegeneration [211, 212]. However, it is crucial to further explore miRNAs expression patterns and profiling in order to better understand the mechanisms underlying PD and, consequently, design novel biomarkers and possible therapeutic targets.

2.2.4 aSyn and alternative splicing

Alternative splicing is an important mechanism in regulation of gene expression. This process takes place when a single gene leads to formation of multiple mRNA transcripts and protein isoforms with different functional properties [213]. It is predictable that this process occurs in 94% of human protein-coding genes and is one of the main contributors to complexity of the organism [214, 215].

Alternative splicing can occur in different ways: one-third of it involve cassette-type alternative exons, while at least one-quarter is related to alternative selection of 5' or 3' splice sites within the sequence of the exons. There are other alternative splicing events that include alternative promoters, mutually exclusive exons, intron retention, and poly-adenylation [216, 217].

Currently, it is possible to have a better understanding of the transcriptomic events including alternative splicing events, with the introduction of some techniques, such as exon arrays and next-generation sequencing (NGS) [218].

Interestingly, the most of splicing events are tissue specific and the brain appears to be the one with more alternatively spliced genes [217, 219, 220]. In line with this, several of these genes have a strong association with neurodegenerative disorders, including PD [221]. There are several splicing variants originated from SNCA gene in addition to the full-length transcript, usually known as SNCA-140. The expression of SNCA splice variants, such as, SNCA-140, SNCA-126, SNCA-112, and SNCA-98 differs between brain regions under normal and pathological conditions [222]. These four

transcripts were found overexpressed in PD frontal cortex, compared to healthy controls [223]. Curiously, only the three shorter transcripts were observed upregulated in PD SNPc [224, 225].

Additionally, differential splicing events were also observed in other PD associated genes, such as PARK2, LRRK2 and PINK1 [222].

Understanding the role of alternative splicing in gene regulation might represent a crucial step toward a better understanding of the PD pathobiology.

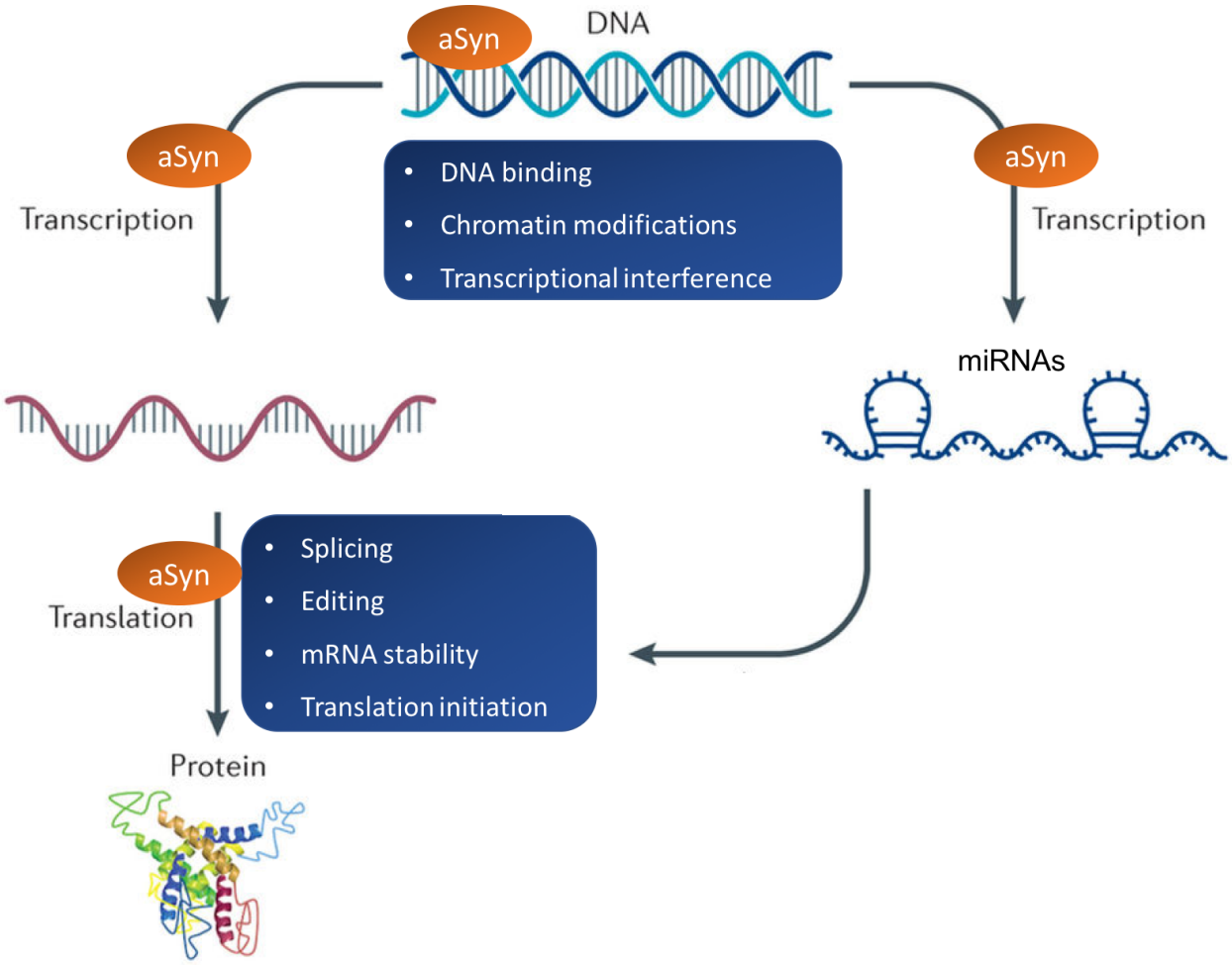

Figure 4. Putative mechanisms implicated in aSyn-induced gene deregulation. aSyn is able to interact with DNA, inducing chromatin and histone modifications and. It can also affect transcriptional deregulation and alter miRNAs expression, affecting their target mRNAs. Also, splicing effects are implicated with aSyn expression, leading to abnormal translation process. (adapted from Wahlestedt *et al*., 2013 [226])

3. Models for studying aSyn-mediated toxicity

3.1 Cell-based models

Cell-based models have been instrumental in our understanding of the molecular mechanisms underlying central biological processes, such as trafficking, protein degradation, and cell division. The models that are currently used to study neurodegeneration in general, and aSyn-mediated toxicity in particular, only recapitulate certain aspects of the diseases they attempt to model [227]. Despite intrinsic limitations, using cellular models provides exclusive opportunities to assess molecular mechanisms and to help identifying therapeutic targets for PD. Thus, it is crucial to explore cellular models that can mimic the pathobiology associated with aSyn, including its aggregation and toxicity. However, it is important to have in mind what are the limitations in the use of cell-based models [227]. These models are not fully able to recapitulate *in vivo* physiology and importantly, they cannot mimic the ageing process [228, 229].

Immortalized cell lines are commonly used in the study of synucleinopathies. These include human neuroglioma (H4), human embryonic kidney 293 (HEK293), and human neuroblastoma SH-SY5Y cells, among many others. The main advantages of using these models are the ease of culture and transfection, and also that these cell lines are useful to study aSyn aggregation and release/secretion.

A powerful tool to study dimerization/oligomerization of aSyn is the Bimolecular Fluorescence Complementation (BiFC) assay [183, 230]. This assay allows the direct visualization of protein-protein interactions in living cells and where they take place within the cell. However, the fact that H4 and HEK293 models do not show dopaminergic phenotype, and cannot be differentiated, can limit the recapitulation of phenotypes that are relevant in the context of dopaminergic neurons, which are important in PD [227]. Thus, differentiated neuronal cells are useful tools to address questions in the context of post-mitotic phenotypes. Neuronal cell lines, such as PC12, derived from rat adrenal medulla, and primary dopaminergic cells that are originated from rat or mouse embryos,

are commonly used in the study of aSyn-mediated toxicity. PC12 cells are also useful to study neurodegeneration induced by toxic molecules, such as rotenone and MPP^+, since they are more vulnerable to these toxins when compared with other cell lines [231, 232].

The main advantage of using human neuronal cell models, such as SH-SY5Y (human neuroblastoma) and Lund Human Mesencephalic (LUHMES) cells, is the fact that they can be differentiated into a dopaminergic cell phenotype. SH-SY5Y cells are commonly used to mimic dopamine homeostasis dysfunction and toxicity induced by MPP+, since they express dopamine transporters (DAT) and receptors [233].

LUHMES cells were generated from embryonic human mesencephalon and were immortalized by using a tetracycline-responsive v-myc gene (TET-off) [234, 235]. After differentiation in the presence of several factors, such as cyclic AMP (cAMP) and glial derived neurotrophic factor (GDNF), they display a dopaminergic phenotype, expressing TH and developing extensive neurites [234, 235]. Furthermore, these post-mitotic cells show spontaneous electrical properties, are able to uptake and release dopamine and reveal MPP+ sensitivity, which are the main characteristics of dopaminergic neurons [234, 236]. A recent study reported that differentiated LUHMES cells are more sensitive to toxic agents compared to other dopaminergic cell lines [237]. This cytotoxicity effect was specific for differentiated cells, showing the high significance of this model in studying toxicity mechanisms. Recently it was shown that LUHMES cells can also be cultured in a three-dimension (3D) format [238]. In order to study aSyn-induced toxicity in dopaminergic neurons, stable lines expressing WT and A30P aSyn were generated via lentiviral infection [239, 240].

Induced pluripotent stem cells (iPSCs) are also greatly used in PD research, since they can be derived from patients and from healthy individuals, and can also be differentiated into a dopaminergic phenotype [241]. These cells, which are able to recapitulate some PD features, were already generated from patients carrying the *SNCA* triplication and differentiated into dopaminergic neurons [242].

Although further efforts need to be undertaken to develop cell models able to fully recapitulate aSyn-induced toxicity [227], the models currently used enabled us to advance in our understanding of the molecular basis of PD and other synucleinopathies.

3.2 Transgenic mouse models

Animal models of PD have improved our understanding of this pathology [243]. Each animal model has its own specific characteristics and limitations and it is important to keep in mind that the use of the most applicable one depends on the purpose and aim of each study.

Genetic mouse models of aSyn try to mimic relevant features of familial forms of PD, and assume that these share similar pathological mechanisms with those in idiopathic cases. Many of these models have provided valuable insight into the biological processes altered in PD. However, the most common motor features of the disease are not observed in many of these models [244]. For example, mice carrying a deletion of the gene encoding for aSyn only show minor phenotypes, such as synaptic dynamics changes, and no behavioral changes [135, 136].

Several aSyn transgenic models, where aSyn expression is driven by promoters such as PDGFß, Thy1, or PrP, have been developed [110, 245, 246]. All these models develop some behavioral phenotype and neuropathological features. Loss of striatal dopamine is one of the main characteristics found in the majority of aSyn transgenic mice [247, 248]. Among those, Thy1 models have been more extensively used since they recapitulate many PD features, such as astroglial and microglial activation [249]. Interestingly, Thy1 model also shows phosphorylated aSyn aggregates throughout the brain that are proteinase K resistant and develop mitochondrial dysfunction [250, 251]. Additionally, non-motor phenotypes are also described in this model, including cognitive disturbances, impaired olfactory dysfunction and disrupted circadian rhythms [250].

Other models expressing mutant aSyn, such as A30P or A53T have also been generated [110, 252]. These models display alterations in motor dysfunction, pathological

inclusions, striatal dopamine and neuroinflammation [110, 246, 252]. While most of aSyn transgenic mice do not show loss of dopaminergic neurons within the SN, models based on aSyn mutations, especially the doubly mutated (A30P/A53T) or truncated aSyn [244], display mild loss of these neurons. Moreover, WT aSyn Thy1 mice showed stronger motor phenotype compared to the other models, including A30P aSyn Thy1 transgenic mice (Table 2).

Table 2. Transgenic mouse models of synucleinopathy based on the expression of human aSyn.

Model	Promoter	Cells in which gene expressed	Corresponding human genetics	Dopaminergic neuron loss in SN	Striatal dopamine loss	aSyn aggregation in SN	Motor phenotype	Non-motor phenotype
WT SNCA	PDGFβ	Neurons (3-fold increase in expression)	Multiplication/poly morphisms	NP	Mild	Mild	Mild	Mild (cognitive)
WT SNCA	mThy-1	Neurons (2–3-fold increase in expression)	Multiplication/poly morphisms	NP	Moderate	Strong	Strong	Strong (olfactory); Moderate (cognitive, gastrointestinal and anxiety)
A53T SNCA	mThy-1	Neurons	Dominant mutation	NP	NP	NP	Moderate	-
A30P SNCA	mThy-1	Neurons	Dominant mutation	NP	NP	NP*	Moderate	Mild (cognitive); Moderate (anxiety)
A30P and A53T SNCA	hThy-1	Neurons	Dominant mutation	..	Moderate	Mild*	Moderate	-
A30P and A53T SNCA	Rat TH	TH+ neurons	Dominant mutation	Mild	Moderate	NP*	Mild	-
Truncated 1-120 SNCA	Rat TH	TH+ neurons	Found in brain of patients with PD	NP	Mild‡	Moderate	Mild	-

(adapted from Chesselet *et al*, 2001 [253])

It is not clear why these transgenic mice models do not recapitulate certain features of PD. One hypothesis might be the fact that the mice do not live enough to develop all PD features, as ageing is the major risk factor for the disease [254]. Another important aspect is the fact that we are only modulating aSyn expression in these mice, and LBs of PD patients have many other proteins, which might have also a relevant impact on the onset and progression of PD. Thus, the development and characterization of new animal models is essential for a greater understanding of PD-related mechanisms, paving the way for the identification of novel therapeutic strategies for the disease.

Aims of the study

The mechanisms underlying aSyn-induced toxicity and PD have been investigated over the years. Knowing the putative effect of aSyn on the modulation of potential pathogenic pathways, led us to focus our attention in the mechanisms associated with transcriptional deregulation. Thus, we aimed to investigate the potential mechanisms involved in WT and A30P aSyn-induced toxicity using cell-based and transgenic mouse models of PD. In this context, the major aims of my studies were to:

Aim 1. (**Publication I)** Investigate the impact of modulating aSyn expression on transcriptional deregulation in a dopaminergic cell line (LUHMES cells):

- Assess the effect of both WT and A30P aSyn in transcriptional deregulation and investigate the most affected pathways related to their expression;
- Determine the role of aSyn in acetylation of H3 and its relevance in neurotoxicity;
- Modulate aSyn-induced transcriptional deregulation using HDACi.

Aim 2. (**Publication II)** Investigate the role of aSyn in transcriptional deregulation in transgenic mouse models of PD:

- Investigate the differential gene regulation in both WT aSyn and A30P aSyn transgenic mice;
- Assess the common deregulated genes/pathways between aSyn transgenic mice and LUHMES cells;
- Identify and investigate the most deregulated pathways associated with aSyn expression;
- Investigate RNA splicing events and effect on miRNAs modulation by aSyn LUHMES cells and transgenic mice.

Publication I

Sodium butyrate rescues dopaminergic cells from alpha-synuclein-induced transcriptional deregulation and DNA damage.

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Original Article

ORIGINAL ARTICLE

Sodium butyrate rescues dopaminergic cells from alpha-synuclein-induced transcriptional deregulation and DNA damage

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Abstract

Alpha-synuclein (aSyn) is considered a major culprit in Parkinson's disease (PD) pathophysiology. However, the precise molecular function of the protein remains elusive. Recent evidence suggests that aSyn may play a role on transcription regulation, possibly by modulating the acetylation status of histones. Our study aimed at evaluating the impact of wild-type (WT) and mutant A30P aSyn on gene expression, in a dopaminergic neuronal cell model, and decipher potential mechanisms underlying aSyn-mediated transcriptional deregulation. We performed gene expression analysis using RNA-sequencing in Lund Human Mesencephalic (LUHMES) cells expressing endogenous (control) or increased levels of WT or A30P aSyn. Compared to control cells, cells expressing both aSyn variants exhibited robust changes in the expression of several genes, including downregulation of major genes involved in DNA repair. WT aSyn, unlike A30P aSyn, promoted DNA damage and increased levels of phosphorylated p53. In dopaminergic neuronal cells, increased aSyn expression led to reduced levels of acetylated histone 3. Importantly, treatment with sodium butyrate, a histone deacetylase inhibitor (HDACi), rescued WT

[†]The authors wish it to be known that, in their opinion, the first two authors should be regarded as joint First Authors.
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aSyn-induced DNA damage, possibly via upregulation of genes involved in DNA repair. Overall, our findings provide novel and compelling insight into the mechanisms associated with aSyn neurotoxicity in dopaminergic cells, which could be ameliorated with an HDACi. Future studies will be crucial to further validate these findings and to define novel possible targets for intervention in PD.

Introduction

Parkinson's disease (PD) is a complex neurodegenerative condition, associated with a broad range of motor (1) and non-motor symptoms (2). The precise molecular mechanisms leading to PD are still elusive, but mounting evidence suggests a crucial role for alpha-synuclein (aSyn), the main protein component of Lewy bodies, a pathological hallmark of the disorder (3). In addition, point mutations as well as multiplications in the gene encoding for aSyn are associated with familial forms of PD.

Since the association of aSyn with PD, the protein has been widely investigated, and has been linked to a multitude of cellular pathways, such as synaptic transmission, mitochondria homeostasis, and protein degradation, but there is still no consensus regarding its precise function. Recent reports suggest a possible role of aSyn on transcription regulation (4,5), a vital cellular mechanism found deregulated in PD patients (6,7) and in mouse models of the disease (8). However, the genes and pathways regulated by aSyn are also unclear. While some studies postulate that aSyn regulates gene expression in order to confer cellular protection (9,10), others report a deregulation of key pro-survival genes (11,12). Additionally, although deregulation of some genes is attributed to both wild-type (WT) and mutant aSyn (13,14), expression changes in other genes occur only in the presence of the WT or A30P aSyn (15). Thus, our primary goal was to assess the effect of increased levels of WT and A30P mutant aSyn on gene expression in dopaminergic cells.

Since aSyn was previously shown to modulate histone acetylation levels, we investigated whether this related to gene expression changes induced by aSyn. As we have recently reviewed (16), aSyn co-localizes with and binds to histones, in mice (17). In response to stress, increased levels of acetylated histone 3 (acH3) are observed in yeast expressing aSyn (18). In contrast, in SH-SY5Y cells and in transgenic flies, both WT and mutant aSyn promote histone 3 hypoacetylation and toxicity, which is reduced upon treatment with histone deacetylase inhibitors (HDACi) (19). HDAC inhibition is used as an efficient therapeutic strategy against cancer. In these cells, HDACi modulate gene expression, promote DNA damage and DNA repair impairment, and increase oxidative stress (20,21). Conversely, in neurons, HDACi confer protection against oxidative stress (20,21) and protect neurons in a mouse model of PD (22). Conflicting effects were found in dopaminergic neurons (23), thereby arguing for the great need for additional studies.

Our study explored the impact of WT and A30P aSyn on gene expression, in dopaminergic cells. We aimed at deciphering the mechanisms associated with aSyn-induced transcriptional deregulation and whether HDACi could be used to prevent neuronal toxicity associated with increased expression of aSyn.

We demonstrate that in Lund Human Mesencephalic (LUHMES) cells, a dopaminergic neuronal cell line (24), WT and A30P aSyn promoted extensive transcription deregulation. Interestingly, cells expressing aSyn exhibited a significant downregulation of key genes involved in DNA repair. We found that increased expression of WT aSyn induces DNA damage, which is not observed upon expression of A30P aSyn, possibly due to distinct biochemical properties of the protein. We also found that histone 3 hypoacetylation may be involved in aSyn-induced DNA damage. Treatment of dopaminergic neurons with the HDACi sodium butyrate (NaB) rescued WT aSyn expressing cells from DNA damage, possibly by restoring the expression of DNA-repair genes.

Our findings provide new insight into a putative role of aSyn in transcription deregulation, and suggest that treatment with HDACi may regulate gene expression changes related to DNA damage response. Additional studies will be crucial to further validate these findings and to define novel possible targets for intervention in PD.

Results

aSyn induces transcriptional deregulation in dopaminergic neurons

Eight days after differentiation naïve LUHMES cells were immunostained in order to verify the expression of neuronal dopaminergic markers. Microtubule-associated protein 2 (MAP2) and neuron-specific class III β-tubulin (TUJ1) staining revealed an extensive axonal/dendritic network, largely positive for tyrosine hydroxylase (TH). At this stage of differentiation, we also observed endogenous expression of aSyn (Fig. 1A). To investigate the impact of WT and A30P aSyn on gene expression, LUHMES cells were infected using equimolar concentrations of lentivirus encoding for WT aSyn-IRES-GFP, A30P aSyn-IRES-GFP or GFP, as a control (Fig. 1B). Each cell line was treated as described in Figure 1B. We then used fluorescence activated cell sorting to select the green fluorescent cells, resulting in highly homogeneous green-positive, TH-positive neuronal populations (Fig. 1C). The level of aSyn immunosignal was stronger in WT and A30P aSyn cells, and was distributed throughout the cell, including the soma and neurites (Fig. 1D). Using immunoblot analyses, we unequivocally confirmed that expression of aSyn was significantly higher in both WT and A30P aSyn, compared to control cells (Fig. 1F and G).

RNA was extracted from differentiated LUHMES cells stably expressing GFP, WT aSyn, or A30P aSyn, and processed in parallel to avoid bias induced by sample handling. The quality of the RNA was assessed using a Bioanalyzer before proceeding with library preparation and RNA-sequencing (RNA-seq). Extensive quality control of the sequencing data was performed prior to differential analysis.

We identified differential gene expression between control cells and those expressing WT or A30P aSyn (Supplementary Material, Tables S1 and S2). As expected, the gene encoding for aSyn, *SNCA*, exhibited the highest significance and fold-change (FC) increase in both comparisons [$\log_2$FC = 4.06, *p-adjusted* value (*padj*)=2.27E-228 for control cells versus WT aSyn; $\log_2$FC = 4.24, *padj* =2.52E-182 for control cells versus A30P aSyn]. FC scatter plots further confirmed that several genes were significantly deregulated in cells expressing WT aSyn, compared to control cells (Fig. 2A). An even more robust deregulation was observed in cells expressing A30P aSyn (Fig. 2A).

