Alpha-synuclein-associated alterations at the synapse in models of Parkinson's disease

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“The important thing is not to stop questioning. Curiosity has its own reason for existing. One cannot help but be in awe when he contemplates the mysteries of eternity, of life, of the marvellous structure of reality. It is enough if one tries merely to comprehend a little of this mystery every day.”

Albert Einstein

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List of abbreviations

A30PaSyn	Human A30P alpha-synuclein mutation
A53TaSyn	Human A53T alpha-synuclein mutation
AD	Alzheimer’s disease
AK	Adenylate kinase
aSyn Mono	Alpha-synuclein monomers
aSyn Oligo	Alpha-synuclein oligomers
aSyn PFF	Alpha-synuclein pre-formed fibrils
aSyn	Alpha-synuclein
CNS	Central nervous system
Co-IP	Co-immunoprecipitation
DAT	Dopamine active transporter
DIV	Day in vitro
DLB	Dementia with Lewy bodies
DOPAC	Dihydroxyphenylacetic acid
EM	Electron microscopy
ENS	Enteric nervous system
ER	Endoplasmic reticulum
ERAD	ER-associated degradation
EVs	Extracellular vesicles
GFP	Green fluorescent protein
GO	Gene ontology
haSyn	Human alpha-synuclein
HVA	Homovanillic acid
ICC	Immunocytochemistry
LBs	Lewy bodies
LRRK2	Leucine-rich repeat kinase 2
MAPT	Microtubule-associated protein tau
MEA	Microelectrode arrays
MPTP	1-methyl-4-phenyl-1,2,5,6-tetrahydropyridine
MSA	Multiple system atrophy
NAC	Nonamyloid-beta component
PD	Parkinson's disease
PDD	Parkinson's disease dementia
PINK1	PTEN-induced putative kinase
PKC	Protein kinase c

PLO	Poly-L-ornithine
PNS	Peripheral nervous system
PrP^{C}	Cellular form of the prion protein
PrP^{SC}	Scrapie isoform of the prion protein
PSD95	Postsynaptic density 95
PSTH	Peristimulus time histogram
PTMs	Post-translational modifications
RBD	REM sleep behaviour disorder
REM	Rapid eye movement
SAA	Seed amplification assays
SNAP25	Synaptosomal associated protein 25
SNARE	Soluble N-ethylmaleimide-sensitive factor attachment protein receptor
SNpc	Substantia nigra pars compacta
SV	Synaptic vesicle
Tg	Transgenic
TH	Tyrosine hydroxylase
WT	Wild-type
WTaSyn	Human wild-type alpha-synuclein

Abstract

Parkinson's disease (PD) is one of the most common neurodegenerative disorders that is characterized by the typical motor features, and also by a multiplicity of non-motor symptoms. The main pathological hallmarks of PD are a progressive, and profound, loss of dopaminergic neurons in the substantia nigra, as well as the accumulation of protein inclusions rich in the protein alpha-synuclein (aSyn). Yet, the exact molecular mechanisms linking aSyn to PD remain unknown. Despite the fact that aSyn is involved in producing synaptic vesicles (SVs) and their transport, in SNARE complex assembly, and in modulating synaptic functions, the effect of aSyn accumulation at the synapses remains unclear. Therefore, we sought to investigate the link between the overexpression of aSyn and the major aSyn-related alterations in the synapse.

In our study, we exposed primary hippocampal neurons to recombinant aSyn species, or to aSyn overexpression using a lentiviral vector encoding for aSyn, and systematically analysed the effects on presynaptic function. Although the neurons exhibited a robust expression of aSyn, aSyn did not result in a direct effect on neurotoxicity, in a change in synaptic protein levels, in dendrite length, or in spontaneous neuronal activity. We used a differential ultracentrifugation protocol to purify SVs from 3-month-old transgenic mice overexpressing human wild-type aSyn (WTaSyn) and from control wild-type (WT) mice and provided a proteomic and functional characterization of these vesicles. The proteomic analysis of SVs from both animals revealed specific protein composition and pathways enrichment.

Furthermore, we analysed the synaptic proteins levels in the brains of Tg and WT mice and we did not observe any significant changes. These results were consistent with the results from the cellular model. Finally, we quantified the aSyn levels in several biofluids from 3 m.o, 6 m.o and 9 m.o old transgenic mice overexpressing A30P mutant aSyn (A30PaSyn), WTaSyn, and WT. Interestingly, serum and plasma aSyn levels were increased in A30PaSyn mice when compared to age-matched controls for the three-time points, suggesting that serum and plasma aSyn could be a reliable biomarker to distinguish PD from healthy controls.

Overall, our work indicates that even if the levels of aSyn are increased, affecting the protein composition and/or biological processes associated with SVs, in the end, overexpression of the protein does not, per se, induce significant synaptic dysfunction in the models used. A clear understanding of the precise mechanisms that correlate some biological effects of neurodegeneration with the accumulation of specific aSyn species

is fundamental for understanding the molecular underpinnings of PD. Overcoming the limitations that have hampered the identification of these mechanisms can contribute to the improvement of diagnostic accuracy and the development of novel targets for therapeutic intervention in PD and other synucleinopathies.