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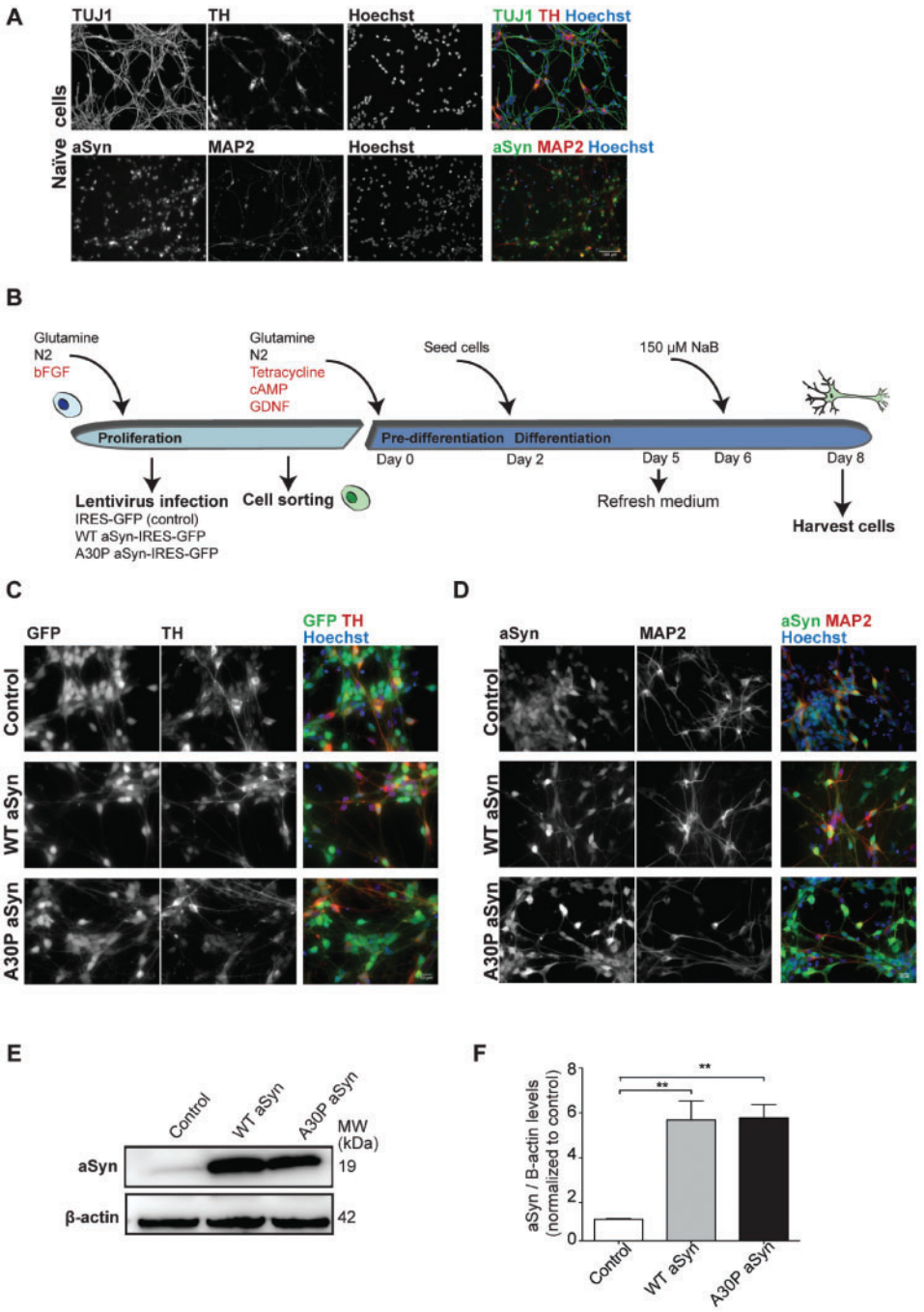

Figure 1. Study design and characterization of the three LUHMES cell lines used. LUHMES cells were differentiated as previously described [44]. Naïve cells grown on glass coverslips were immunostained for TUJ1, MAP2, TH or aSyn. Nuclei were stained with Hoechst. At day 8, differentiated cells exhibited an elaborate neurite network, strongly immunopositive for TH (**A**). Three cell lines were generated: control (IRES-GFP), WT, and A30P aSyn expressing LUHMES cells. Cells were treated according to the schematics and experiments were performed at differentiation day 8 (**B**). Differentiated LUHMES cells infected with viruses encoding for GFP, WT aSyn, or A30P aSyn, were immunostained for TH and co-stained with Hoechst. Images were acquired for each cell line showing highly homogeneous green-positive neuronal cells (**C**). aSyn was present both in the cell bodies and neurites, and stronger immunosignal was detected for WT and A30P aSyn cells (**D**). Immunoblot analyses were performed to assess the levels of aSyn protein in the three cell lines (**E**). Quantification of the immunoblot signals and immunostainings showed that WT and A30P aSyn expressing cells had significantly higher aSyn protein levels, compared to control cells (**F**). Data are expressed as mean ± SD of at least three replicates. One-way ANOVA, with Bonferroni correction, was used for statistical analysis with significance level of $P < 0.05$.

Considering $\log_2FC > 0.5$ and $padj < 0.01$, we detected 647 downregulated genes and 355 upregulated genes in cells expressing WT aSyn. In cells expressing A30P aSyn, we found 813 downregulated and 657 upregulated genes. Analysis of differentially expressed genes ($padj < 0.05$) between control cells and those expressing WT aSyn showed a significant overlap to those deregulated between control and A30P aSyn expressing cells. In particular, we identified 710 upregulated and 914 downregulated genes in both conditions (Fig. 2B). Furthermore, we found distinct expression profiles when comparing WT aSyn to A30P

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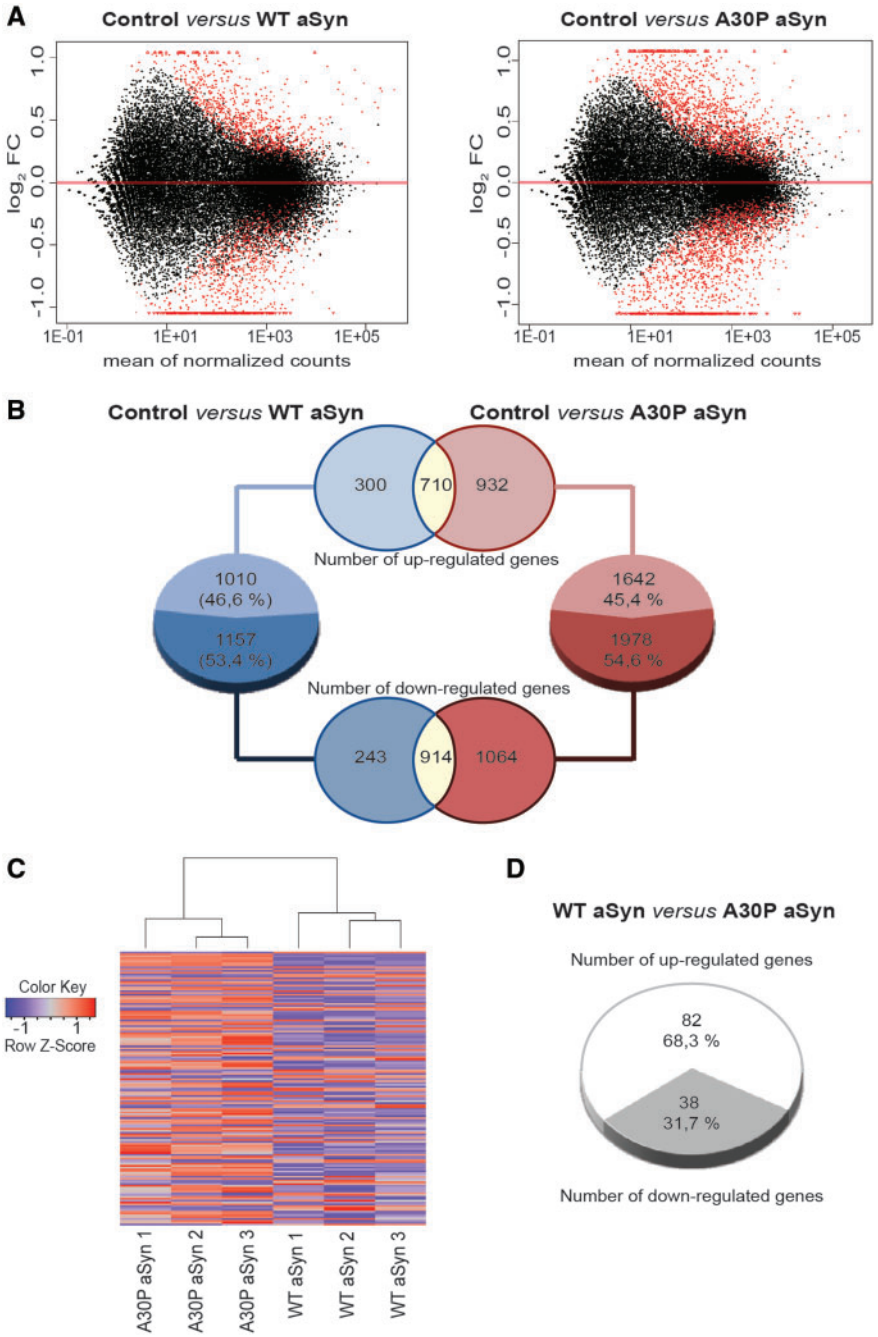

Figure 2. Gene expression changes associated with the expression of WT or A30P aSyn. (**A**) MA-plots were performed for differential gene expression data obtained when comparing RNA-seq data of cells expressing either WT or A30P aSyn. The log_2FC for each comparison is plotted on the y-axis and the average counts normalized by size factor is shown on the x-axis. The expression of aSyn promoted the differential expression of several genes with low expression levels. Each gene is represented with a dot. Genes with *padj* <0.01 are shown in red. (**B**) Pie-charts indicating the number of genes that are significantly (*padj* <0.05) up and downregulated upon expression of WT (blue) or A30P (red) aSyn, compared to control cells. Venn diagrams displaying the number of genes that are simultaneously upregulated (upper diagram) or downregulated (lower diagram) in both conditions. (**C**) Heatmaps of differentially expressed genes between WT and A30P aSyn. Color-spectrum codes the level of expression: blue color for low expression, and red color for high expression. Each column represents one independent experiment and each row one gene, in a total of 120 genes. (**D**) Pie chart showing how many genes are significantly up- and downregulated when comparing cells expressing WT aSyn versus A30P aSyn.

aSyn expressing cells (Fig. 2C). From a total of 120 differentially expressed genes, 82 were upregulated and 38 downregulated when comparing A30P aSyn to WT aSyn (Fig. 2D).

Consistently with previous studies (25), we observed that WT and A30P aSyn expression led to a significant reduction in the levels of NOTCH1. We also detected that NR4A2, the gene encoding Nurr1, was downregulated in both WT and A30P aSyn expressing cells, in agreement with other studies (12,26). However, the GDNF receptor alpha 2 was only significantly downregulated in cells expressing A30P aSyn. In these cells, we also detected significant downregulation of PRKCZ, but in contrast to previous reports (27), other members of the PRKC family, such as PRKCB (also known as Akt), PRKCE and PRKD1 where upregulated. Tyrosine 3-monooxygenase/tryptophan

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5-monooxygenase activation protein eta (14-3-3θ), downregulated upon aSyn overexpression (28), was downregulated in cells expressing A30P aSyn. Importantly, downregulation of the dopamine receptor (DAT, SLC6A3), a putative early event in PD (29), was observed in LUHMES cells expressing either WT or A30P aSyn.

aSyn overexpression downregulates DNA repair genes

In order to determine the molecular pathways specifically affected by aSyn expression, we performed Ingenuity Pathway Analysis (IPA). The 500 most significant genes were used, rendering a *padj* cut off of 1.5E-3 for control versus WT aSyn, and of 1.6E-6 for control versus A30P aSyn. From the 500 genes, 156 were upregulated and 344 were downregulated in WT aSyn-expressing cells. Similar numbers were observed for A30P aSyn-expressing cells: 157 up- and 343 downregulated genes. IPA analysis revealed that a high number of differentially expressed genes were associated with 'cell cycle', 'DNA damage checkpoint', and 'ATM signalling' in cells expressing WT or A30P aSyn (Fig. 3A and B).

Next, we performed additional network analysis of differentially deregulated genes using ToppGene Suite (Supplementary Material, Table S3). Upregulated and downregulated genes were analysed separately. Consistently with IPA analysis, the main biological processes downregulated upon expression of WT or A30P aSyn were 'cell cycle' and 'cellular response to DNA damage stimulus' (Supplementary Material, Table S3). On the other hand, analysis of upregulated pathways revealed differences between cells expressing WT and A30P aSyn: WT aSyn induced upregulation of genes involved in mitochondrial energy metabolism, whereas A30P aSyn induced upregulation of genes linked with neuron differentiation and synaptic transmission.

The effect of aSyn on DNA-repair associated genes was further corroborated by gene ontology analysis using David Functional Annotation database (Supplementary Material, Table S4). Protein association network analysis of genes involved in DNA repair, using STRING, revealed a strong interaction between DNA-damage response molecules (Fig. 4A and B). A large number of DNA repair-associated genes was downregulated in both WT and A30P aSyn expressing cells (genes in red), whereas, approximately, one-third of the genes were exclusive to one of the conditions. Three of the most downregulated DNA repair-associated genes (BRCA2, TOP2A and FOXM1) were selected for further validation by real-time PCR (qPCR). Differential expression analysis from the RNA-seq results showed that TOP2A exhibited the strongest downregulation in both WT (Fig. 4C) and A30P aSyn expressing cells (Fig. 4D). The analysis by qPCR confirmed a significant downregulation of these genes in cells expressing WT aSyn, but only a trend, that did not reach significance, for BRCA2 and FOXM1 in A30P aSyn cells (Fig. 4E).

WT, but not A30P aSyn, induces DNA damage and impairs miROS handling in dopaminergic neurons

To investigate whether WT and A30P aSyn promoted DNA damage, we performed comet assay analysis in order to detect DNA single-strand breaks, double-strand breaks, and alkali-labile lesions. Tail moment analysis demonstrated that cells expressing WT aSyn displayed the highest tail moment compared to control and those expressing A30P mutant (Fig. 5A and B). To further characterize the DNA damage induced by aSyn, we assessed the levels of phosphorylated H_2AX (p-H_2AX) and phosphorylated p53 (p-p53) using immunoblot analyses. Consistently, we found that only cells expressing WT aSyn displayed increased levels of p-p53 compared to the cells. Curiously, no significant changes were observed in the levels of p-H_2AX (Fig. 5C and D).

Damage of genomic DNA may occur in the presence of high levels of reactive oxygen species (ROS) (30). Thus, we asked whether the DNA damage was associated with the levels of mitochondrial ROS (miROS). Using MitoSOX probe, we found no differences between the three cell lines at basal levels. Thus, we investigated whether stressing the cells with 5% H_2O_2, as a 'second hit', would unbalance cells and expose differences in ROS handling. Indeed, 30 min after exposure to H_2O_2, cells expressing WT aSyn showed a significant increase in miROS production compared to control. No significant differences were detected for cells expressing A30P aSyn (Fig. 5E). To assess overall redox changes in the cell, we quantified the levels of oxidized 2′,7′-dichlorofluorescein diacetate (DCFDA). No significant differences were observed between the three cell lines (Fig. 5F), suggesting the effect was stronger at the level of mitochondria. Since mitochondria are key modulators of apoptotic pathways, we investigated the impact of aSyn on the activation of caspase 3, a key player in apoptosis. We found that both the WT and A30P aSyn significantly increased the percentage of cells immunopositive for cleaved caspase 3 (Fig. 5G). Interestingly, we found no significant differences in cytoplasmic membrane integrity between the three cell lines, suggesting that miROS alterations and activation of apoptosis may be early effects of increased levels of expression of WT aSyn, which do not directly result in strong cytotoxicity (Fig. 5H).

Sodium butyrate alleviates WT aSyn-induced DNA damage

It has been suggested that aSyn may impact on gene transcription via modulation of acH3. Thus, we next investigated the levels of acH3 in whole cell lysates (Fig. 6A). Immunoblot analysis demonstrated a highly significant reduction in the levels of acH3 in cells expressing WT aSyn. A milder, but also significant, effect was observed in cells expressing A30P aSyn (Fig. 6B). We then asked whether modulation of histone deacetylase activity, using NaB, would revert the reduction in the levels of acH3 induced by aSyn. After determining the optimal concentration of NaB (150 μM, Fig. 6C), we treated the three cell lines with the compound for 48 h and observed an increase in the levels of acH3 levels in the presence of aSyn, but only significant in the presence of the WT aSyn (Fig. 6D and E).

Given the strong effect observed, we then asked whether NaB could rescue cells from DNA damage induced by aSyn, and performed comet-assay analysis. NaB treatment led to a reduction on tail moment values in all the conditions, but the effect was particularly striking in WT aSyn LUHMES cells (Fig. 7A and B). Additionally, we also found a significant reduction in the levels of p-p53 in cells expressing WT aSyn (Fig. 7C and 7D), and a reduction in miROS, compared to the control cells (similar to Fig. 7E).

Next, to assess whether the protective effects of NaB would derive from modulation of gene expression, we investigated the levels of the three DNA-repair genes selected above using qPCR. Strikingly, we verified that NaB restored FOXM1 and BRCA2 expression levels in cells expressing WT aSyn. Curiously, the levels of FOXM1 in cells expressing WT aSyn and treated with NaB

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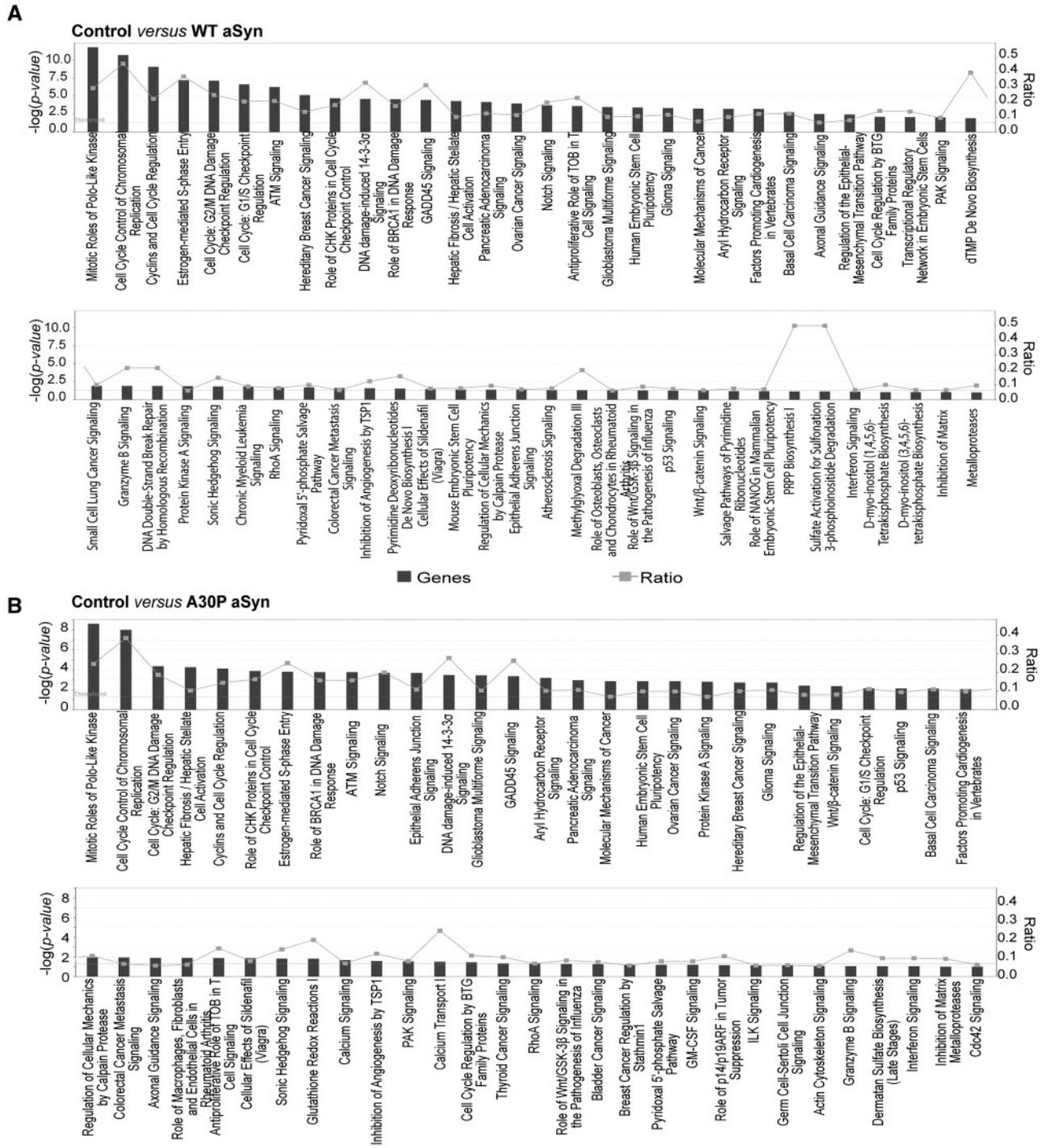

Figure 3. Ingenuity pathway analysis (IPA) of RNA-seq data comparing gene expression profiles of cells expressing WT or A30P aSyn. IPA graphs showing the predominant canonical pathways of differentially expressed genes in cells expressing the WT (**A**) or A30P (**B**) aSyn, compared to control cells. The ratio (gray line) indicates the number of genes within each pathway divided by the total number of genes in the canonical pathway. IPA showed that both WT and A30P aSyn impacts the expression of genes associated with a multitude of pathways, suggesting a pivotal role of aSyn in the expression of genes associated with cell cycle and DNA damage checkpoints.

were significantly higher than those in cells expressing A30P aSyn. Although we found also increase in the levels of TOP2A upon NaB treatment, this did not reach significance. The apparent increase in the levels of TOP2A upon NaB treatment in cells expressing WT or A30P aSyn did not reach statistical significance; indeed, the only significant effect was the reduction in TOP2A levels in control cells, suggesting there might have been a protective effect in cells expressing aSyn (Fig. 7F).

Finally, to assess the overall protective effect of NaB on cellular pathologies associated with PD, we used a different model based on the treatment of naïve LUHMES cells with the neurotoxin 1-methyl-4-phenylpyridinium (MPP^+), a known inducer of

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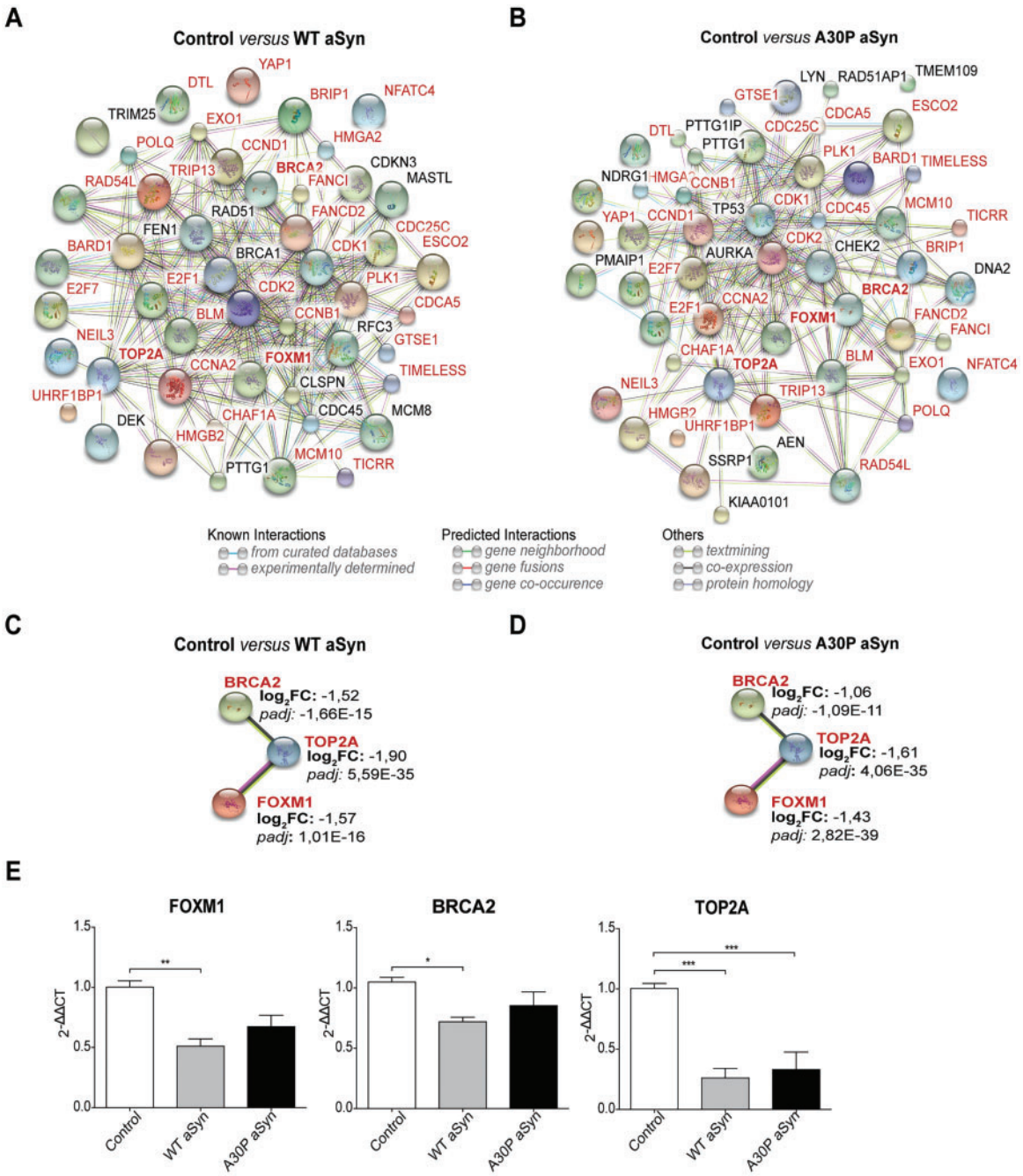

Figure 4. Downregulation of DNA repair-associated genes in dopaminergic cells expressing WT or A30P aSyn. STRING protein network analysis of DNA repair genes downregulated in WT (**A**) or A30P (**B**) expressing cells, compared to control cells. Approximately two-thirds of the genes are deregulated in both conditions (genes in red). Genes exhibiting highly significant downregulation in WT (**C**) and A30P (**D**) aSyn expressing cells were selected for qPCR validation (**E**). qPCR analysis confirmed a strong deregulation of TOP2A in both WT and A30P aSyn expressing cells, while FOXM1 and BRCA2 were only significantly downregulated in cells expressing WT aSyn. Data are expressed as mean ± SD of at least three replicates. One-way ANOVA, with Bonferroni correction, was used for statistical analysis with significance level of $P < 0.05$.

cellular changes characteristic of PD, including oxidative stress and DNA fragmentation (31). Importantly, we confirmed the protective effects of NaB in naïve LUHMES cells treated with MPP^+. Since the toxin is known to induce strong gene expression changes, we focused on the rescue of cytotoxicity. TUJ1 staining revealed a strong rescue of neurite integrity (Supplementary Material, Fig. S1A), and of membrane integrity, as measured by the adenylate kinase (AK)-release assay (Supplementary Material, Fig. S1B), upon NaB treatment in cells exposed to MPP^+.

Discussion

Our study provides new evidence for a role of aSyn on transcription deregulation in dopaminergic cells, and uncovers new pathways underlying aSyn toxicity. Both WT and A30P aSyn

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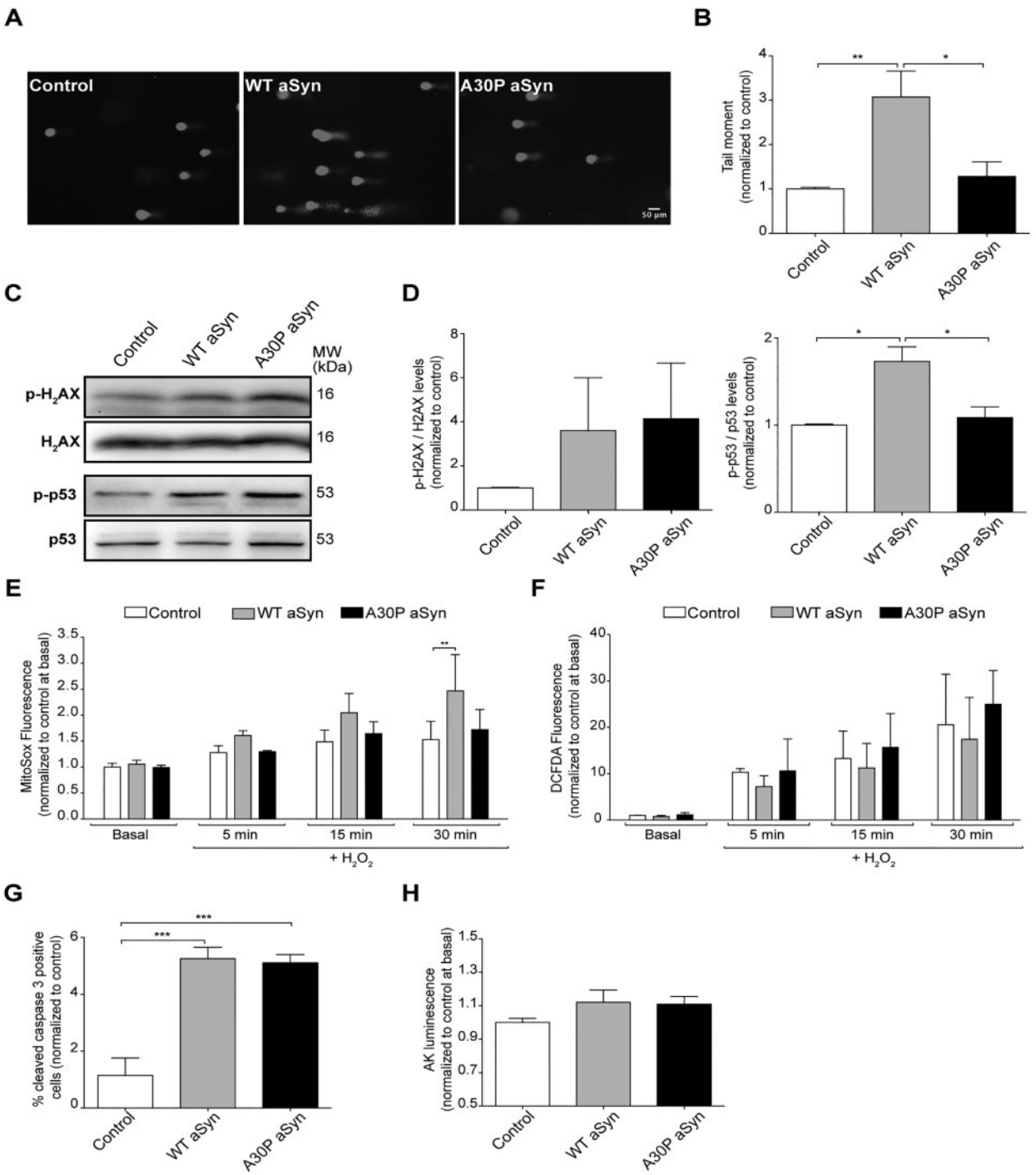

Figure 5. WT aSyn induces DNA damage and impairs miROS handling. The comet assay alkali method was used to detect DNA single-strand breaks, double-strand breaks, and alkali-labile lesions. Following PI staining, comets were imaged using epifluorescence microscopy. Representative images are presented for each condition (**A**). Tail moments were calculated using the Comet Score software. Statistical analysis showed that tail moments from cells expressing WT aSyn were significantly higher than in control or A30P aSyn expressing cells (**B**). Levels of p-H2AX and p-p53 were analysed by immunoblot analyses (**C**). Quantification of the immunoblots confirmed a trend towards an increase of p-H2AX levels, and a significant increase in the levels of p-p53 (**D**). miROS were measured using MitoSOX Red. At basal levels, no differences in miROS between the three cell lines were observed. About 30 min after treatment with 5% H_2O_2, miROs levels were significantly higher in LUHMES cells expressing WT aSyn (**E**). Similar experiments performed with DCFDA revealed no significant differences in the total cellular levels of ROS (**F**). The percentage of cleaved caspase 3 immunopositive cells was calculated. (**G**). Bioluminescence measurement of AK performed with Toxilight assay showed no differences in cytotoxicity between the cell lines (**H**). Data are expressed as means ± SD of at least three replicates. One-way ANOVA (B and D) or two-way ANOVA (E and F), with Bonferroni correction, was used for statistical analysis with significance level of $P < 0.05$.

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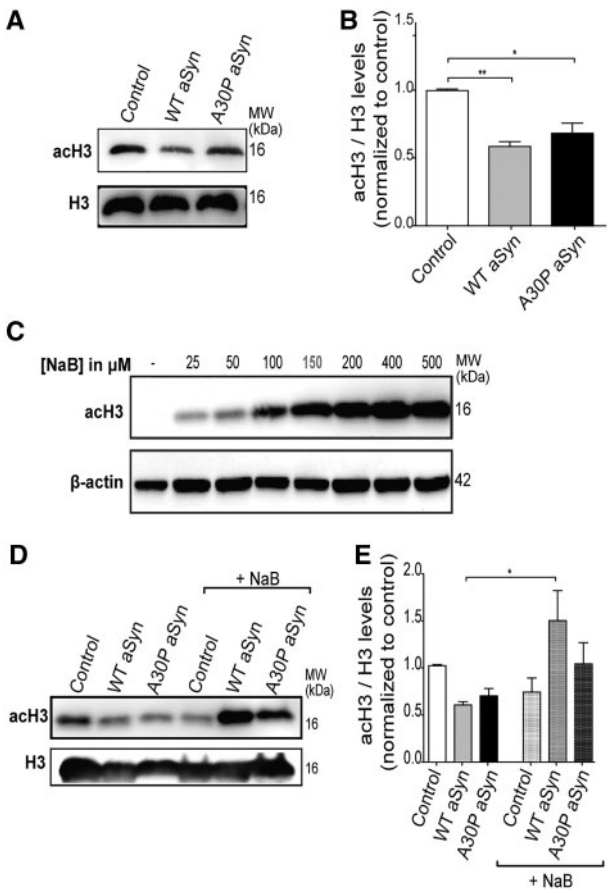

Figure 6. aSyn-mediated reduction in the levels of acH3 is rescued NaB. An evident reduction of acH3 levels (lysine 9) was observed in cells expressing either WT or A30P aSyn (**A**). Quantification of the immunoblots revealed a significant decrease of acH3 levels in cells expressing the WT aSyn, and a more modest, although still significant, reduction in cells expressing A30P aSyn (**B**). acH3 levels were assessed in cells treated with different concentrations of NaB, for 48 h. An evident increase in acH3 levels was already observed for cells treated with 150 µM NaB (**C**). The three cell lines exhibited higher levels of acH3 upon treatment with 150 µM NaB for 48 h (**D**). This effect was particularly strong in cells expressing WT aSyn (**E**). Data are expressed as mean ± SD of at least three replicates. One-way ANOVA, with Bonferroni correction, was used for statistical analysis with significance level of $P < 0.05$.

downregulated the expression of genes involved in DNA repair, which we suggest may be an early indication of toxicity promoted by aSyn. Interestingly, we identified effects induced by WT aSyn but not by the A30P mutant, underlining their distinct biochemical and biological properties (Fig. 8A–F). Finally, we demonstrated that treatment with the general HDACi NaB ameliorated aSyn-induced transcription deregulation and prevented DNA damage (Fig. 8G and H).

The interplay between transcription deregulation and neurodegenerative diseases has been a subject of interest in the last decade (7,32,33). However, the involvement of aSyn on transcription was only recently suggested (4,5). Through RNA-seq analyses of dopaminergic LUHMES cells infected with viruses encoding for either WT or A30P mutant aSyn, we evaluated the impact of these aSyn variants on transcription. We observed a distinct and highly significant transcription deregulation in aSyn expressing cells. Our results are in line with previous findings, where genes such as NR4A2 (12,26), NOTCH1 (34) and 14-3-3θ (28) were identified. However, we now identify novel pathways that shed new light into our understanding of the effects of aSyn on gene expression. In our dopaminergic cell model, most downregulated genes were involved in cell cycle and DNA repair. Two previous studies reported that aSyn can act as a cell cycle regulator (35) and is an important player on the initiation of DNA replication (18). A close link between unrepaired DNA damage and neurodegeneration process was recently suggested (36,37). Although mitochondrial impairment and oxidative stress are common causes of DNA damage in neurons, deregulation of DNA repair genes may constitute the tipping point. In Alzheimer's Disease, p53 and BRCA1, a crucial gene in DNA repair that was also downregulated in our WT aSyn cells, have been recently put forward as central players in the disease pathogenesis and are now considered promising therapeutic targets (38). In PD, mutations in ATM, a gene encoding for an important protein involved in DNA repair, are a risk factor (39). Interestingly, we have also recently discovered that alterations in DNA repair genes may be linked to PD progression (7). Last year, it was also shown that Ercc1 deficient mice, having impaired DNA excision repair, displayed typical PD-like pathological alterations, such as decreased striatal dopaminergic innervation, defects in mitochondrial respiration, and increased levels of phosphorylated aSyn (40). In differentiated neuroblastoma cells, aSyn expression led to increased DNA damage upon exposure to Fe(II) (41). However, to the best of our knowledge, aSyn has not been previously associated with transcriptional deficits on DNA repair genes.

We selected three major genes involved in DNA repair (FOXM1, BRCA2 and TOP2A), and validated the expression changes observed in the RNA-seq experiments by qPCR. Consistently, the expression levels of all three genes were significantly lower in cells expressing WT aSyn. In cells expressing A30P aSyn, the expression of TOP2A was significantly lower, but for FOXM1 and BRCA2 we observed only a slight downregulation. We then used a combination of established reporters for DNA damage to understand how aSyn-induced transcription deregulation impacts on cell viability and, specifically, on DNA damage. Increased tail-moment values, measured via comet assay (42), and p-p53 levels confirmed DNA damage in cells expressing WT aSyn. Surprisingly, we did not detect a significant increase on p-H_2AX levels, suggesting that H_2AX-independent DNA damage mechanisms might be taking place.

aSyn is involved in mitochondrial dysfunction, a molecular mechanism implicated in the onset and progression of PD (43,44). Although the localization of aSyn in mitochondria is still not consensual, overexpression of aSyn promotes several mitochondrial abnormalities, including decreased complex I activity and unbalanced miROS handling, resulting in increased oxidative stress and activation of apoptosis (45–48). The nuclear genome is susceptible to damage by increased levels of ROS generated during mitochondrial respiration or due to exposure to exogenous toxins (30). While we did not observe differences in total ROS levels, we detected a significant increase of miROS in cells expressing WT aSyn treated with H_2O_2, suggesting that aSyn causes impairs the handling of ROS species, specifically at the mitochondrial level. Although it is possible that other mechanisms also play a role in aSyn-induced DNA damage, the impact of aSyn on miROS handling may be a major culprit in this process. Future studies, addressing the effect of both WT and mutant aSyn on mitochondrial homeostasis and morphology, will be important to elucidate the mechanisms associated with the impact of aSyn on mitochondrial biology.

In the event of increased DNA damage (e.g. due to increased oxidative stress), the preferred survival strategy of a cell is to

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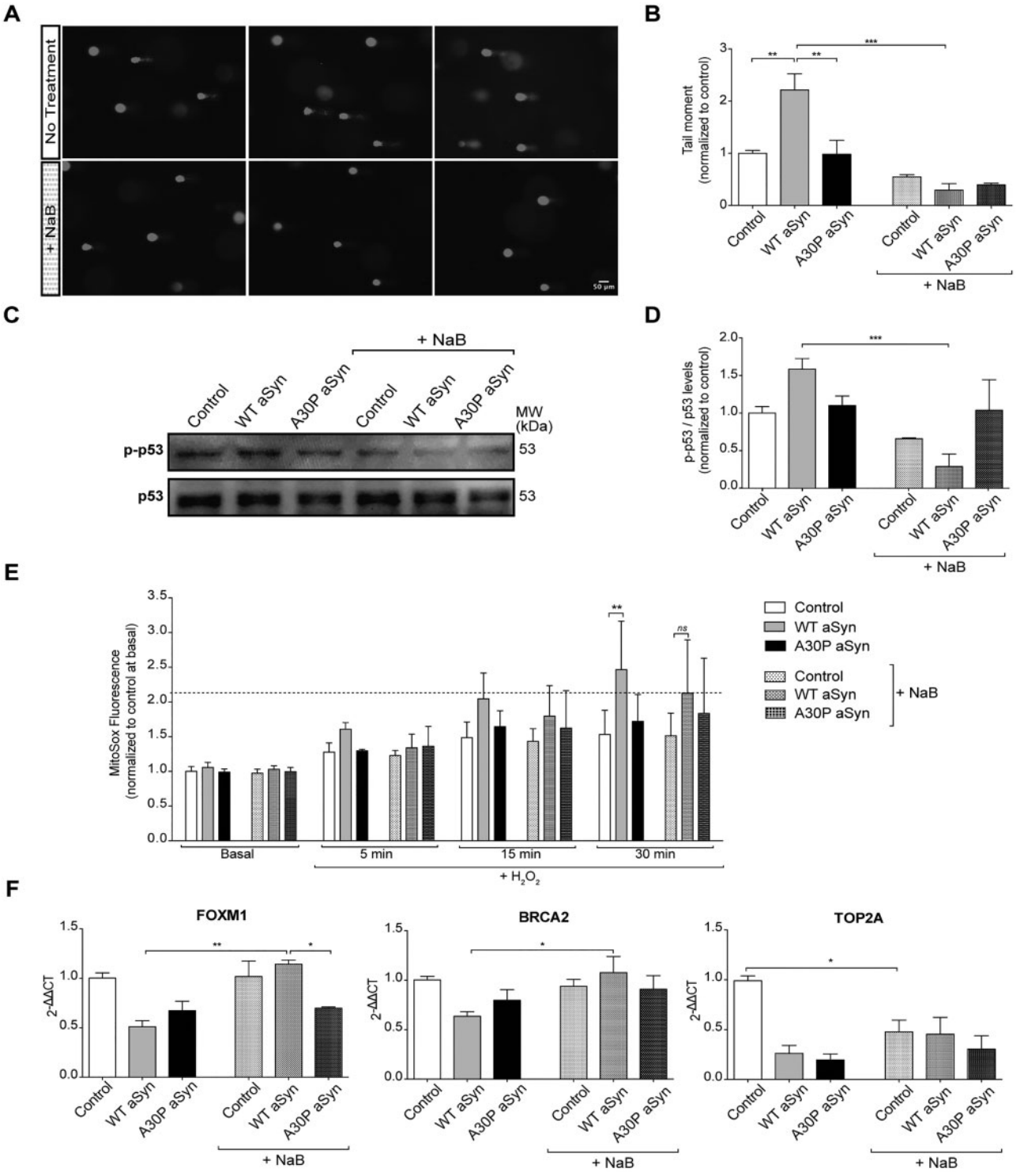

Figure 7. NaB rescues the DNA damage induced by WT aSyn expression. Representative images of comets obtained using the alkaline comet assay are shown for each condition (A). Comet assay analysis showed a significant decrease in tail moment in cells expressing WT aSyn after treatment with 150 µM of NaB (B). Levels of p-p53 were measured using immunoblot analysis (C). Quantification of the immunoblots showed a reduction in the levels of p-p53 in WT aSyn expressing cells treated with NaB (D). Assessment of miROS using MitoSOX Red indicated that the levels of miROS in cells expressing WT aSyn treated with H_2O_2 were reduced upon treatment with 150 µM NaB (E). qPCR analysis of DNA repair-associated genes upon treatment with 150 µM NaB showed the normalization of the expression FOXM1 and BRCA2 in cells expressing WT aSyn (F). Data are expressed as mean ± SD of at least three replicates. One-way ANOVA (B, C and E) or two-way ANOVA (E and F), with Bonferroni correction, were used for statistical analysis with significance level of $P < 0.05$.

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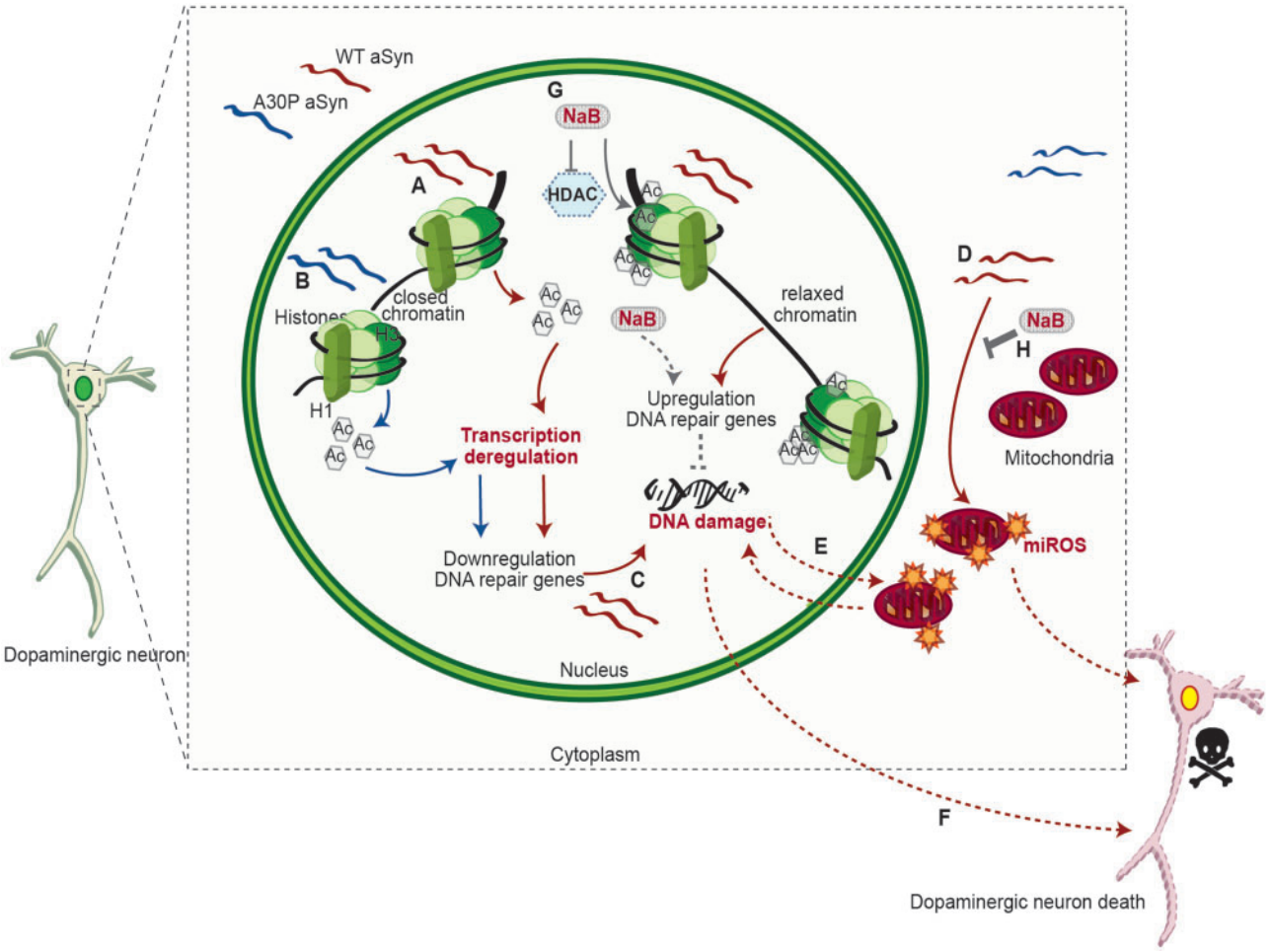

Figure 8. Proposed model for the neurotoxicity associated with increased levels of expression of aSyn in dopaminergic cells. Increased levels of WT (**A**) or A30P aSyn (**B**) reduce the levels acH3. H3 hypoacetylation causes chromatin condensation and transcription repression. Thus, we propose that altered acH3 levels may be, at least partially, involved in the transcriptional deregulation mediated by WT (**C**) or A30P aSyn (**D**) in dopaminergic neurons. Gene expression analysis revealed downregulation of genes involved in DNA repair in cells expressing both aSyn variants. Interestingly, our data indicate that only WT aSyn promotes DNA damage in these neuronal cells (**C**), highlighting a distinct biological effect of the two aSyn variants. Although several mechanisms may underlie these differences, the negative impact of WT aSyn on mitochondrial ROS handling (**D**) represents an additional source of DNA damage (**E**). We propose that these alterations may be early effects of increased levels of WT aSyn (**F**). Excitingly, increasing acH3 levels, via NaB treatment, rescued dopaminergic neurons from WT aSyn-induced DNA damage and restored the expression levels of specific DNA repair genes (**G**). In addition to the effect on transcription, NaB improved the handling of ROS (**H**), which further contributes to alleviate the neurotoxic effects promoted by WT aSyn.

activate cell cycle arrest and a complex set of DNA repair pathways (49). However, as WT aSyn leads to a downregulation of DNA repair genes, damage to DNA becomes excessive and, ultimately, may lead to apoptosis. Consistently with previous reports showing that aSyn overexpression activates apoptosis (48,50), we found an increase in the percentage of cells that are positive for cleaved caspase 3. Interestingly, activation of apoptosis was also induced in cells expressing A30P aSyn, although no changes in DNA integrity and miROS species handling were detected. However, the A30P aSyn mutant displays different biochemical properties than WT aSyn, such as differential affinity to bind membranes (51), and reduced cytotoxicity in yeast models (52), producing distinct effects on cells. Our results confirm the idea that aSyn mutants might affect cellular homeostasis via distinct mechanisms. On the other hand, since mitochondria are key modulators of apoptosis (53), these results further corroborate the hypothesis that, at least in our model, aSyn may impact mitochondrial function. Future studies will be necessary to further understand whether other mechanisms are underpinning aSyn-induced DNA damage and toxicity.