CHAPTER 1

INTRODUCTION

1.1. Neurodegenerative diseases

Neurodegenerative diseases are a diverse group of disorders generally characterized by the progressive loss of nerve cells of the central nervous system (CNS) and/or peripheral nervous system (PNS). Tauopathies, amyloidosis, synucleinopathies, and TAR DNA binding protein-43 (TDP-43) proteinopathies are the most common neurodegenerative diseases. They are classified according to three different parameters such as (i) clinical features, (ii) anatomic distribution of neurodegeneration, or (iii) principal molecular abnormality (Dugger and Dickson, 2017). Despite the typical classification of these diseases, they share several processes associated with the spreading of misfolded proteins, neurodegeneration, neuroinflammation, mitochondrial and axonal transport dysfunction, intracellular protein degradation systems dysfunction, and oxidative stress (Dugger and Dickson, 2017). Moreover, the misfolded proteins associated with these disorders are commonly found before the onset of the typical clinical symptoms. Clinical symptoms occur years after the neurodegenerative processes have started, and loss of neurons is irreversible. For that reason, the need for early biomarkers is crucial. Several neurodegenerative processes can be present in a patient, leading most patients to have a mix of clinical features (Adler et al., 2010; Dugger et al., 2014b, 2014a; Eisenberg and Jucker, 2012). This is one of the motives why the complete diagnosis is only made at autopsy after a meticulous neuropathological assessment. There have been significant advancements in treatments and therapies that enhance the quality of life for people with neurodegenerative diseases. Nevertheless, these interventions are not entirely effective, and finding a cure remains a crucial challenge. Therefore, it is imperative that we prioritize research efforts towards uncovering the mechanisms underlying neurodegenerative disorders. To this end, researchers should continue investigating novel biomarkers such as biofluid and molecular imaging markers (Kolb and Andrés, 2017; Seeley, 2017), and developing innovative therapeutic strategies. By doing so, we can take a step closer towards finding a permanent solution to these debilitating conditions.

1.2. Synucleinopathies

Neurodegenerative diseases are generally characterized by the accumulation of pathological proteins, such as alpha-synuclein (aSyn) in synucleinopathies (Spillantini and Goedert, 2016). Synucleinopathies are a group of disorders featured by progressive aggregation in amyloid-like filamentous inclusions of insoluble, misfolded aSyn protein

in neurons and glial cells, associated with the neurodegeneration of multiple systems. Parkinson's disease (PD), PD dementia (PDD), multiple system atrophy (MSA), and dementia with Lewy bodies (DLB) are some of the most common synucleinopathies (Gai et al., 1998; Spillantini et al., 1997; Wakabayashi et al., 1997). Both genetic (e.g., mutations in the aSyn gene) and environmental factors are involved in the aetiology of synucleinopathies. However, nongenetic factors, as disturbances in the aSyn metabolism (e.g., increase in aSyn synthesis and formation of fibrillary forms due to a degradation impairment), also play a role in most cases, probably in interaction with susceptibility genes (Chiti and Dobson, 2017; Jellinger, 2010). Protofibrillar rather than fibrillar forms of aSyn have been found to be cytotoxic. However, aSyn contained in Lewy bodies (LBs) may act as a structural cytoprotective mechanism, confining or eliminating toxic species. Nevertheless, significant levels of intracellular and neuritic protein aggregation may ultimately contribute to dysfunction and neuronal death. The question of whether LBs and other aSyn aggregates are harmful or protective remains unsolved. Albeit their formation may reflect one of many CNC response patterns to upstream dysregulation of aSyn metabolism, the aSyn pathway appears to be a crucial element in the selective loss of neuronal and glial in multiple systems of synucleinopathies, such as PD.

1.3. Parkinson's disease

PD is the second most common neurodegenerative disease, after Alzheimer's disease (AD), and is the most common movement disorder. In 1817, PD was described for the first time by James Parkinson in his "An Essay on the Shaking Palsy" (Parkinson, 2002). Unfortunately, the molecular mechanisms causing this disorder remain unclear. Clinical diagnosis of PD still relies on the identification of motor symptomatology, such as resting tremor, rigidity, bradykinesia, and postural instability (Jankovic, 2008; Moore et al., 2005). These symptoms are a consequence of deregulation in the basal ganglia activity due to the progressive loss of dopaminergic neurons in the *substantia nigra pars compacta* (SNpc) (Dauer and Przedborski, 2003). However, PD patients also experience a multitude of non-motor symptoms such as hyposmia, constipation, fatigue, depression, anxiety, speech and swallowing difficulties, rapid eye movement (REM) sleep disorder, and cognitive and behavioural problems (Pandya et al., 2008; Ziemssen and Reichmann, 2007) which, together, have a tremendous impact on the quality of life (Figure 1).