Importantly, we did not observe significant alterations in cytoplasmic membrane integrity upon aSyn expression. This suggests that transcriptional deregulation and DNA damage may be early events in the cascade of aSyn-induced neurotoxicity, which helps us delineate the order of the cascade of events involved.

We then investigated how aSyn promotes transcription deregulation and whether it could be reversed, as this would have strong interest for therapeutic purposes. aSyn was previously shown to interact with DNA (14) and to regulate acH3 levels (17–19). Post-translational modifications of histones are essential in the regulation of transcription activation/repression, DNA replication, and repair of DNA damage (20). Consistently with previous findings (19), we found that increased levels of aSyn significantly reduce the levels of acH3. This effect was particularly strong in cells expressing WT aSyn. This prompted us to

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test whether increasing acH3 levels, using HDACi, could be a useful strategy for mitigating aSyn-induced cellular pathologies. Intriguingly, the effect of NaB on acH3 levels were only significant in WT aSyn-expressing cells, suggesting that the mechanism of action may be different than that observed in cells expressing A30P aSyn. The beneficial effects of HDAC inhibition are still controversial, as both neuroprotective (22) and neurotoxic (23) effects were reported. NaB is a short chain fatty acid that preferentially affects HDACs from classes I and IIa and other non-histone targets.

Our study showed that treatment of dopaminergic cells expressing WT aSyn with NaB strongly reduces DNA damage, as assessed by comet assay and levels of p-p53. We also observed a reduction in the levels of miROS. Interestingly, previous studies showed mitochondrial function improvement upon treatment with NaB in Huntington's disease models (54).

In the context of cancer, HDACi are used to promote DNA damage through transcription downregulation, impairment of DNA repair proteins, and increase in oxidative stress. In contrast, HDACi confer protection against oxidative stress in neuronal cells (20,21). In our model, in addition to the effect on miROS handling, NaB appears to modulate the transcription of genes involved DNA repair. When we evaluated the expression of major genes involved in DNA repair, we found that protection by NaB involves the restoration of the levels of expression of DNA repair genes, including FOXM1 and BRCA2. Interestingly, the effect of NaB on FOXM1 was significantly stronger in WT aSyn versus A30P aSyn-expressing cells, further corroborating our hypothesis that NaB may act via distinct mechanisms on cells expressing WT and A30P mutant aSyn. Altogether, these findings suggest that WT aSyn may cause transcriptional deregulation and cytotoxicity via chromatin remodeling, and that this can potentially be rescued by increasing histone acetylation levels (eg. treatment with HDACi). On the other hand, although A30P aSyn also promotes histone 3 hypoacetylation, it is likely that other molecular events are also associated with its cytoxicity.

Given the beneficial effects of NaB on aSyn-mediated DNA damage, we asked whether this compound was also protective on a distinct model of PD, also characterized by mitochondrial impairment, increased oxidative stress and DNA fragmentation (31). Importantly, we observed a clear neuroprotective effect of NaB in naïve LUHMES cells exposed to the neurotoxin MPP^+.

In conclusion, although several questions demand additional investigations, we provide novel evidence suggesting that transcriptional deregulation and nuclear DNA damage are connected with the toxicity of increased levels of aSyn in dopaminergic cells, opening novel avenues for the development of future intervention strategies in PD.

Materials and Methods

Cell culture

LUHMES cells were maintained and differentiated as previously described (24). Briefly, cells were cultured in flasks (Corning) pre-coated with 50 μg/mL poly-L-ornithine and 1 μg/mL fibronectin (Sigma–Aldrich), and grown at 37°C in a humidified 5% CO_2 atmosphere. Advanced Dulbecco's modified Eagle's medium/F12 (DMEM/F12, Gibco) supplemented with 1× N2 (Gibco), 2 mM L-glutamine (Gibco) and 40 ng/mL recombinant basic fibroblast growth factor (bFGF, R&D Systems) was used to maintain proliferating LUHMES cells. Differentiation was achieved by replacing the proliferating medium with DMEM/F12 supplemented with 1× N2, 2 mM L-glutamine, 1 mM dibutyryl cAMP (cAMP, Sigma Aldrich), 1 μg/mL tetracyclin (Sigma–Aldrich) and 2 ng/mL recombinant human GDNF (R&D Systems). Two days after adding differentiation medium, cells were trypsinized [(138 mM NaCl, 5.4 mM KCl, 6.9 mM NaHCO3, 5.6 mM D-Glucose, 0.54 mM EDTA, 0.5 g/L trypsin from bovine pancreas type-II-S (Sigma–Aldrich)] and seeded into poly-L-ornithine/fibronectin pre-coated plates (Corning) or glass coverslips. On day 5, one-third of the medium was refreshed. Experiments were performed 8 days after differentiation. A schematic representation of the overall differentiation process is provided on Figure 1A.

Generation of aSyn expressing LUHMES cells

Full-length human aSyn c-DNA (*SNCA*, NM_000345) or A30P aSyn were subcloned into a second generation bicistronic lentiviral vector, pWPI (Tronolab, Switzerland), under the chicken/β-actin (CBA) promoter. All cloned sequences were verified by direct-sequencing (StarSeq). pWPI vector containing only IRES-GFP cassette was used as infection control in all the experiments. Second-generation lentiviral particles were generated as previously described (55). Briefly, 293T cells were transiently co-transfected with the modified transfer vector plasmids and second-generation packing system (Tronolab). Supernatant was collected 48-h post-transfection, concentrated by PEG-it Virus Precipitation Solution (System Bioscience) and resuspended in Panserin 402 (PAN). Lentiviral vector titers were quantitatively measured by qPCR using primer sequences specific for the woodchuck hepatitis virus post-transcriptional regulatory element (WPRE), as previously described (56). To generate cells expressing aSyn, proliferating LUHMES cells were infected with equimolar amounts of IRES-GFP, WT aSyn-IRES-GFP or A30P aSyn-IRES-GFP lentivirus. Positive green fluorescent cells were selected by fluorescence activated cell sorting (Calibur flow cytometer, BD Biosciences).

Sodium butyrate treatment

The concentration of NaB (Sigma) was determined by treating differentiated LUHMES cells with increasing concentrations (0–500 μM) of the drug. NaB was dissolved in DMEM/F12 medium and added to LUHMES cells on differentiation day 6, for 48 h. On differentiation day 8, cells were harvested for western-blot analysis of acH3 levels. For subsequent experiments, cells were treated on day 6 with 150 μM NaB (Fig. 1A), as it represented the lower NaB concentration leading to a high increase of H3 acetylation.

Treatment with MPP^+

LUHMES cells were treated with 2.5 μM MPP^+, dissolved in water, on day 5 of differentiation. Subsequent experiments were performed 3 days after starting the treatment (Fig. 1A).

Cytotoxicity assay

Cell viability was assessed by quantitatively measuring AK content in the supernatants of cells. ToxiLight bioassay kit (Lonza) was used according to the manufacture recommendations.

Immunocytochemistry

Cells on coverslips were fixed with 4% paraformaldehyde, for 15 min, at room temperature (RT). About 1× saline phosphate

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buffer (PBS) was used to wash the cells before permeabilization with 0.5% Triton/PBS, for 15 min at RT. After blocking with 3% BSA/PBS for 1 h at RT, cells were incubated, overnight at 4°C with primary antibodies diluted in blocking solution: anti-TUJ1 1:3000 (Covance, mouse); anti-TH 1:1000 (Millipore, rabbit); anti-MAP2 1:1000 (Abcam, rabbit), anti-aSyn 1:2000 (BD Transduction, mouse) and anti-cleaved caspase 3 1:2000 (Cell Signaling). Cells were washed with 1× PBS before incubation with secondary Alexa Fluor antibodies (rabbit or mouse, 488 or 555, Life Technologies), prepared at 1:10 000 in blocking buffer, for 2 h at RT. Before mounting the coverslips with Moviol, nuclei were stained with Hoechst. Immunofluorescence images were acquired using an epifluorescence microscope (Leica DMI 6000B microscope, Leica). To assess the percentage of cells displaying cleaved caspase 3, images were acquired on Olympus IX81-ZDC microscope system, using a 40× magnification objective. The percentage of cleaved caspase 3 positive cells was calculated based on the total number of cells (DAPI). For each cell line, at least 150 cells ($N \geq 167$) were counted from seven or eight different optical fields. Cells were manually counted using Image J software. The relative amount of cleaved caspase 3 positive cells was calculated by normalizing to control cells expressing GFP.

Western blot

For protein analysis, differentiated LUHMES cells were incubated with RIPA buffer [0.1% Triton X-100, 0.15 M NaCl, 50 mM Tris pH 7.5 and a protease inhibitor cocktail tablet (Roche)], on ice, for 30 min. Lysates where then collected, sonicated on ice for 10 s at 40–50% power, and centrifuged at 14 000 rpm for 30 min, at 4°C. Protein concentration was determined using the Bradford assay (BioRad), in an Infinite M200 PRO plate reader (Tecan Lta). Total lysates (40–60 μg) were mixed with 5× Laemmli buffer (250 mM Tris pH 6.8, 10% sodium dodecyl sulfate, 1.25% Bromophenol Blue, 5% β-mercaptoethanol, 50% glycerol), boiled at 95°C for 5 min, and loaded on 12% sodium dodecyl sulfate polyacrylamide (SDS–PAGE) gels. After electrophoresis, proteins were transferred on a Trans-blot Turbo system to either nitrocellulose or PVDF membranes (Trans-blot Turbo Nitrocellulose/PVDF membranes, BioRad). About 5% skim milk, prepared with 1× Tris Base Solution/0.05% Tween (TBST), was used for blocking. After 1 h at RT, membranes were incubated overnight at 4°C with primary antibodies diluted in 4% BSA/TBST. The following antibodies and dilutions were used: anti-aSyn 1:2000 (BD Transduction, mouse); anti-p-p53 Ser46 1:1000 (Cell Signalling, rabbit), anti-p53 1:1000 (Cell Signalling, rabbit); anti-p-H_2AX 1:1000 (Cell Signalling, rabbit); anti-H_2AX 1:1000 (Cell Signalling, rabbit); anti-acH3K9 1:2000 (Millipore, rabbit); anti-H3 1:5000 (abcam, rabbit) and β-actin 1:5000 (Sigma, mouse). Membranes were then incubated with horseradish peroxidase-conjugated secondary antibodies 1:10 000 (mouse or rabbit, GE Healthcare), for 2 h at RT. Between steps, membranes were washed 3 times with TBST, for 5 min at RT. Chemiluminescent signals on the blots were detected using Immobilon Western Chemiluminescent HRP Substrate (Millipore Corporation) and Fusion FX (Vilber Lourmat). Quantification of bands was performed using Image J software.

RNA extraction

Total RNA from differentiated LUHMES cells was extracted and purified using the RNeasy mini kit (Qiagen), according to the instructions of the manufacturer. Three biological replicates were used for RNA-seq experiments. Sequencing and RNA quality analysis was performed as described previously (57). In brief, RNA quality was assessed using RNA 6000 Nano chips run on a 2100 Bioanalyzer (Agilent). Libraries were prepared using the TruSeq RNA Sample Preparation v2 kit (Illumina). The library quality was checked using High Sensitivity DNA chips on a Bioanalyzer. The sample concentration was measured with a Qubit dsDNA HS Assay Kit and adjusted to 2 nM before sequencing (50 bp) on a HiSeq 2000 (Illumina) according to the manufacturer's instructions.

Differential gene expression analysis

The differential gene expression analysis was performed as previously described (57). Briefly, RNA-seq data were aligned to the genome using STAR with default options, generating mapping files (BAM format). Differential expression Read counts were generated using FeaturesCount (http://bioinf.wehi.edu.au/featureCounts/) and samples were compared for differential expression using DESeq2 (58). Genes with a P-value ≤ 0.05 and a mean read count ≥ 10 were considered to be differentially expressed.

Networks analysis

Additional network analysis was performed for the 500 most significant genes using the bioinformatics tool ToppGene Suite (59). For this analysis, we considered upregulated and downregulated genes separately. Functional enrichment analysis was then conducted on those genes through DAVID (60).

Real-time PCR

To assess gene expression via qPCR, RNA was reverse transcribed with a QuantiTect Reverse Transcription kit (Qiagen), following the protocol provided by the manufacture. cDNA was then added to a qPCR reaction mixture, according to the Mesa Blue qPCR MasterMix Plus for SYBR Assay (Eurogentec) instructions. Custom primers for genes of interest were designed and checked for target specificity using the bioinformatics tool Primer-Blast (61). The following sequences of primers were used: FOXM1 5′-GCACTTGGAATCACAGCAGA-3′/5′-CACCGGGAACTGGATAGGTA-3′; BRCA2 5′-AGCAGATGATGTTTCCTGTCC-3′/5′-TCTGACGACCCTTCACAAAC-3′; TOP2A 5′-GCCCATTGGTCAGTTTGGTA-3′/5′-AATCGAGCCAAAGAGCTGAG-3′; and B-actin 5′-GCGAGAAGATGACCCAGATC-3′/5′-CCAGTGGTACGGCCAGAGG-3′. qPCRs were carried out in a Mx300P cycler (Agilent Techonolgy), equipped with a MxPro software, with the following settings: 5 min at 95°C, 40 cycles of 30 s at 95°C and 1.5 min at 60°C. cDNA from at least three independent experiments was tested and each sample was ran in triplicates. The Livak method ($2^{-\Delta\Delta Ct}$) was used to perform relative quantification of genes (62).

Single cell gel electrophoresis

DNA single- and double-strand breaks were detected via single cell gel electrophoresis (comet) assay, according to the guidelines provided in (54). Adherent slides were coated with 1% low-gelling temperature agarose (PeqLab) and allowed to dry. LUHMES cells harvested in PBS were mixed with 1% agarose and placed on a precoated slide. After agarose gelling, slides were submerged in alkaline lysis buffer (1.2 M NaCl, 100 mM Na_2EDTA, 0.1% sodium lauryl sarcosinate, 0.26 M NaOH,

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pH > 13) and placed overnight at 4°C. Slices were then washed 3 times with rinse solution (0.03 M NaOH, 2 mM Na_2EDTA, pH = 12.3). Electrophoresis was carried out for 25 min at a constant voltage of 0.6 V/cm. Slides were removed from the electrophoresis chamber, neutralized with distilled water and stained with 2.5 μg/mL of propidium iodide, for 20 min at RT. Excess of stain was removed by rinsing the slides with distilled water. For each slide, at least 50 comet images were acquired in an epifluorescence microscope (Leica DMI 6000B microscope, Leica). Tail moment (the product of the percentage of DNA in the tail and the tail length) of each individual comet was determined using the CometScore software (TriTek Corp).

Reactive oxygen species detection

Differentiated LUHMES cells seeded in 96-well plates were washed once with warm PBS. For measuring total ROS production, cells were incubated with 25 μM DCFDA (Sigma), for 25 min at 37°C, whereas miROS was measured by incubating cells with 1 μM MitoSOX Red probe (Invitrogen), for 20 min at 37°C. After incubation time, excess of probe was removed by washing the cells with PBS. Fluorescence measurements were performed using an Infinite M2000 PRO (Tecan Ltd). To detect total ROS, the excitation and emission wavelengths were set to 485 and 535 nm, respectively. miROS was measured by using 510 nm for excitation and 580 nm for emission wavelength. Following three basal measurements, cells were challenged with 5% H_2O_2 and fluorescence values were recorded up to 30 min, at indicated time points.

Statistical analysis

Data are presented as mean ± standard deviation (SD) of, at least, three independent experiments. One- or two-way ANOVA test, with Bonferroni correction, were applied to determine significance of differences using GraphPad Prism 5 software. Unless otherwise, stated P-value < 0.05 was considered to indicate a significant difference.

Supplementary Material

Supplementary Material is available at *HMG* online.

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Conflict of Interest statement. None declared.

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Author contribution agreement

We confirm that **Isabel Paiva** contributed in a prominent way to the planning and experiments, analyzes an interpretation of the data, as well as writing the manuscript entitled **"Sodium butyrate rescues dopaminergic cells from alpha-synuclein-induced transcriptional deregulation and DNA damage (2017)** ***Human Molecular Genetics,*** **https://doi.org/10.1093/hmg/ddx114"**. Thus, we agree that this article can be included in her doctoral thesis.

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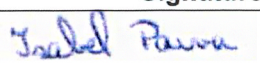
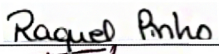
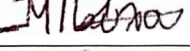
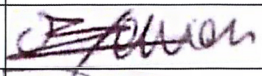
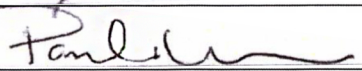
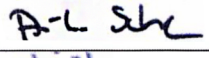
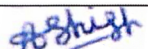
Isabel Paiva	
Raquel Pinho	
Maria Angeliki Pavlou	
Magali Hennion	
Pauline Wales	
Anna-Lena Schütz	
Ashish Rajput	
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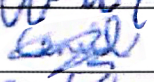
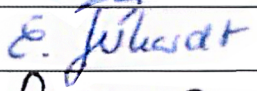
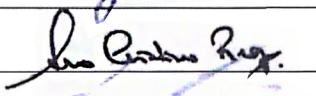
Cemil Kerimoglu	
Ellen Gerhardt	
Ana Cristina Rego	
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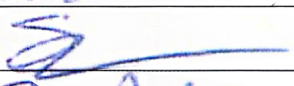
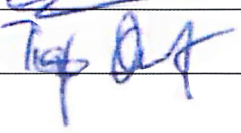
Stefan Bonn	
Tiago F. Outeiro	

Publication II

The A30P alpha-synuclein mutation induces transcriptional deregulation and ER-Golgi impairment

(submitted)

The A30P alpha-synuclein mutation induces transcriptional deregulation and ER-Golgi impairment

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Abstract

Alpha-synuclein (aSyn) is the major protein component of Lewy bodies and Lewy neurites, the typical pathological hallmarks in Parkinson's disease (PD). Although it has been shown that aSyn plays a role in transcriptional deregulation, the effect of specific aSyn mutants associated with familial forms of PD, remains unclear. We used transgenic mice overexpressing human wild-type (WT) or A30P aSyn to compare the transcriptional profiles of the two animal models. We observed that A30P aSyn promotes strong transcriptional deregulation and increases DNA binding. Interestingly, COL4A2, a pro-apoptotic gene, was found to be upregulated in both A30P aSyn transgenic mice and in dopaminergic neurons expressing A30P aSyn. Finally, we observed that A30P aSyn alters Golgi morphology and increases endoplasmic reticulum (ER) stress in dopaminergic cells. In total, our findings provide novel insight into a putative role of aSyn on transcription, opening novel avenues for future therapeutic interventions in PD and other synucleinopathies.

Key words: A30P alpha-synuclein, COL4A2, ER stress, Golgi fragmentation, Transcription deregulation

Introduction

PD is the second most common neurodegenerative disease after Alzheimer's disease and is associated with several motor and non-motor features [1, 2]. The motor features arise due to the loss of dopaminergic neurons in the *substantia nigra pars compacta*. Although the precise molecular mechanisms associated with the development of PD are not clearly understood the protein aSyn plays a central role in the pathology as it is the major component of Lewy bodies and Lewy neurites, typical pathological inclusions found in the brains of PD patients [3]. In addition, several autosomal dominant mutations in the gene encoding aSyn (*SNCA*), including duplication or triplication of the gene, and some point mutations, such as A30P, have been linked to familial forms of PD. Previous reports indicate that the A30P mutation alters some important properties of aSyn. One alteration is the reduction of membrane affinity that might be involved in aSyn aggregation and neurotoxicity [4, 5]. Physiologically, aSyn has been linked to several biological processes such as synaptic vesicle release and biology and mitochondrial function [6, 7]. Interestingly, recent studies revealed transcriptional deregulation in PD patients and animal and cellular models of the disease, suggesting aSyn may also influence gene expression [8-11].

Here, we investigated the effect of increased WT or A30P mutant aSyn levels on gene expression. We used transgenic mice overexpressing either human WT or A30P mutant aSyn and found the A30P mutant to promote robust transcriptional deregulation, in agreement with our previous findings using a cell model of PD [10]. The mechanisms underlying aSyn-induced transcriptional deregulation are not well understood. However,

nuclear aSyn binds to histones and specific DNA regions [12]. Furthermore, alternative splicing modulation is considered an important mechanism of gene expression regulation [13] and it has been proven as a relevant mechanism to understand PD molecular etiology [14, 15]. Importantly, the microRNA (miRNA) pool is also a significant player in the regulation of gene expression that is increasingly drawing attention. MiRNAs contribute to post-transcriptional regulation via pairing with the 3'-untranslated region (3'UTR) of mRNAs and have been previously implicated in aSyn-induced toxicity and PD pathogenesis [16, 17].

Several studies have shown a connection between ER stress and the etiology of neurodegenerative diseases [18]. It has been reported that aSyn can induce ER stress and that this might be associated with aSyn-induced toxicity [19]. Moreover, signs of ER stress are observed in post-mortem tissue from sporadic cases of PD and in most animal models of the disease [20].

Our findings showed that A30P mutant aSyn directly promotes robust transcriptional deregulation via binding to numerous DNA regions, and also affects differential exon usage of several genes. Interestingly, we found that A30P aSyn upregulated COL4A2, a pro-apoptotic gene, and downregulated miR-29a-3p, a known COL4A2 miRNA target, in transgenic mice. Furthermore, we demonstrate that A30P aSyn overexpression in dopaminergic neurons, leads to increased levels of ER stress and induces Golgi fragmentation.

To the best of our knowledge, this is the first study directly addressing the effect of A30P aSyn on transcriptional deregulation in transgenic mice. Our findings provide new insight into the effect of A30P mutant aSyn on transcription and identifies downstream effects on

ER and Golgi systems, opening novel avenues for future therapeutic intervention in PD and other synucleinopathies.

Materials and Methods

Transgenic mice

Transgenic mice expressing human WT aSyn (C57BL/6xDBA), A30P aSyn (C57Bl/6) under Thy1 promoter [21, 22] and littermate controls were used for the experiments. Animals were housed and handled according to institutional and national guidelines. Half midbrains of 6-month-old transgenic mice were used for RNA-sequencing (RNA-seq), ChIP-seq (Chromatin immunoprecipitation-sequencing) and real-time PCR (qPCR) procedures.

LUHMES cell culture

LUHMES cells were maintained and differentiated as previously described [23]. In short, proliferating cells were cultured in Advanced Dulbecco's modified Eagle's medium/F12 (DMEM/F12, Gibco) supplemented with 1xN2 (Gibco), 2 mM L-glutamine (Gibco) and 40 ng/mL recombinant basic fibroblast growth factor (bFGF, R&D Systems), at 37ºC in a humidified 5% CO_2 atmosphere. Differentiation was performed by the addition of 1 μg/mL tetracyclin (Sigma-Aldrich), 1 mM dibutyryl cAMP (cAMP, Sigma Aldrich) and 2 ng/mL recombinant human GDNF (R&D Systems) in the aforementioned culture medium. After two days in culture with differentiation medium, cells were trypsinized [(138 mM NaCl, 5.4 mM KCl, 6.9 mM NaHCO3, 5.6 mM DGlucose, 0.54 mM EDTA, 0.5 g/L trypsin from bovine pancreas type-II-S (Sigma-Aldrich)] and seeded into plates pre-coated with 50

μg/mL PLO and 1μg/mL fibronectin (Sigma-Aldrich). On the fifth day of differentiation one third of culture medium was refreshed and experiments were performed on the eighth day of differentiation. Tunicamycin (1 ug/mL) and DMSO (0.1%) treatment as control were performed on the seventh day of differentiation.

Generation of WT and A30P aSyn-expressing LUHMES cells

Full-length human WT aSyn c-DNA (SNCA, NM_000345) or A30P mutant aSyn were subcloned into the pWPI, second generation bicistronic lentiviral vector (Tronolab, Switzerland), under the chicken/β-actin (CBA) promoter. pWPI vector containing only IRES-GFP cassette was used as infection control in all experiments. Direct sequencing (StarSeq) was used to verify all cloned sequences. Second-generation lentiviral particles were generated as previously described [24]. In short, modified transfer vector plasmids and second-generation packing system (Tronolab) were transiently co-transfected in 293T cells. 48h post-transfection, culture medium was collected, concentrated by PEG-it Virus Precipitation Solution (System Bioscience), and resuspended in Panserin 402 (PAN). Lentiviral vector titration was performed by qPCR using primer sequences specific for the woodchuck hepatitis virus post-transcriptional regulatory element (WPRE) as previously described [25]. Finally, proliferating LUHMES cells were infected with equimolar amounts of IRES-GFP, WT aSyn-IRES-GFP, or A30P aSyn-IRES-GFP lentivirus. Fluorescence-activated cell sorting (Calibur flow cytometer, BD Biosciences) was used to select positive green fluorescent cells.

RNA extraction

RNA was extracted from half midbrain of mice using Tri Reagent (Sigma) following the protocol provided by the manufactures. RNA was treated with 2U of DNase I for 20 min at 37 °C and extracted using phenol-chloroform. RNA quality analysis and sequencing were performed as previously described [26]. In summary, RNA quality was assessed using RNA 6000 Nano chips run on a 2100 Bioanalyzer (Agilent). The preparation of the libraries was done using the TruSeq RNA Sample Preparation v2 kit (Illumina). The quality of the library was checked using High Sensitivity DNA chips on a Bioanalyzer and the concentration of the sample was measured using a Qubit dsDNA HS Assay Kit. Finally, sample concentration was adjusted to 2 nM before sequencing (50 bp) on a HiSeq 2000 (Illumina) according to the manufacturer's instructions.

Gene expression analysis (RNA-seq)

Differential gene expression analysis was performed as previously described [26]. In summary, a customized in-house software pipeline was used to process the sequencing data. Illumina's bcl2fastq (v1.8.4) was used to convert the base calls in the per-cycle BCL files to the per-read FASTQ format from raw images. Along with base calling, adapter trimming and demultiplexing were performed. FastQC (v 0.11.5) was used to assess the quality control of raw sequencing data. Reads were then mapped to the mouse transcriptome (Mus_musculus.GRCm38.86) along with the human *SNCA* gene (Ensembl_id:ENSG00000145335). The alignment of the RNA-seq data was done using

rna-STAR (version STAR_2.5.2b), generating mapping files (BAM format) [27]. All the reads were mapped in the non-splice-junction-aware mode and all other parameters were used in default settings by rna-STAR.

In order to identify differentially expressed genes, RUVSeq (v. 1.8.0) was used to identify unwanted sources of variation (RUVs) and correct them. Then, DESeq2 was used to compare differential expression of genes between experimental groups [28]. RNA-seq analysis of LUHMES cells is described in our previous report [10]. Thresholds were set as $p\text{adj} < 0.01$. Differential exon-level usage was analyzed with *DEXSeq* (v1.20.0) [29].

Gene ontology analysis on differentially expressed genes was performed using the ToppGene Suite [30].

qPCR experiments

For qPCR experiments, total RNA from LUHMES cells was isolated using the RNeasy mini kit (Qiagen, Germany). cDNA was generated from RNA using the QuantiTect Reverse Transcription kit (Qiagen, Germany) following the protocol provided by the manufacturer.

qPCR was performed with the Mesa Blue qPCR MasterMix Plus for SYBR Assay (Eurogentec) according to the manufacturer's instructions. Primer Blast was used to design and validate target specificity of custom primers for the genes of interest [31].

The following sequences of primers were used: COL4A2 5'-ATTCCTTCCTCATGCACACG -3'/5'- ACTTGTTGGCGTAGTAGTGG -3'; *TNFRS10B* 5'-TGCTCTGATCACCCAACAAG -3'/5'- CTGAGATATGGTGTCCAGGTG -3'; and B-actin

5’-GCGAGAAGATGACCCAGATC-3’/5’-CCAGTGGTACGGCCAGAGG-3’. Triplicates from at least three independent experiments were assessed using the Mx300P cycler (Agilent Techonolgy), equipped with a MxPro software with the following settings: 5 minutes at 95°C, 40 cycles of 30 seconds at 95°C and 1.5 minutes at 60°C. Relative quantification of the genes was performed with the Livak method ($2^{-\Delta\Delta Ct}$) [32].

To assess miRNA expression in mice samples, total RNA from half of midbrain was extracted as described in the “RNA extraction” section. cDNA was generated using miScript II RT Kit (Qiagen) following the protocol provided by the manufacturer. qPCR was performed using miScript Primer for miRNA-29a-3p (mmu-miR-29a-3p miScript Primer Assay - Qiagen) and the miScript SYBR Green PCR Kit (Qiagen). MiRNA expression was normalized to the endogenous control RNU6.

ChIP-seq experiments

Sample preparation

Brain tissue was harvested from 6-month-old transgenic mice and their respective controls. Midbrain was dissected and half of the region was cut in ~1 mm^3 pieces and cross-linked in 1% formaldehyde (Sigma) for 10 minutes at RT agitating. Fixation reaction was stopped with addition of 0.125M glycine followed by 5 min incubation at RT agitating. Samples were subjected to mild centrifugation for 10 min at 4ºC and washed twice with ice-cold PBS supplemented with Protease Inhibitor Cocktail (Roche). Pellets were snap frozen and kept on -80ºC. All samples were lysed using a specific lysis buffer (1.0% SDS, 10 mM EDTA, 50 mM Tris-HCl, pH 8.1, and Protease Inhibitor Cocktail (Roche)).

Chromatin shearing and library preparation

Chromatin was sheared using a Diagenode Bioruptor sonicator. In order to ensure an average DNA fragment size of 100-500bp, chromatin was assayed by both agarose gel electrophoresis and Bioanalyzer DNA 1000 kit (Agilent). A fraction of chromatin was saved as total input control (Input) and the rest was subjected to pre-clearing with 45 µl Protein G-Agarose solution (Millipore). Chromatin immunoprecipitation (IP) was performed overnight at 4ºC in mild agitation, using 4 µg of monoclonal aSyn antibody (Syn-1, BD Bioscience #610787). Beads were washed sequentially with Buffer 1 (0.1% SDS, 1% Triton X-100, 2 mM EDTA, 20 mM Tris-HCl pH 8.1, 100 mM NaCl), Buffer 2 (Buffer 1 with 500 mM NaCl) and Buffer 3 (0.25 M LiCl, 1% NP-40, 1% $C_{24}H_{39}NaO_4$, 1 mM EDTA, 10 mM Tris-HCl), and two times with TE Buffer. Precipitated chromatin was eluted in 1% SDS, 0.1M $NaHCO_3$ and incubated at 65ºC overnight in NaCl (to 0.2 M) to reverse the crosslinks. The DNA fragments with 200±25 bp in length were then selected for the construction of ChIP-seq DNA library. DNA was purified with the QIAquick PCR Purification Kit (Qiagen) and quantified using PicoGreen or NanoDrop Spectrophometer for immunoprecipitated (IP) and Input DNA, respectively. TruSeq ChIP Library preparation kit (Illumina) was used according to manufacturer´s instructions for library preparation of all IP and Input samples. Quality and size of the libraries was checked using Agilent Bioanalyzer HS DNA kit, showing DNA fragments with 200±25 bp and expected concentration. High-throughput sequencing of those fragments was performed with MiSeq (Illumina).

ChIP-seq analysis

Mice samples were aligned to UCSC mm10 using BaseSpace Sequence Hub (Illumina). The Partek Genomics Suite (Partek) ChIP-seq analysis workflow was used to identify enriched aSyn binding regions. Peaks were determined using a 100 bp window and the zero-truncated binomial model with a peak cut-off p-value of 0.001. Additionally, ChIP-seq samples were subjected to differential binding analysis using the DiffBind Bioconductor package with an built-in option of DESeq2 [28]]. For the analysis with Diffbind, peaks were called with MACS2 [33] using the q-value of 0.1 (“q 0.1” option).

Immunoblotting

Proteins were extracted using assay buffer (50 mM Tris, pH 8.0, 0.15 M NaCl, 0.1% SDS, 1.0% NP-40, 0.5% Na-Deoxycholate, 2 mM EDTA, supplemented with Complete Protease Inhibitor Cocktail [Roche, Mannheim, Germany]. Samples were centrifuged at 14.000 rpm for 30 min, at 4°C. Protein concentration was determined by the Bradford assay (BioRad) using an Infinite M200 PRO plate reader (Tecan Lta). Total lysates (mice samples: 5ug; LUHMES: 30ug) were mixed with 5x Laemmli buffer (250 mM Tris pH 6.8, 10% SDS, 1.25% Bromophenol Blue, 5% β-Mercaptoethanol, 50% Glycerol), boiled at 95°C for 5 minutes, and loaded on 12% SDS-polyacrylamide (SDS-PAGE) gels. Proteins were transferred onto the membrane (Trans-blot Turbo Nitrocellulose/PVDF membranes, BioRad) using the trans-blot Turbo system. Blocking was with 5% skim milk, 1xTris Base Solution/0.05% Tween (TBST) for 1 hour at RT. Membranes were probed with the primary

antibodies diluted in 4% BSA/TBST overnight at 4°C. The following antibodies were used: anti-aSyn (1:2000, BD Transduction, mouse); anti-COL4A2 (1:1000, Thermo Scientific, rabbit); anti-calnexin (1:1000, abcam, rabbit); anti-GAPDH (1:5000, Cell Signaling, rabbit). Membranes were then probed for 2h at RT with horseradish peroxidase-conjugated secondary antibodies (1:10.000, mouse or rabbit, GE Healthcare). Between steps, membranes were washed 3 times with TBST for 5 minutes at RT. Proteins were visualized based on the chemiluminescent signal that was detected using Immobilon Western Chemiluminescent HRP Substrate (Millipore Corporation) and Fusion FX (Vilber Lourmat). Quantification of bands was performed using ImageJ.

Immunocytochemistry

Cells were grown on coverslips were fixed with 4% paraformaldehyde for 15 minutes at RT, washed 3 times with 1x phosphate buffer saline (PBS), permeabilized with 0.5% Triton/PBS for 15 minutes, and finally blocked with 3% BSA/PBS (blocking buffer) for 1 hour at RT. Cells were incubated overnight at 4ºC with the primary antibodies diluted in blocking buffer. The following antibodies were used: anti-aSyn 1:2000 (BD Transduction, mouse), anti-COL4A2 1:1000 (Thermo Scientific, rabbit) anti-Giantin 1:2000 (Abcam, rabbit). Washing was with PBS and then probed for 2 hours at RT with the secondary Alexa Fluor antibodies (rabbit or mouse 488 and 555, Life Technologies) diluted 1:1000, in blocking buffer. Nuclei were stained with DAPI. Images were obtained using an epifluorescence microscope (Leica DMI 6000B microscope, Leica) with a 63x magnification objective.

Golgi morphology

LUHMES cells were immunostained for Giantin to assess Golgi morphology. For each condition, at least three independent experiments were performed. Immunofluorescence images were randomly acquired using an epifluorescence microscope (Leica DMI 6000B microscope, Leica). Golgi morphology was classified as normal, diffuse, or fragmented as we previously described [34]. At least 50 cells were counted and scored per experiment and per condition.

Statistical analysis

Data are presented as mean ± SD of at least N=3 unless stated otherwise in the figure legend. Each N represents one independent experiment. GraphPad Prism 5 (California, USA) was used to calculate significance of differences. One-way/Two-way ANOVA with Bonferroni correction was used when two or more groups were considered. Unpaired t-test with equal SD was considered when comparing two groups. Unless stated otherwise, p-value < 0.05 was considered to indicate a significant difference.

Results

A30P aSyn transgenic mice display stronger transcriptional deregulation than WT aSyn transgenic mice

To investigate the effect of aSyn expression on transcriptional deregulation we used the human WT aSyn and A30P aSyn transgenic mouse models of PD [21, 22]. 6-month-old transgenic mice express more aSyn than the respective littermate controls (Fig 1A,B). At this age, mice expressing human WT aSyn or A30P aSyn display mild cognitive impairment [35-37]. RNA from midbrain of 6-month-old mice and littermate controls was extracted and RNA-seq analyses were performed. Principal Component Analysis (PCA) plots from RNA-seq data revealed a clear separation between samples derived from control or A30P mice, while those derived from WT aSyn transgenic mice were similar to controls (Fig 1C,D). As expected, differential expression analysis of WT aSyn and A30P aSyn compared to the respective control mice showed upregulation of the gene encoding for aSyn (SNCA) [log_2Fold-Change(log_2FC)=1.80, p-adjusted value (p_{adj})=1.23E-27 for control versus WT aSyn; log_2FC=2.80, p_{adj} =1.75E-22 for control versus A30P aSyn]. MA plots showed that only 18 genes were significantly deregulated in WT aSyn transgenic mice compared to control mice (p_{adj}<0.1) (Fig 1E) (Table S1). Among the 18 genes, 6 were found upregulated and 12 downregulated (Fig 1 G). Among those, *HSPA1b*, a gene encoding for a heat shock protein, revealed high deregulation in WT aSyn transgenic mice (log_2FC=-1.09). In contrast, a robust transcriptional deregulation was observed in A30P aSyn expressing mice, as the number of deregulated genes was significantly higher (Fig 1F,H) (Table S2). Considering p_{adj}<0.01, 2165 genes were found deregulated in the

A30P aSyn transgenic mice compared to control. Among those, 886 genes were upregulated and 1279 downregulated (Table S2).

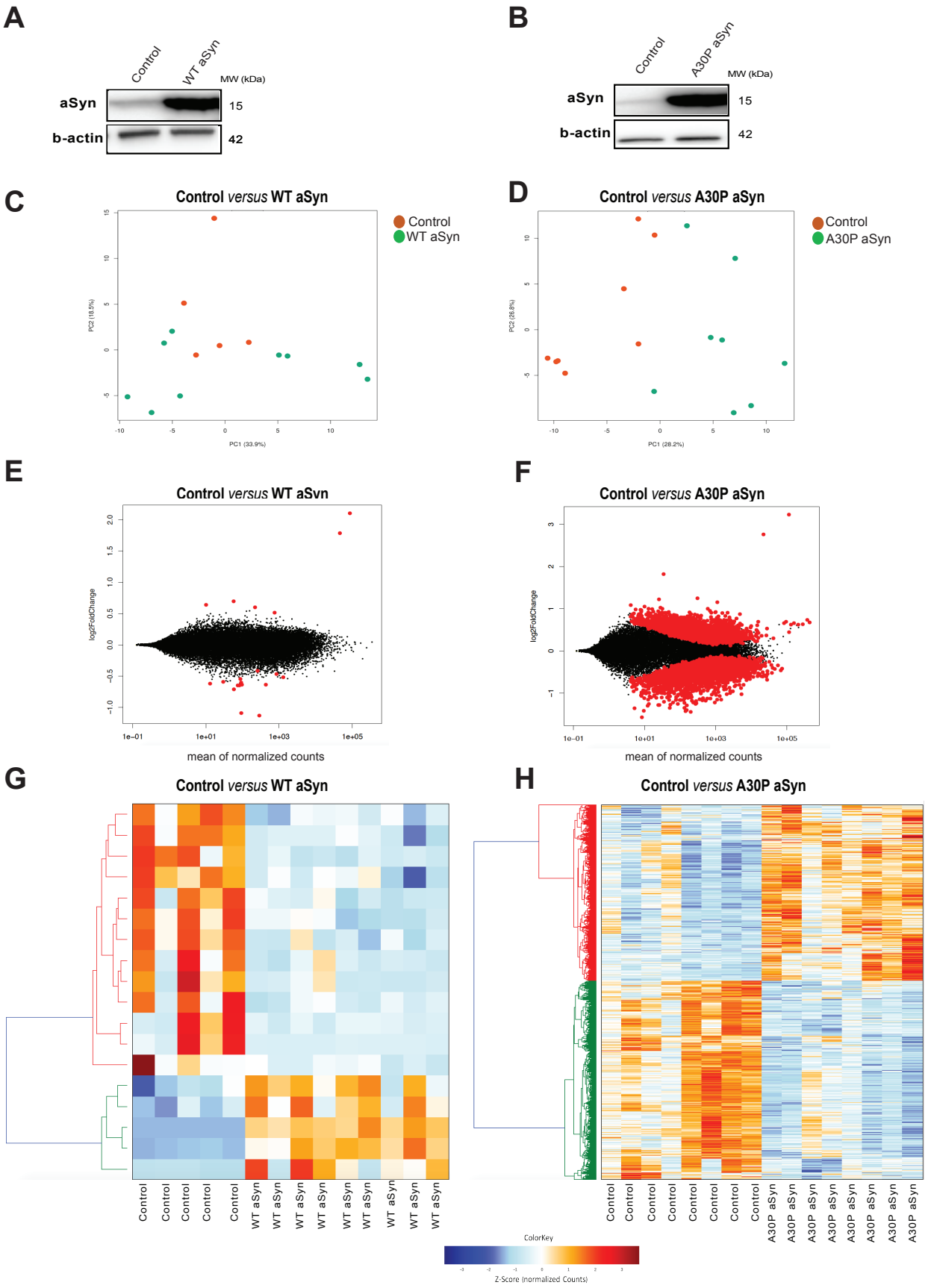

A
Control
WT aSyn
MW (kDa)
aSyn
15
b-actin
42
B
Control
A30P aSyn
MW (kDa)
aSyn
15
b-actin
42
C
Control versus WT aSyn
Control
WT aSyn
PC2 (18.5%)
PC1 (33.9%)
D
Control versus A30P aSyn
Control
A30P aSyn
PC2 (26.8%)
PC1 (28.2%)
E
Control versus WT aSyn
log2FoldChange
mean of normalized counts
F
Control versus A30P aSyn
log2FoldChange
mean of normalized counts
G
Control versus WT aSyn
Control
WT aSyn
H
Control versus A30P aSyn
Control
A30P aSyn
ColorKey
Z-Score (normalized Counts)

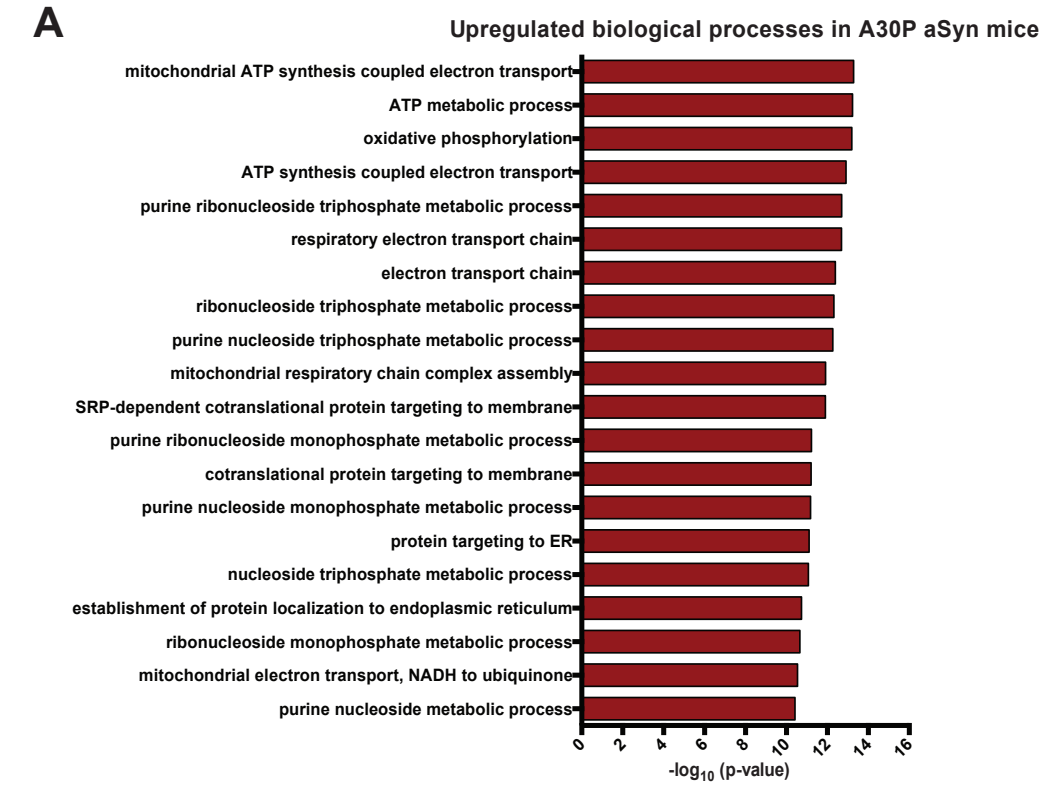
Figure 1. Transcriptional deregulation in aSyn WT and A30P mutant transgenic mice.

aSyn expression levels in the midbrain of 6-month-old transgenic mice expressing human aSyn WT or A30P mutant aSyn (A,B). PCA-plots obtained from RNA-seq show the correlation between WT aSyn (C) or A30P aSyn (D) mice with the respective controls. MA-plots were generated for differential gene expression data obtained for WT aSyn (E) or A30P mutant aSyn (F) transgenic mice. The log2FC for each comparison is plotted on the y-axis and the average counts normalized by size factor are shown on the x-axis. Each dot represents a gene. Genes with p_{adj} bellow 0.1 are shown in red. Heatmaps showing the differentially expressed genes in WT aSyn (G) or A30P mutant aSyn transgenic mice (H). Warm colors represent the upregulated genes and cool colors refer to the downregulated ones. Heatmaps were generated with the genes showing $|\log_2 FC| \geq 0.25$, base mean ≥ 25.00 and $p_{adj} \leq 0.05$. The expression of A30P aSyn affected a large number of genes while WT aSyn showed an effect on only 18 genes.

A30P aSyn affects several biological processes in transgenic mice

To determine the molecular pathways affected by the A30P mutant aSyn in transgenic mice, we performed pathway analyses using the ToppGene Suite. With a cut-off of $p_{adj}<0.01$ for significantly deregulated genes in A30P aSyn transgenic mice relative to control, we identified several pathways affected. Upregulated and downregulated genes were analyzed separately. In total, upregulated genes were involved in 127 biological processes, and downregulated genes were involved in 483 biological processes (qvalue FDR<0.05) (Table S3). Interestingly, in A30P aSyn transgenic mice we found upregulation of several pathways associated with mitochondria metabolism, such as ATP synthesis and oxidative phosphorylation, as well as ER related-mechanisms, like

targeting and establishment of protein localization to the ER (Fig 2 A). Likewise, we found downregulation of pathways related to neuronal development and differentiation, synaptic signaling, and cell-adhesion (Fig 2 B).


A
Upregulated biological processes in A30P aSyn mice
mitochondrial ATP synthesis coupled electron transport
ATP metabolic process
oxidative phosphorylation
ATP synthesis coupled electron transport
purine ribonucleoside triphosphate metabolic process
respiratory electron transport chain
electron transport chain
ribonucleoside triphosphate metabolic process
purine nucleoside triphosphate metabolic process
mitochondrial respiratory chain complex assembly
SRP-dependent cotranslational protein targeting to membrane
purine ribonucleoside monophosphate metabolic process
cotranslational protein targeting to membrane
purine nucleoside monophosphate metabolic process
protein targeting to ER
nucleoside triphosphate metabolic process
establishment of protein localization to endoplasmic reticulum
ribonucleoside monophosphate metabolic process
mitochondrial electron transport, NADH to ubiquinone
purine nucleoside metabolic process
0 2 4 6 8 10 12 14 16
-log10 (p-value)

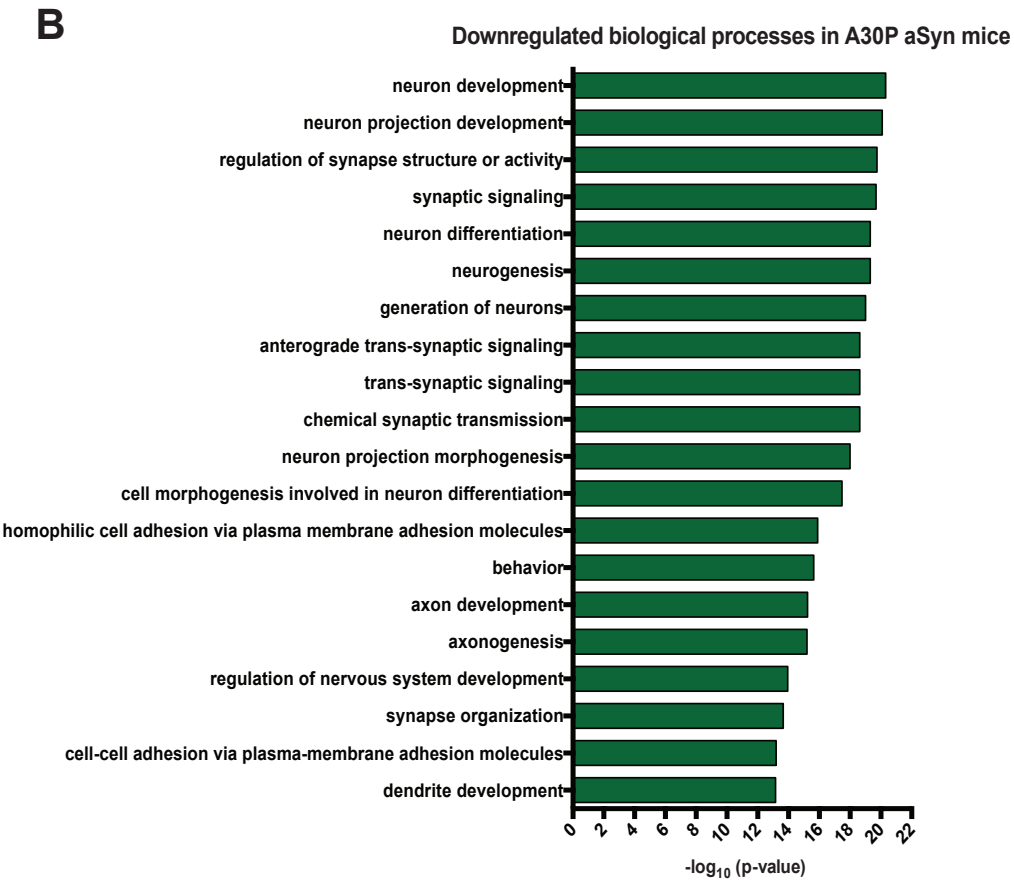

B
Downregulated biological processes in A30P aSyn mice
neuron development
neuron projection development
regulation of synapse structure or activity
synaptic signaling
neuron differentiation
neurogenesis
generation of neurons
anterograde trans-synaptic signaling
trans-synaptic signaling
chemical synaptic transmission
neuron projection morphogenesis
cell morphogenesis involved in neuron differentiation
homophilic cell adhesion via plasma membrane adhesion molecules
behavior
axon development
axonogenesis
regulation of nervous system development
synapse organization
cell-cell adhesion via plasma-membrane adhesion molecules
dendrite development
0 2 4 6 8 10 12 14 16 18 20 22
-log10 (p-value)

Figure 2. Biological processes affected in A30P aSyn mutant transgenic mice. ToppGene Suite analyses of deregulated genes found in the RNA-seq data ($p_{adj} < 0.01$) show upregulation (A) and downregulation (B) of numerous pathways. The graphs represent the top 20 biological processes with greater statistical significance.

A30P aSyn binds several regions of DNA and promotes differential exon usage

We performed aSyn ChIP-seq in A30P aSyn transgenic mice to investigate whether aSyn could bind DNA. Midbrain preparations of 6-month-old A30P aSyn transgenic mice and littermate controls were processed for chromatin IP using an anti-aSyn antibody and then sequenced. The sequenced fragments were analyzed and aligned with the mouse genome in order to identify the genomic regions recruited by endogenous or human A30P mutant aSyn. Our differential ChIP-seq data analysis revealed that A30P mutant aSyn was differently bound to 5400 gene body regions when compared to the endogenous mouse aSyn (control) (FDR < 0.1) (Table S4). The majority of these gene body regions showed higher binding affinity for A30P aSyn than for the endogenous mouse aSyn (Fig 3A). Specifically, we identified 5395 regions that A30P aSyn bound with higher affinity and only 5 regions that showed lower binding affinity (Table S4). In parallel, we asked whether aSyn bound to DNA promoter regions and identified 429 promoter regions bound by A30P mutant and by endogenous mouse aSyn (p-value ≤ 0.05) (Table S4). All promoter regions showed increased binding by A30P mutant aSyn compared to endogenous mouse aSyn (Fig 3B). Among the 429 promoter regions, 39 of their corresponding genes were found differentially expressed and, among them, we found

COL11A1 (Fig 3C). In summary, our differential analyses of ChIP-seq data generated from A30P aSyn transgenic mice revealed that A30P aSyn exhibited increased propensity for binding to DNA than endogenous mouse aSyn.

To determine whether A30P mutant aSyn affected exon usage, we performed differential exon usage analysis using our RNA-seq data of both A30P mutant aSyn-expressing LUHMES cells (previously described [10]) and transgenic mice. Interestingly, changes in RNA exon usage were found in several genes associated with A30P mutant aSyn expression in both models. 5235 genes identified in A30P aSyn transgenic mice showed differential exon usage but no gene expression changes. However, there was an overlap of 850 genes that showed both differential exon usage and differential expression (Fig. 3D, left side). Our analysis identified 136 upregulated and 714 downregulated genes that were also affected by differential exon usage in A30P aSyn transgenic mice. Curiously, in A30P aSyn-expressing LUHMES cells, the number of genes affected by differential exon usage was significantly lower compared to that in the A30P aSyn transgenic mice. In total, 191 genes showed differential exon usage with no change on gene expression. Moreover, 20 upregulated and 37 downregulated genes revealed differential exon usage upon A30P mutant aSyn expression (padj $\leq$ 0.01) (Fig 3D, right side). Our previous findings in LUHMES cells show that A30P aSyn promotes transcriptional deregulation, and a more significant downregulation of gene expression [10]. Consistently, we observed a similar effect in A30P mutant aSyn transgenic mice, as 886 genes were upregulated and 1279 genes were downregulated (p_{adj} < 0.01). Correlating data from A30P aSyn expressing LUHMES cells and transgenic mice, we identified 29 commonly upregulated and 57 commonly downregulated genes. Among the upregulated genes, 4 showed

differential exon usage in A30P aSyn transgenic mice, including *COL4A1*, and only *PHLDB1* showed differential exon usage in LUHMES cells. Regarding the commonly downregulated genes, 37 genes showed differential exon usage in A30P aSyn transgenic mice, including *CCND1* and *CLVS1*, important regulators of cell cycle and autophagic processes. None of the commonly downregulated genes showed differential exon usage in A30P aSyn-expressing LUHMES cells. Overall, our data suggest that A30P mutant aSyn promotes differential exon usage in several genes, but that this does not necessarily result in altered gene expression.

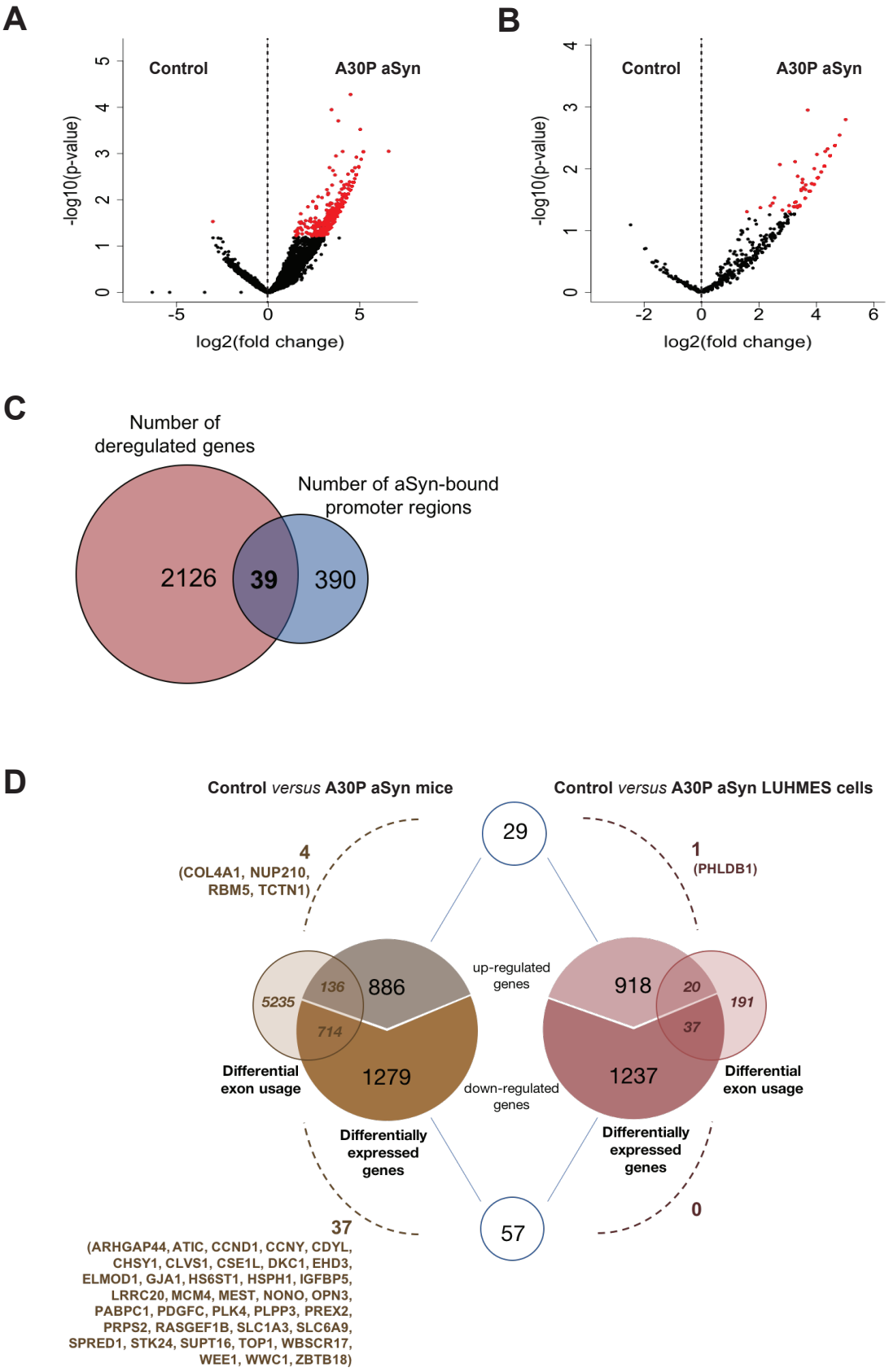

A
Control
A30P aSyn
-log10(p-value)
log2(fold change)
B
Control
A30P aSyn
-log10(p-value)
log2(fold change)
C
Number of deregulated genes
Number of aSyn-bound promoter regions
2126
39
390
D
Control versus A30P aSyn mice
Control versus A30P aSyn LUHMES cells
29
4
(COL4A1, NUP210, RBM5, TCTN1)
1
(PHLDB1)
5235
136
886
714
1279
up-regulated genes
down-regulated genes
918
20
191
37
1237
Differential exon usage
Differentially expressed genes
Differential exon usage
Differentially expressed genes
57
37
(ARHGAP44, ATIC, CCND1, CCNY, CDYL, CHSY1, CLVS1, CSE1L, DKC1, EHD3, ELMOD1, GJA1, HS6ST1, HSPH1, IGFBP5, LRRC20, MCM4, MEST, NONO, OPN3, PABPC1, PDGFC, PLK4, PLPP3, PREX2, PRPS2, RASGEF1B, SLC1A3, SLC6A9, SPRED1, STK24, SUPT16, TOP1, WBSCR17, WEE1, WWC1, ZBTB18)
0

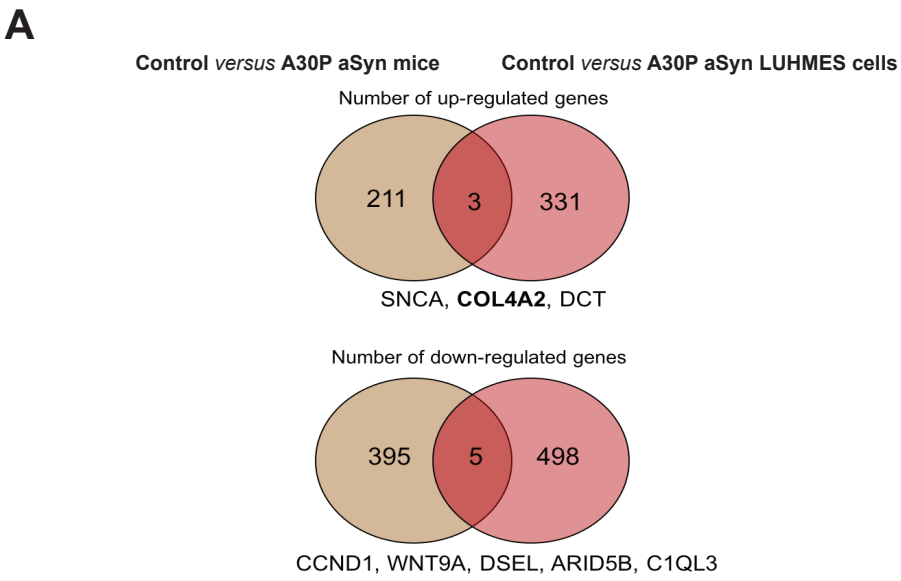
Figure 3. DNA binding and differential exon usage in A30P aSyn mutant transgenic mice. ChIP-seq data showed that A30P mutant aSyn bound more DNA regions compared to endogenous mouse aSyn in control mice (A) (cut-off of FDR<0.1). Considering a *p*-value<0.05, A30P mutant aSyn transgenic mice also showed binding to a much larger number of DNA promoter regions (B). Significant binding to all regions of DNA and promoter regions is shown in red (A30P aSyn mice: N=2; control: N=3)). (C) Venn-diagram showing the overlapping genes between differentially deregulated genes and aSyn-bound promoter regions in A30P aSyn transgenic mice. (D) Venn-diagrams showing the overlapping genes with differential exon usage and expression in A30P mutant aSyn transgenic mice (left side) and in LUHMES cells expressing A30P aSyn (right side). A large number of genes (5235) showed only differential exon usage in A30P mutant aSyn transgenic mice. Additionally, 4 upregulated and 37 downregulated genes in both A30P mutant aSyn transgenic mice and in A30P aSyn-expressing LUHMES cells showed also differential exon usage (left side). In A30P aSyn-expressing LUHMES cells, 191 genes showed only differential exon usage. Only *PHLDB1* gene was commonly upregulated in mice and LUHMES cells and showed also differential exon usage. No overlap was found between differential exon usage and commonly downregulated genes in both models (right side). The cut-off used for differentially expressed genes was p_{adj}<0.01 and for genes with differential exon usage it was $p_{adj} \leq 0.01$.

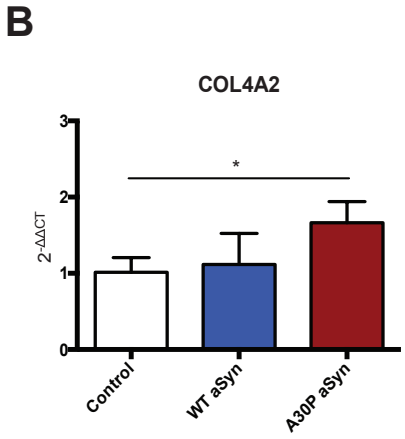
A30P aSyn upregulates *COL4A2* and downregulates miR-29a-3p in transgenic mice

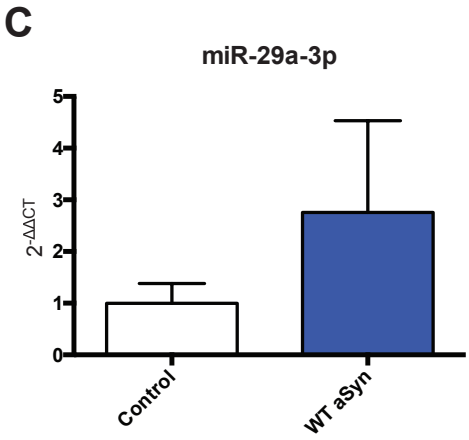
To further explore the transcriptional deregulation associated with A30P mutant aSyn in transgenic mice and in LUHMES cells, we investigated the most deregulated genes in both models. Using $|\log_2 FC| \geq 0.6$ and $p_{adj} \leq 0.01$ as cut-offs, we identified 211

upregulated and 395 downregulated genes in A30P aSyn transgenic mice and 331 upregulated and 498 downregulated genes in A30P aSyn-expressing LUHMES cells. Of those, 5 genes were found commonly downregulated in LUHMES cells and in transgenic mice, including *CCND1* and *WNT9A*. We identified 3 commonly upregulated genes, including *SNCA* and *COL4A2*. Collagen related proteins, such as collagen VI, have previously been associated with neuroprotection during physiological aging [38]. However, other studies report on associations of abnormal expression of collagen IV with increased levels of ER stress [39]. Since we identified several ER stress related pathways upregulated in A30P aSyn transgenic mice (Fig 2A), we assessed the expression of *COL4A2* in A30P aSyn-expressing LUHMES cells. Using qPCR, we confirmed the increased levels of *COL4A2* expression (two-fold), as expected from the RNA-seq experiments (Fig 4B). By RNA-seq, *COL4A2* showed a log2FC = 0.70 in A30P aSyn transgenic mice, and log2FC = 0.6 in A30P LUHMES cells (Table S2).

Next, we investigated the mechanism involved in the upregulation of *COL4A2* in A30P aSyn transgenic mice. Thus, we asked whether A30P mutant aSyn affected the expression of miR-29a-3p, an experimentally validated miRNA that targets *COL4A2* mRNA. Interestingly, we confirmed the downregulation of miR-29a-3p in A30P aSyn transgenic mice by qPCR (Fig 4D). The same experiment was performed in midbrain samples from human WT aSyn transgenic mice, but no significant difference was observed (Fig 4C). This result is consistent with the upregulation of *COL4A2* in A30P mutant aSyn transgenic but not in the human WT aSyn transgenic mice.


A
Control versus A30P aSyn mice
Control versus A30P aSyn LUHMES cells
Number of up-regulated genes
211
3
331
SNCA, COL4A2, DCT
Number of down-regulated genes
395
5
498
CCND1, WNT9A, DSEL, ARID5B, C1QL3


B
COL4A2
2-ΔΔCT
Control
WT aSyn
A30P aSyn


C
miR-29a-3p
2-ΔΔCT
Control
WT aSyn

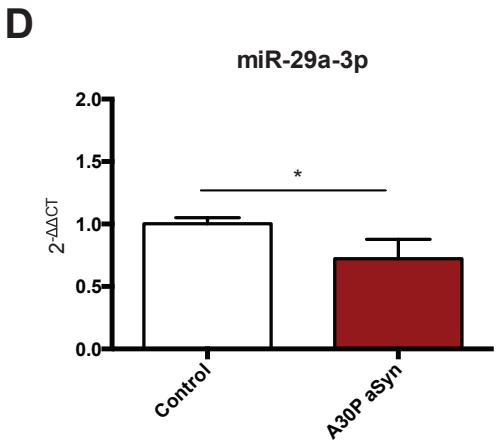

D
miR-29a-3p
2-ΔΔCT
Control
A30P aSyn

Figure 4. *COL4A2* and miR-29a-3p expression in A30P mutant aSyn transgenic mice. (A) Venn-diagrams showing deregulated genes found in common between A30P mutant aSyn transgenic mice and A30P aSyn-expressing LUHMES cells. The upper part of the diagram shows the commonly upregulated genes (log2FC>0.6) and the lower part shows the commonly downregulated genes (log2FC<-0.6). *COL4A2* was found highly upregulated in both models. (B) qPCR results confirmed the upregulation of COL4A2 in A30P aSyn-expressing LUHMES cells found by RNA-seq (N=4). (C) qPCR analysis confirmed downregulation of miRNA-29a-3p in A30P mutant aSyn transgenic but not in human WT aSyn transgenic mice (N=4). (D). Data is expressed as mean ± SD and significance level of $p<0.05$ two-tailed was used for statistical analyses. Statistical analyses were performed using one-way ANOVA, with Bonferroni correction (B) and unpaired t-test (C,D).

A30P aSyn increases COL4A2 protein levels in dopaminergic neurons

Next, we investigated the levels of COL4A2 protein in dopaminergic neurons using differentiated LUHMES cells expressing GFP (control), WT aSyn, and A30P mutant aSyn. On the eighth day of differentiation, protein levels where assessed by SDS-PAGE and immunoblotting experiments. Consistently with the mRNA expression results obtained by RNA-seq and qPCR experiments, we observed an increase in the protein levels of COL4A2 only in A30P mutant aSyn-expressing cells compared to the control (Fig 5A,B). To determine the subcellular distribution of COL4A2, we performed immunocytochemistry. COL4A2 was distributed throughout the cell, including in the soma and neurites. Moreover, we observed the increase of COL4A2 levels in A30P aSyn cells comparing to cells expressing WT aSyn or to control cells.

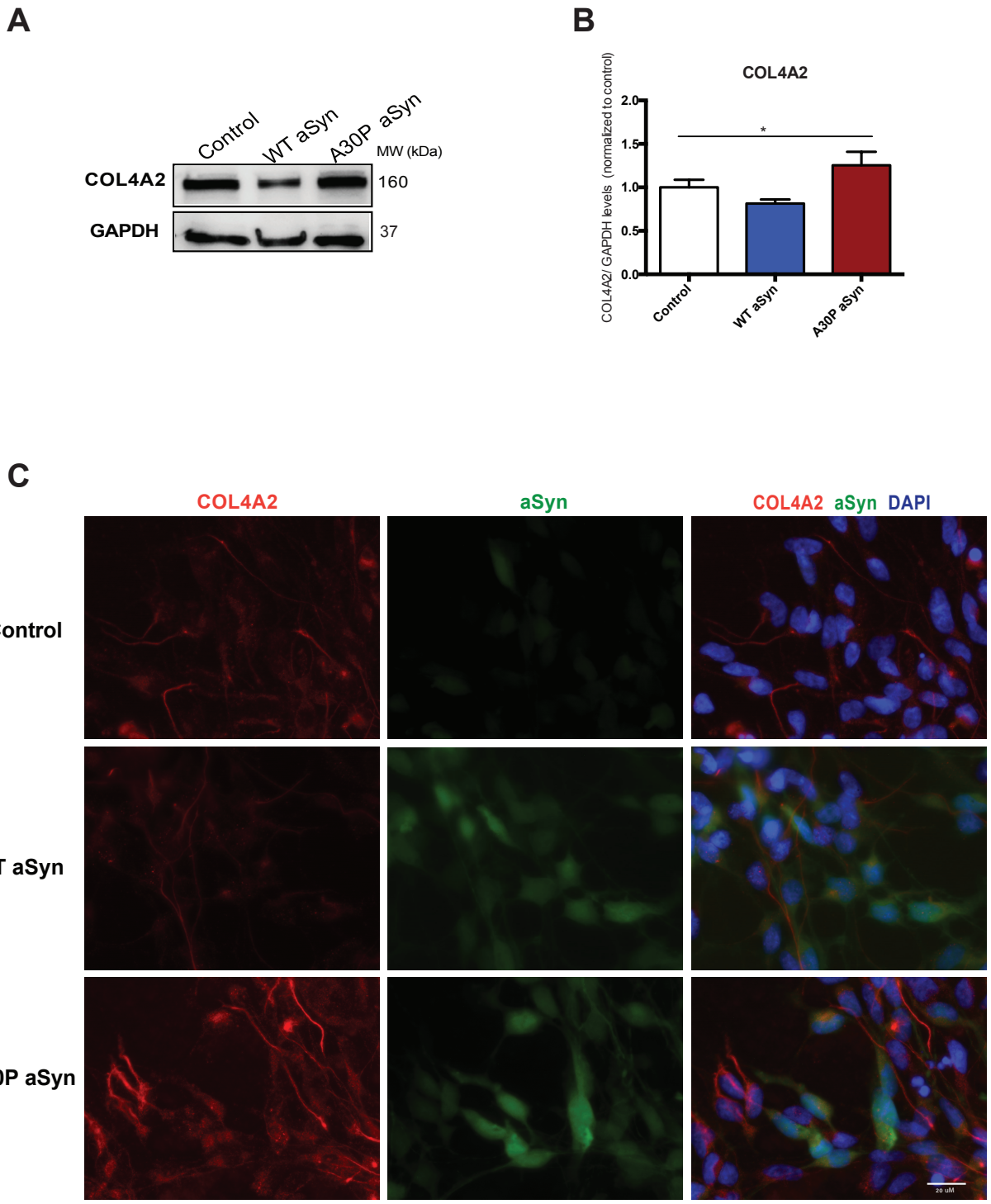

A
Control
WT aSyn
A30P aSyn
MW (kDa)
COL4A2
160
GAPDH
37
B
COL4A2
COL4A2/ GAPDH levels (normalized to control)
2.0
1.5
1.0
0.5
0.0
*
Control
WT aSyn
A30P aSyn
C
COL4A2
aSyn
COL4A2 aSyn DAPI
Control
WT aSyn
A30P aSyn

Figure 5. COL4A2 protein levels in A30P aSyn-expressing dopaminergic neurons. Immunoblot analysis of the levels of COL4A2 in differentiated control, WT and A30P aSyn-expressing LUHMES cells (A). Quantification of immunoblots reveals the upregulation of COL4A2 in A30P aSyn-expressing LUHMES cells but not in cells expressing WT aSyn (B) (N=3). Data is expressed as mean ± SD and significance level of $p<0.05$ two-tailed was used for statistical analyses. Statistical analyses were performed using one-way ANOVA, with Bonferroni correction. (C) Differentiated LUHMES cells were immunostained for COL4A2 and aSyn, and co-stained with DAPI (to label nuclei). Images were acquired for each cell line and show the distribution of COL4A2 both in neurites and cell bodies. The levels of COL4A2 are higher in A30P aSyn-expressing LUHMES cells, confirming the immunoblot data.

A30P aSyn increases ER stress and induced Golgi fragmentation in dopaminergic neurons

As described above, we found several ER-associated pathways deregulated in A30P mutant aSyn transgenic mice (Fig 2). Given the role of COL4A2 in ER stress [40] and the observed upregulation of the gene in A30P aSyn-expressing LUHMES cells and transgenic mice, we asked whether the expression of A30P aSyn affected ER stress. In order to investigate whether aSyn-expressing cells were more sensitive to ER stress, we used LUHMES cells expressing GFP (control), WT aSyn, or A30P mutant aSyn and treated the cells with sub-toxic concentrations of tunicamycin (1ug/mL) at day six of differentiation. Interestingly, A30P mutant aSyn-expressing cells showed in immunoblots elevated levels of calnexin, an ER stress marker [41]. However, WT aSyn expressing cells did not show significant differences in calnexin levels after treatment with

tunicamycin (Fig 6A). Next, we used qPCR to assess the levels of the death receptor 5 (DR5), which is known to be upregulated upon unmitigated ER stress [42]. Consistently with the previous result, we observed increased DR5 levels in A30P mutant aSyn-expressing cells after treatment with tunicamycin, but not in WT aSyn-expressing cells (Fig 6B). These findings indicate that dopaminergic cells expressing A30P aSyn are more sensitive to ER stress than cells expressing WT aSyn.

ER and Golgi are interconnected organelles and are key players in intracellular homeostasis. Thus, we next asked whether Golgi morphology was also affected by aSyn expression. For this, we immunostained differentiated LUHMES cells using an antibody against Giantin, an endogenous transmembrane protein of the cis and medial Golgi complex. The number of cells expressing normal, diffuse, and fragmented Golgi was determined by fluorescence microscopy (Fig 6C) and calculated as percentage of cells (Fig 6D). Interestingly, we found both WTaSyn and A30P expressing cells to have significantly more Golgi fragmentation compared to the control cells expressing GFP. This change in morphology was more robust in A30P aSyn expressing cells. Cells expressing WT aSyn showed a higher percentage of cells with diffuse Golgi morphology. Finally, the percentage of cells with normal Golgi morphology was significantly lower in both in WT and A30P aSyn-expressing cells compared to control cells (Fig 6D). These results demonstrate that both, WT and A30P aSyn, alter Golgi morphology, however, Golgi fragmentation was higher in cells expressing the A30P mutant aSyn.

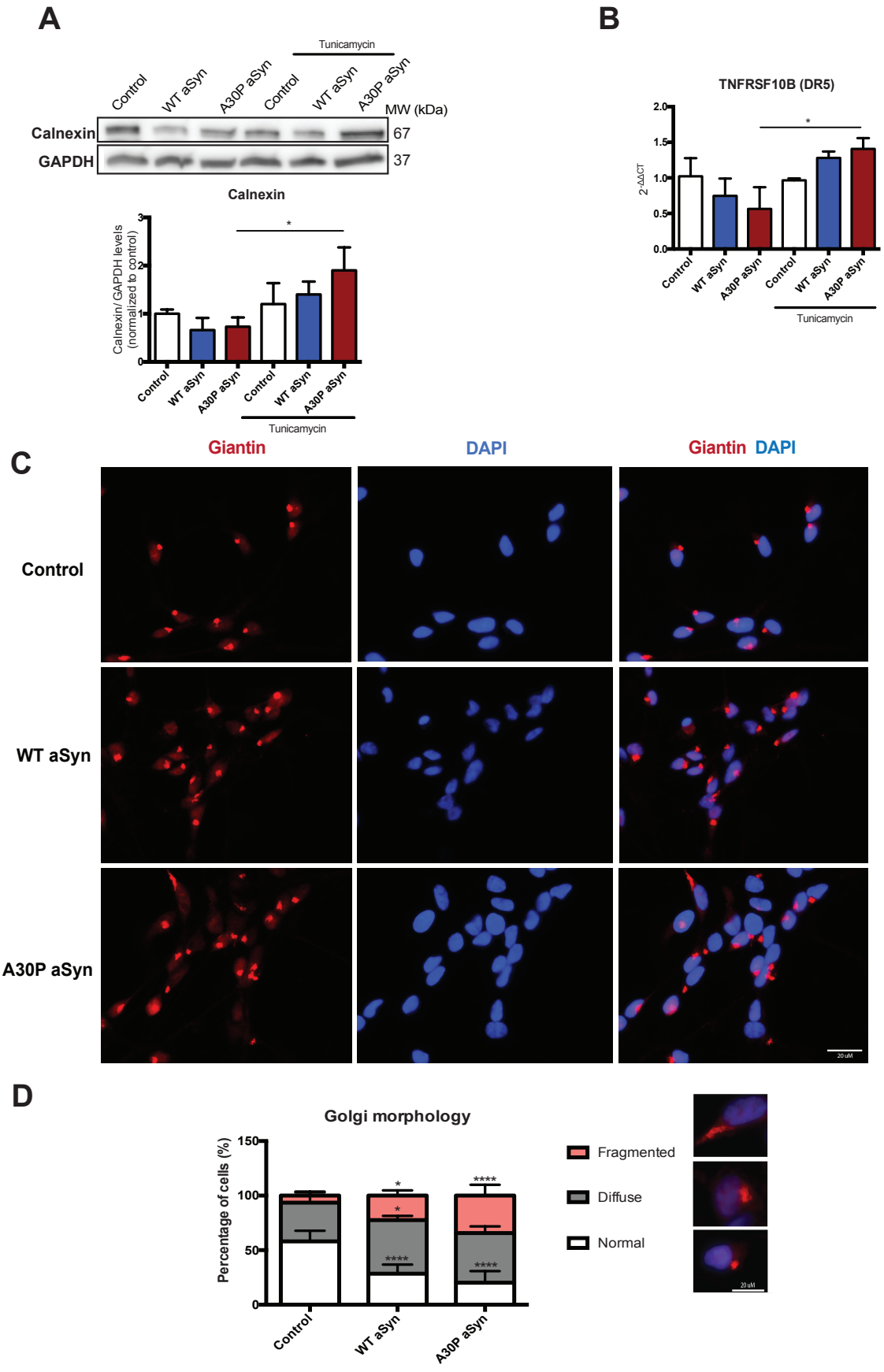

A
Tunicamycin
Control
WT aSyn
A30P aSyn
Control
WT aSyn
A30P aSyn
MW (kDa)
Calnexin
67
GAPDH
37
Calnexin
Calnexin/ GAPDH levels (normalized to control)
Tunicamycin
B
TNFRSF10B (DR5)
2 -ΔΔCT
Tunicamycin
C
Giantin
DAPI
Giantin DAPI
Control
WT aSyn
A30P aSyn
D
Golgi morphology
Percentage of cells (%)
Fragmented
Diffuse
Normal

Figure 6. ER stress and Golgi fragmentation in A30P mutant aSyn-expressing dopaminergic neurons.

Differentiated LUHMES cells expressing GFP (control), WT, or A30P aSyn were treated with tunicamycin (1ug/mL) in order to increase the ER stress. Controls were treated with vehicle (0.1% DMSO). Immunoblots show that, upon treatment with tunicamycin, A30P aSyn-expressing cells display increased levels of calnexin, a marker of ER stress (A) (N=3). No changes were observed in control or in WT aSyn-expressing cells (A). qPCR analysis showed increase of TNFRSF10 (DR5) levels, a marker of ER stress, in A30P aSyn-expressing cells upon treatment with tunicamycin (1ug/mL). No changes were observed in control or in WT aSyn-expressing cells (B) (N=4). (C) Golgi morphology in differentiated LUHMES cells was assessed by immunostaining using an anti-Giantin antibody. Nuclei were visualized with DAPI staining (blue). Representative images are provided. (D) Golgi morphology was classified as normal, diffuse, or fragmented. Statistical analyses showed that both WT and A30P mutant aSyn increase the percentage of cells with fragmented Golgi, but this increase is stronger in cells expressing A30P aSyn. WT aSyn increased the percentage of cells displaying diffuse Golgi comparing to the control cells. The percentage of cells with normal Golgi was significantly decreased in both WT and A30P aSyn-expressing cells (N=3). At least 50 cells were counted and scored per experiment and per condition.

Discussion

In the present study, we provide compelling evidence for a role of aSyn in transcriptional deregulation, uncovering novel biological mechanisms associated with aSyn toxicity. Transcriptional deregulation is attracting increasing attention in the field of neurodegenerative diseases [9, 10, 43, 44]. We previously reported that gene expression profile of peripheral blood may enable to distinguish rapid or slow PD progression in patients [9]. Furthermore, deregulation of gene expression in AD was also found to either increase AD risk or accelerate the disease progression [44].

Using RNA-seq, we found that both WT and A30P mutant aSyn transgenic mice have altered gene expression. This effect was stronger in animals expressing A30P mutant aSyn, and was not due to different expression levels of aSyn, as the protein levels were identical in both lines of transgenic mice. This was consistent with our previous report describing a more robust deregulation in dopaminergic neurons expressing A30P mutant aSyn than those expressing WT aSyn [10]. In order to further understand the possible mechanisms linking transcriptional deregulation with the expression of A30P aSyn, we asked whether A30P aSyn was able to interact with DNA. Consistently with previous studies showing that aSyn binds to DNA [12], we found that A30P mutant aSyn binds to more regions of DNA, including promoter regions, compared to the endogenous mouse aSyn. Interestingly, some of the promoter regions bound by aSyn corresponded to genes whose expression was altered, suggesting that the binding of aSyn to DNA caused transcriptional deregulation. We also speculate that this binding of A30P aSyn to DNA is not simply due to increased expression of the protein, since we observed that WT aSyn binds to fewer sites of DNA despite being expressed at similar levels.

Differential splicing has been observed in several PD-related genes [14, 15]. Thus, we hypothesized that modulation of splicing due to aSyn expression might also contribute to the molecular etiology of PD. Our data showed that A30P mutant aSyn affects exon usage of multiple genes in transgenic mice and in dopaminergic neurons, suggesting possible effects on/interferences with the slicing machinery. Interestingly, a significant number of genes showing differential exon usage did not display differences in expression. This finding suggests that the A30P mutant aSyn affects exon usage in a manner that is not necessarily correlated with gene expression.

We also investigated the possible mechanisms underlying transcriptional deregulation by correlating genes and pathways affected by A30P mutant aSyn expression in transgenic mice. Interestingly, we observed that expression of A30P aSyn in mice and in dopaminergic LUHMES cells resulted in stronger downregulation than upregulation of gene expression. Moreover, A30P aSyn expression in mice downregulated several pathways, including neuronal development and synaptic signaling. This is consistent with previous reports showing that the A30P mutation impairs the physiological synaptic localization of aSyn by blocking lipid raft association, which may contribute to the pathogenesis of familial forms of PD [45]. Furthermore, we found upregulation of genes related to mitochondrial and ER-related pathways in A30P mutant aSyn transgenic mice. This is consistent with previous studies reporting that aSyn promotes mitochondrial dysfunction and ER stress, processes implicated in the onset and progression of PD [7, 19, 46]. In particular, we found that *COL4A2* was upregulated. The *COL4A2* gene encodes for collagen IV, the major protein component of the basement membrane, a specialized extracellular matrix structure that provides support and influences cell

signaling. Moreover, collagen IV has previously been implicated in neurite outgrowth [47]. Mutations in *COL4A2* and *COL4A1* are pleiotropic and contribute to a broad spectrum of disorders, such as myopathy, glaucoma, and hemorrhagic stroke [40, 48]. Interestingly, a possible relation between cerebrovascular diseases, as are stroke and PD, has previously been suggested [49]. Heterotrimers composed of one COL4A2 and two COL4A1 peptides are assembled and modified within the ER and interact with resident proteins to ensure proper folding. Then, they track to the Golgi and are packaged into vesicles for secretion into the vascular basement. Mutations in COL4A1 and COL4A2 result in the accumulation of the heterotrimers within the cell and, in some cases, lead to activation of ER stress responses [39, 40, 50]. Collagen VI, another member of the collagen family, was shown to play a critical role in modulating oxidative stress and autophagy in neurons has previously been reported [51]. Interestingly, although aSyn did not seem to bind to the DNA sequence of the COL4A2 gene, it bound the promoter region of *COL11A1*, which was also found highly upregulated in A30P aSyn transgenic mice. This suggests a putative role of aSyn in regulating expression of collagen-related genes. Furthermore, in order to assess another possible mechanism underlying the upregulation of *COL4A2* in A30P mutant aSyn transgenic mice, we assessed the levels of miR-29a-3p, a miRNA known to target COL4A2 mRNA. Excitingly, we observed that miR-29a-3p was downregulated in A30P mutant aSyn transgenic but not in human WT aSyn transgenic mice. This is consistent with the observed upregulation of COL4A2 in cells and mice expressing A30P mutant aSyn. Interestingly, previous studies correlated the loss of miR-29a with increased levels of BACE1 and Aβ in sporadic AD [52]. Most importantly,

miR-29a-3p is found upregulated in the blood of L-dopa-treated PD patients, and considered as a candidate biomarker for PD [53].

Several studies have been implicated ER stress in the etiology of neurodegenerative diseases [18]. In particular, aSyn can induce ER stress which, in turn, further promotes aSyn aggregation and toxicity [19]. aSyn aggregates can also accumulate in the ER lumen and induce ER stress [54]. Moreover, aSyn inhibits ER to Golgi trafficking, affecting protein maturation and leading to ER stress [55]. Interestingly, we found that dopaminergic neurons expressing A30P mutant aSyn were more prone to ER stress than cells expressing WT aSyn, in line with gene expression changes in ER-related pathways observed in transgenic mice.

Fragmentation of the Golgi apparatus is also a common feature in neurodegenerative diseases, including PD [56-59]. Considering the biological connection between ER and Golgi, we investigated whether Golgi morphology was affected upon aSyn expression. We found that both WT and A30P mutant aSyn increase Golgi fragmentation, but the effect to be more pronounced in A30P aSyn-expressing dopaminergic neurons.

In conclusion, our study underscores the effects of aSyn on transcription, suggesting that the familial mutant A30P aSyn induces stronger gene expression deregulation, leading ER and Golgi dysfunction. Ultimately, our findings provide novel insight into the putative role of aSyn on transcriptional deregulation, thereby uncovering novel targets for therapeutic intervention in PD and other synucleinopathies.

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Conflict of Interest Statement

The authors declare that there is no conflict of interest.

Author contribution

Conceived and designed the project: IP, TO. Performed experiments: IP, KGJ, ES, SB, RH, LG, AR. Analyzed data: IP, GJ, TH, CM, RP, VC, RI, MX. Wrote the manuscript: IP, TFO, PL, JMSH, FB, AF.

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Supporting Information Legends

Table S1. Differential expression analysis between control and aSyn WT transgenic mice. List of genes detected in differential expression analysis from RNA-seq experiments comparing RNA samples from control and WT aSyn transgenic mice.

Table S2. Differential expression analysis between control and A30P aSyn transgenic mice. List of genes detected in differential expression analysis from RNA-seq experiments comparing RNA samples from control and A30P aSyn transgenic mice.

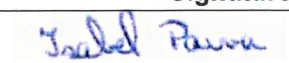
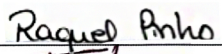
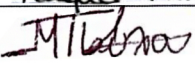
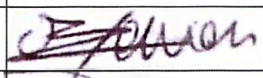
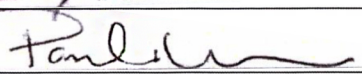
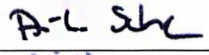
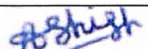
Table S3. ToppGene Suite analysis. Network analysis was performed using ToppGene Suite software. Separate analyses were conducted according to the direction of differential deregulation (up or downregulated) pathways between control *versus* WT aSyn and control *versus* A30P aSyn transgenic mice.

Table S4. aSyn ChIP-seq data in A30P aSyn transgenic mice. ChIP-seq results comparing A30P aSyn transgenic mice with the respective control. Gene body, promoter, and all-gene regions are shown separately.

Supplementary tables: https://owncloud.gwdg.de/index.php/s/kmLTN9l05RA2zyT

Author contribution agreement

We confirm that **Isabel Paiva** contributed in a prominent way to the planning and experiments, analyzes an interpretation of the data, as well as writing the manuscript entitled **"Sodium butyrate rescues dopaminergic cells from alpha-synuclein-induced transcriptional deregulation and DNA damage (2017)** ***Human Molecular Genetics,*** **https://doi.org/10.1093/hmg/ddx114"**. Thus, we agree that this article can be included in her doctoral thesis.

Author	Signature
Isabel Paiva	
Raquel Pinho	
Maria Angeliki Pavlou	
Magali Hennion	
Pauline Wales	
Anna-Lena Schütz	
Ashish Rajput	
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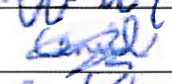
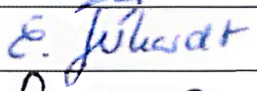
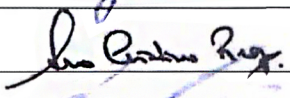
Cemil Kerimoglu	
Ellen Gerhardt	
Ana Cristina Rego	
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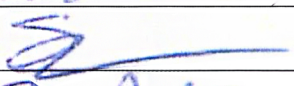
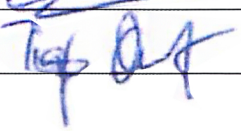
Stefan Bonn	
Tiago F. Outeiro	

Discussion

After >200 years since the original description of PD, the diagnosis and treatment of the disease are still not as developed as necessary. We still lack established biomarkers, and current therapeutics are only symptomatic. Thus, the identification of mechanisms and molecular pathways involved in disease onset and progression are crucial in order to select new targets for therapeutic intervention.

One of the potential mechanisms underlying PD is the process of transcription deregulation, which is receiving increasing attention in the field of neurodegenerative diseases [51, 240, 255, 256]. As shown in a previous study from our group, gene expression profile in peripheral blood of PD patients is distinct between slow and rapid PD progression [51]. Furthermore, other neurodegenerative diseases, such as AD, were also associated with changes on gene expression, that might dictate disease progression [256].

In the present studies, we provide new evidence for the role of aSyn on transcriptional deregulation, using dopaminergic cell lines and transgenic mouse models of PD **(Publications I and II)**.

We observed that, in both LUHMES cells and transgenic mice midbrain, the expression of WT and A30P aSyn promoted transcription deregulation. Interestingly, the mutant A30P aSyn caused a more robust effect in gene deregulation compared to the WT aSyn, in both models **(Publications I and II)**. Consistently, it is already known that both proteins show different biological properties, including differential binding affinity to membranes [257] and different cytotoxicity effect in yeast models [85]. These biological differences can, possibly, be explained by changes in gene expression that we found more significant in A30P aSyn models.

Although we identified many common pathways affected by WT and A30P aSyn in LUHMES cells, other biological processes, including neuronal differentiation and synaptic transmission, were exclusively affected by A30P aSyn. We observed that, LUHMES cells, both WT and A30P aSyn downregulate genes associated to DNA repair

and cell cycle pathways **(Publication I)**. Consistently, previous studies showed that aSyn can regulate cell cycle [258] and has an important role in DNA replication [178]. Although these processes remain unclear in the context of differentiated neurons, such as LUHMES cells, it is known that enhanced oxidative stress and AD-related mutations cause abnormalities in cell cycle re-entry in post-mitotic neurons [259]. Additionally, downregulation of genes related to protein synthesis and cell cycle were identified in PD patients [260].

Furthermore, our RNA-seq analysis showed that expression of both WT and A30P aSyn in LUHMES cells led to downregulation of DNA damage/repair related genes, including BRCA2, FOXM1 and TOP2A. Curiously, the validation of the expression of these genes by qPCR showed a stronger downregulation associated with WT aSyn, while only trend for BRCA2 and FOXM1 in the presence of A30P aSyn. This might indicate that WT aSyn displays a stronger repression of DNA repair genes. Consistently, we observed that aSyn WT increased DNA damage, unlike mutant A30P aSyn, in dopaminergic neurons. Biological markers of DNA damage, such as DNA fragmentation and phosphorylation of p53, were only increased in WT aSyn cells **(Publication I)**. Interestingly, the connection between DNA damage and neurodegenerative diseases has been already described [261-266]. Although the link between transcriptional deregulation of DNA repair genes and PD progression was not extensively investigated, previous study showed that mutations in DNA repair genes, such as ATM, are involved with PD onset [267]. Moreover, mice showing impairment of DNA repair develop typical PD alterations, including dysfunction on mitochondrial respiration, increased levels of phosphorylated aSyn and decreased striatal dopaminergic innervation [57]. Additionally, aSyn expression was shown to increase DNA damage after Fe(II) exposure [268]. We hypothesize that increased DNA damage by WT aSyn expression is caused by lack of DNA repair efficiency and this might be due to changes in transcriptional deregulation, leading to PD development **(Publication I)**.

The impact of aSyn on mitochondrial function, and on oxidative stress, have been studied over the years, since these mechanisms are strongly linked to several neurodegenerative diseases [138, 143, 269, 270]. Furthermore, it is known that high

concentrations of miROS can increase nuclear DNA damage [271]. Interestingly, we found that WT aSyn impaired miROS handling after H_2O_2 exposure, and this was not observed in A30P aSyn cells. These results are consistent with our initial observations, showing that DNA damage promoted by WT aSyn could be associated with the impairment of miROS handling within the cell. This also suggests that besides the lower DNA repair efficiency, WT aSyn affects also mitochondria, leaving dopaminergic cells more prone to oxidative damage **(Publication I)**. Accordingly, in WT aSyn transgenic mice, we identified some downregulated genes related to oxidative stress response, including the heat shock proteins HSPA1A and HSPA1B, and JUN (AP-1 Transcription Factor) **(Publication II)**.

In line with several studies relating aSyn with increased neurotoxicity, we found that aSyn LUHMES cells exhibited increased activation of caspase 3, a marker of apoptosis. Although A30P aSyn did not show increased DNA damage or miROS handling dysfunction, these cells showed higher levels of cleaved caspase 3. These findings suggest that WT and A30P aSyn may induce toxicity through different molecular mechanisms, consistent with other studies demonstrating that A30P aSyn shows distinct biological properties compared to the WT aSyn, as stated above [85, 257]. Furthermore, we also hypothesize that the transcriptional deregulation and increased DNA damage caused by WT aSyn expression are early events in the toxicity process, since no differences were observed in membrane integrity **(Publication I)**.

Our study was the first to connect aSyn expression with transcriptional deregulation associated with lower expression of DNA repair genes and increased DNA damage **(Publication I)**. Nevertheless, it is crucial to explore the mechanisms that might underlie transcriptional deregulation induced by aSyn. Several hypotheses have been considered, including the fact that aSyn is able to interact with DNA [175, 199, 272] and histones [173, 182], affecting chromatin remodeling and modulating transcription. Conformational alterations in the core histones, caused by reversible PTMs, are the main responsible for the switch between transcriptionally active and inactive DNA, by promoting its relaxation and condensation. These epigenetic events are the main physiological players in expressing and silencing specific genes. Interestingly, in line with

previous studies showing that aSyn is able to modulate acH3 levels [173, 178, 182], we observed that both WT and A30P aSyn decrease the levels of acH3 in dopaminergic neurons. We hypothesize that these alteration in acH3 levels might be an intermediary event connected with the overall transcriptional deregulation observed in aSyn cells **(Publication I)**.

Changes in histone conformation and its PTMs are attractive targets for therapeutic strategies in many disorders. This is due to the fact that these changes are common to neurodegenerative diseases and cancer. Among histone modification, the acetylation of lysine is dependent on the antagonistic activity of HATs and HDACs. The use of HDACi has been already approved for clinical trials in several diseases [59] and showed improvements in some neurodegenerative diseases, such as AD [198, 273]. Furthermore, an ongoing Phase I clinical trial using glycerol phenylbutarte (NCT02046434), an HDACi that was shown to decrease aSyn levels in the brain, highlights the potential benefit of using HDACi for synucleinopathies. Other studies showed that two HDACi, SAHA and NaB, rescue the toxicity induced by aSyn [182]. The majority of the HDACi studies do not describe a direct link between aSyn and histone modifications, they rather investigate the effect of the use of HDACi in PD-like features and aSyn-induced toxicity. Interestingly, it was shown that HDACi increase DNA damage in cancer cells, by downregulating transcription and increasing oxidative stress, promoting cell death [59]. In contrast, treatment of neuronal cells with HDACi induced neuroprotection, decreasing the oxidative stress and apoptosis [60]. These contradictory findings suggest that the effect of HDACi depends on the cell type and its concentration and exposure time is a crucial aspect to be considered when assessing toxicity or neuroprotection effects. We showed that, in dopaminergic neurons, the HDACi NaB seems to exert a protective effect in WT aSyn-expressing cells. Treatment with NaB was able to decrease DNA damage in WT aSyn cells, probably by upregulating DNA repair genes, such as FOXM1 and BRCA2. Additionally, an improvement of miROS handling was observed in these cells upon treatment with NaB. Curiously, we observed that NaB restored the levels of acH3 in cells expressing WT aSyn, although we did not find the same effect in A30P aSyn cells **(Publication I)**. This finding is consistent with the different behaviors and cell effects that we have observed between expressing WT or A30P aSyn,

suggesting that the molecular mechanisms behind NaB treatment are distinct between both proteins. Our study suggests that WT aSyn is able to modulate histone acetylation and this might lead to transcriptional changes and DNA damage, which can be rescued by increasing histone acetylation levels using HDACi treatment **(Publication I)**. Nevertheless, it is important to have in mind that given the pleiotropic roles of HDACs and their involvement in cell function, the treatment with HDACi need to be careful studied as they might interfere with several cellular processes, promoting side effects.

In addition to the influence of aSyn expression on histone modification, we also demonstrated that aSyn binds to the DNA, and this might be an additional process leading to transcriptional deregulation **(Publication II)**. We observed that, in transgenic mice, the mutant A30P aSyn binds to more DNA regions compared to the endogenous aSyn **(Publication II)**, while WT aSyn shows less binding (Pinho and Outeiro, under revision). This interesting finding might explain the differences observed in the number of genes affected by WT and A30P aSyn, since we observed, in both models, a higher number of genes affected by the mutant aSyn **(Publications I and II)**. This suggests that, in mice, the binding of A30P aSyn to DNA might affect directly or indirectly the transcription of many genes, while WT aSyn showing less binding only affects a few number of genes. As previously mentioned, other studies also reported an interaction between aSyn and DNA [175, 199, 272] and this binding was found increased in PD brains [175]. Furthermore, other neurodegenerative related proteins, such as Tau, was also implicated in DNA binding and maintenance [174], corroborating the hypothesis that aSyn-DNA binding might be a relevant mechanism underlying neurotoxicity. Nevertheless, the nuclear localization of aSyn is still debatable, with more evidences supporting its localization in the nucleus [92, 175, 183, 274, 275]. Another article that we recently submitted reports extensively the nuclear aSyn and shows that aSyn is present in the nucleus of PD brains, as well as in cellular and mice models of PD (Pinho and Outeiro, under revision).

Besides the aSyn-DNA binding and histone modifications, there are many other processes that can also influence gene regulation. One of them is the alternative splicing which is known to be a crucial mechanism on gene expression modulation [276]. Several

studies refer a connection between alternative splicing and PD, since many PD-related genes were found affected by this process [221, 277]. A possible tool to investigate splicing events is the assessment of exon usage. Interestingly, we observed that A30P aSyn interferes with the exon usage of many genes in transgenic mice and LUHMES cells. Intriguingly, a significant number of those genes did not show any difference in gene expression **(Publication II)**. This suggests that mutant A30P aSyn to interferes with exon usage of several genes but this does not necessarily lead to changes their expression. However, we can hypothesize that this effect on exon usage might be associated with the overall and robust transcriptional deregulation found in the A30P aSyn transgenic mice.

As stated above, we observed that A30P aSyn promotes robust transcriptional deregulation in a much higher level than WT aSyn in transgenic mice. We hypothesize that this could be due to the fact that WT aSyn mice are homozygous while A30P aSyn mice are heterozygous, which could influence the levels of aSyn and, consequently, lead to changes in transcription. However, we observed that 6-months old WT aSyn and A30P aSyn transgenic mice show similar aSyn protein levels, excluding the previous hypothesis. Thus, we further investigated the mechanisms underlying A30P aSyn-induced transcriptional deregulation. Consistently with our findings using A30P aSyn LUHMES cells, we observed that A30P aSyn mice showed higher number of downregulated genes compared to upregulated ones **(Publication I and II)**. This suggests that the mutant aSyn exerts a more robust effect in transcription repression. Furthermore, we observed that expression of A30P aSyn led to changes in expression of genes related to many biological processes previously linked to neurodegenerative diseases, including PD, as previously observed in aSyn LUHMES cells **(Publications I and II)**. In A30P aSyn mice, we identified some deregulated genes related to synaptic signaling and neuronal development, and, interestingly, these pathways were also found deregulated in A30P aSyn LUHMES cells **(Publications I and II)**. This might be one possible explanation that associates this mutant aSyn with the development of early onset PD, since synaptic dysfunction is considered a strong feature of the disease. Previous reports also showed that A30P aSyn alters the physiological synaptic localization by blocking lipid rapid association, which might contribute to neurodegeneration [278]. Moreover, several upregulated genes found in A30P aSyn transgenic mice were related

to mitochondrial and ER-associated biological processes. This finding is consistent with many studies showing that aSyn affects mitochondria mechanisms and increases ER stress, processes previously linked with PD onset and progression [279-281]. Moreover, our approach in selecting the genes that were commonly deregulated in both A30P aSyn models, led us to identify COL4A2 as one of the highest upregulated genes **(Publication II)**. This gene encodes the major protein component of the basement membrane, the collagen IV protein. This specialized extracellular matrix provides support and is crucial in cell signaling mechanisms. Major alterations in the molecular composition of the vascular basement membrane were already associated with neuropathological settings. Interestingly, changes in thickness and composition of the vascular membrane basement are observed during normal aging and in AD [282]. This suggests that components of the basement membrane might be involved in the neurodegeneration process. Additionally, mutations in COL4A2 were already linked to several diseases, including glaucoma, myopathy, and haemorrhagic stroke [283, 284]. An interesting aspect that needs to be further explored is the possible association between PD and cerebrovascular diseases, such as stroke, since previous reports supported this connection [285]. In this line, a recent study showed that focal ischemia, which is related to ischemic stroke, upregulates aSyn expression and its nuclear translocation, indicating aSyn as a promising target for stroke therapy [286]. The heterotrimers composed of one collagen type IV alpha 1 (COL4A1) and alpha 2 (COL4A2) are assembled and modified within the ER, interacting with the resident proteins to ensure proper folding. After tracking to the Golgi, they are packaged into vesicles for secretion into the vascular membrane. In line with the interaction with ER, mutations in COL4A1 and COL4A2 were previously reported to promote accumulation of their heterotrimers within the ER, and this lead to ER stress [284, 287, 288]. Other members of the collagen family, such as collagen VI, were already shown to influence autophagy and oxidative stress in neurons [289]. A previous study demonstrated increased expression of collagen VI in a transgenic mouse model of familial AD, as well as in AD patients, and the authors suggest a neuroprotective role of this protein against Aβ peptides-induced toxicity [290]. These findings combined with our data, showing upregulation of COL4A2 upon A30P aSyn expression, suggest that collagen

proteins might be important targets for further investigation in the field of neurodegenerative diseases **(Publication II)**.

Furthermore, we investigated other mechanisms that could lead to upregulation of COL4A2 upon A30P aSyn expression. Excitingly, we observed that miR-29a-3p, which is known to target COL4A2 mRNA, was highly downregulated in midbrain of A30P aSyn transgenic mice **(Publication II)**. Considering the role of miRNAs in negatively regulating the expression of target genes, the downregulation of miR-29a-3p might decrease the silencing/degradation of COL4A2 mRNA, promoting its upregulation. Contrarily, WT aSyn transgenic mice did not show differences in miR-29a-3p expression, and this might also explain the physiological levels of COL4A2 expression found in these mice **(Publication II)**. The inherent ability of miRNAs to regulate many targets makes them potential candidates for designing therapeutic strategies. Also, understanding the roles that miRNAs play in complex disorders, such as PD, will contribute to improve our understanding of the cellular mechanisms linked to the disease onset and progression. In line with our findings, previous studies linked the expression of miR-29a with sporadic AD [291]. The authors observed that the loss of miR-29a was associated with increased levels of BACE1 and Aβ in sporadic AD patients [291]. Most importantly, upregulation of miR-29a-3p was found in the blood of PD patients treated with L-DOPA and it is considered a potential biomarker for PD [200]. Our findings suggest that miR-29a-3p may play an important role in A30P aSyn-induced toxicity and this might be linked with upregulation of many important targets, such as COL4A2.

As stated above, in A30P aSyn transgenic mice, we observed several deregulated genes that play a role in ER-related pathways **(Publication II)**. Several studies pointed out a strong link between ER stress and etiology of neurodegenerative diseases [292]. In PD, signs of ER stress were observed in post-mortem tissue from cases of sporadic PD and in many animal models of PD [69]. We further investigated whether the upregulation of the ER-related genes could be associated with increased ER stress in aSyn cells. As we have hypothesized, we found A30P aSyn LUHMES cells more prone to ER stress, by modulating the expression of calnexin and DR5 - two important players in ER stress mechanism. This effect was observed only when the cells were challenged with

tunicamycin which is known to induce ER stress **(Publication II)**. Our data suggests that, although A30P aSyn does not seem to increase ER stress in normal conditions, the susceptibility to induce ER stress becomes higher when these cells are exposed to ER stressors. Previous studies also reported a strong connection between aSyn and ER stress. It was previously shown that aSyn aggregates accumulate in the lumen of ER and this leads to ER stress [293]. Other reports stated that aSyn is able to induce ER stress which, in turn, further induces its aggregation and toxicity [279]. aSyn is also known to affect ER to Golgi trafficking, and this disturbs the protein maturation process inducing ER stress [294].

Considering the known association between Golgi disassembly and the progression of some neurodegenerative diseases, such as PD, we asked whether aSyn could influence Golgi morphology. We observed that expressing of both WT and A30P aSyn in dopaminergic neurons altered Golgi morphology, inducing its fragmentation. However, Golgi fragmentation observed was more prominent in A30P mutant aSyn cells **(Publication II)**. This fragmentation can be a result of several mechanisms, including perturbation of microtubules and modifications of Golgi structural proteins, as cleavage or phosphorylation [295].

Our second study provides evidence that A30P aSyn induces stronger gene expression deregulation, compared to the WT protein, leading to dysfunction of ER and Golgi systems.

Overall, both studies demonstrated a strong effect of aSyn on transcription, leading to deregulation, and highlighting different cellular mechanisms modulated by WT and A30P aSyn, such as DNA damage and ER-Golgi systems, respectively.

Conclusion and Future Perspectives

A major goal in PD research is the identification of novel therapeutic strategies towards a cure. Great effort towards this goal has been done within the scientific community, however not successfully. Despite progression in understanding the molecular mechanisms of PD, the complex disease etiology and progression remains unclear. Thus, deciphering the mechanisms linked with aSyn-induced toxicity is critical to further explore the pathobiology underlying PD, as well as other synucleinophaties. Our findings provide new insight into a putative role of aSyn on gene expression, exploring distinct molecular mechanisms associated with genetic alterations in aSyn that are linked to PD familial forms, such as multiplication of WT aSyn and the A30P aSyn point mutation. We observed that both WT and A30P aSyn promoted transcriptional deregulation. However, this effect was more pronounced in A30P aSyn models, in both dopaminergic neurons and transgenic mice. It is currently known that, in PD, not only dopaminergic neurons are affected. Thus, the use of both LUHMES cells and mice midbrain gave us different perspectives on the putative mechanisms specifically deregulated in dopaminergic neurons or in a more complex environment. Single-cell RNA-seq from transgenic mice midbrain would help us to further investigate the cell type-specific transcriptional signatures underlying aSyn expression. Furthermore, the use of aSyn knockout models would help us to further investigate the impact of aSyn on transcription. We observed that aSyn knockdown LUHMES cells exhibited strong transcriptional deregulation. Although this effect needs to be further explored, we hypothesize that this might be explained by the upregulation of bSyn, another synuclein member found in these cells (data not shown).

Furthermore, the higher impact on gene deregulation associated to the mutant A30P aSyn needs to be further investigated. It remains unclear if this effect is directly linked to neuronal toxicity, as we need to consider putative occurrence of compensatory mechanisms. Moreover, we identified some distinct pathways affected by WT and A30P aSyn. While WT aSyn seems to display higher effect on DNA damage and ROS handling,

the mutant A30P aSyn appears to have a stronger impact on ER-Golgi systems. In addition to this, we also aimed to prevent WT aSyn-induced neurotoxicity by using HDACi treatment (NaB). Excitingly, we observed amelioration of DNA damage and mitROS handling impairment caused by WT aSyn. Although this approach allows us to investigate the impact of HDACi in aSyn-induced toxicity in dopaminergic cells, the effect of this treatment in other cell types may be different. For this, the use of transgenic mice of PD could help us to further explore the impact of HDACi in a complex environment as well as possible side effects.

Our study provides additional insight into the use of HDACi in PD, exploring their putative effect in ameliorating cellular mechanisms such as DNA damage. Although we found that NaB upregulates DNA repair genes, which might explain the decrease of DNA damage, the overall effect on transcription should be explored. This would help us to decipher other cellular mechanism involvement with the treatment.

Our findings add significant new knowledge to our current view on the effect of the A30P aSyn familial mutation on transcriptional deregulation, DNA binding, RNA splicing, and ER-Golgi systems. Although the origin of the gene deregulation effect need to be further investigated, we observed that A30P aSyn binds to many DNA sites, including promotor regions. Ideally, the use of different aSyn antibodies would help us to strengthen this finding. Moreover, we observed that A30P aSyn affects differential exon usage of many genes and this is not necessarily correlated with changes in their expression. We also provide evidence that A30P aSyn increases Golgi fragmentation and increase susceptibility to ER stress. Although we found many ER related genes altered upon A30P aSyn expression, the question whether the ER-Golgi alterations observed are due to the transcriptional deregulation needs to be further investigated.

Additionally, the use of both models, LUHMES cells and transgenic mice, led us to find COL4A2 gene, a pro-apoptotic gene that was upregulated upon A30P aSyn expression. Although the mechanisms of COL4A2 upregulation need to be further explored, we observed a downregulation of one of its miRNA target, miR-29a-3p, in A30P aSyn transgenic mice. Since COL4A2 is strongly related with cerebrovascular diseases, such as stroke, our findings strength the connection between PD and these disorders.

However, the modulation of COL4A2 or miR-29a-3p expression in our models of PD would bring us a deeper understanding of their putative function in aSyn-induced toxicity.

Our findings provide novel and compelling insight into the different effects of both WT and A30P aSyn on different cellular processes, including gene deregulation, histone modification, DNA damage, miROS handling and ER-Golgi systems. By uncovering new pathways involved in aSyn pathology, our findings may help define future therapeutic strategies, as well as identify putative biomarkers for PD and other synucleinopathies.

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