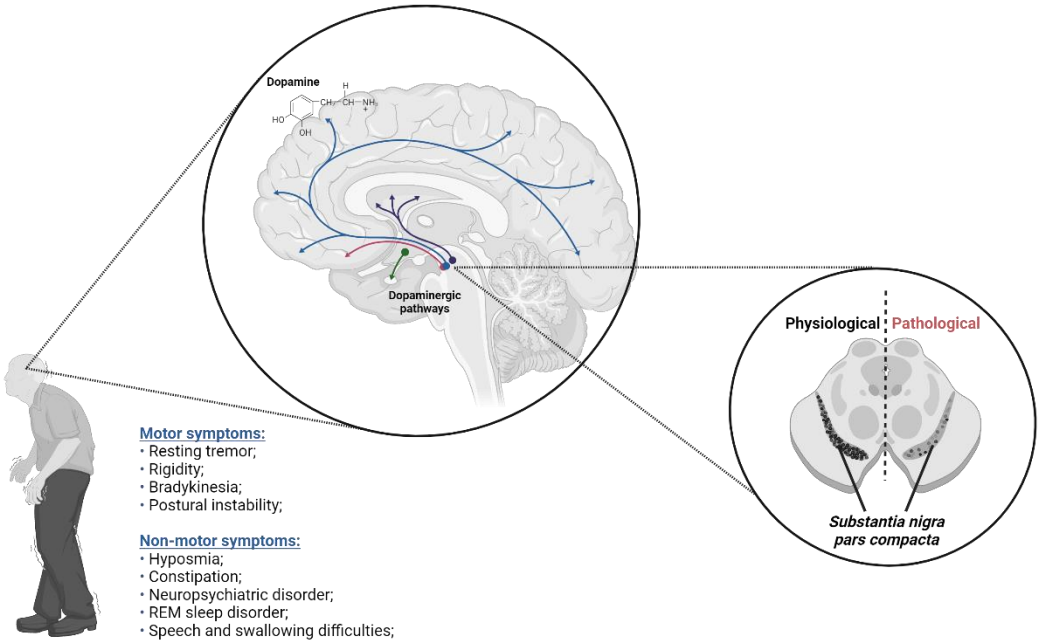


Figure 1 | Pathophysiology of Parkinson's disease (PD). PD is primarily identified by the presence of motor symptomatology, such as resting tremor, rigidity, bradykinesia, and postural instability. These symptoms are a consequence of the progressive loss of dopaminergic neurons in the *substantia nigra pars compacta* (SNpc). In addition to the motor symptoms, non-motor symptoms, such as hyposmia, constipation, neuropsychiatric disorder, rapid eye movement (REM) sleep disorder, and speech and swallowing difficulties are also linked to PD. The image was created using BioRender.com.

The first pathological alterations in PD dates back to 1912 when Friedrich Lewy described the presence of intracellular inclusion bodies in post-mortem brain tissue of patients who had suffered from shaking palsy (Lewy, 1912). These protein inclusions enriched in aSyn are known as LBs and Lewy neurites (LNs) and are considered the major hallmark of PD and are involved in the pathogenesis of synucleinopathies (Spillantini et al., 1997, 1998). In addition to aSyn, these aggregates also contain neurofilaments, ubiquitin, synphilin-1, torsin A, and heat shock proteins (Jellinger, 2012; Olanow et al., 2004; Spillantini et al., 1998).

Even though genetic and environmental factors favour neurodegenerative diseases, the exact molecular and cellular mechanism(s) that initiated these diseases are still unclear. Moreover, the diagnosis of these diseases is still based on the identification of motor symptoms, which can begin years after the first neuropathologic alterations emerge. This highlights the need to find biomarkers for an early diagnosis. Since aSyn misfolding and aggregation occur before the clinical features, the study of the mechanisms behind it, maybe would allow us to do an earlier diagnosis at a prodromal stage. Different hypotheses have been proposed to explain these mechanisms, such as the dual-hit hypothesis, which correlates the LB pathology with the temporal PD features, suggesting

that the pathology starts from the periphery (nose and gut) and spreads through the brain (Braak et al., 2003a). Recent research has also shown that PD pathology can spread from damaged to healthy neurons, as evidenced by Lewy pathology in PD patients who had received fetal nigra tissue grafts (Kordower et al., 2008a; Li et al., 2008). Moreover, aSyn has been found to exhibit prion-like behavior (Visanji et al., 2014). Given its key role in synucleinopathies and Lewy pathology, aSyn has been the subject of extensive research as it is also involved in other biological processes that contribute to the progression of PD pathology (Brás et al., 2020).

1.4. Aetiology of Parkinson's Disease

PD has a multifactorial aetiology that involves aging, genetics, and environmental factors (Riess and Krüger, 1999). It is estimated that 90% of PD cases are sporadic/idiopathic (Collier et al., 2011), with advancing age being the main risk factor. Moreover, exposure to certain environmental factors, such as 1-methyl-4-phenyl-1,2,5,6-tetrahydropyridine (MPTP), paraquat, or rotenone promotes dopaminergic neurons degeneration and chronic parkinsonism, mimicking similar effects to those observed in sporadic PD cases (Di Monte et al., 2002). Additionally, PD has been associated with exposure to iron, pesticides, and channel blockers (Delamarre and Meissner, 2017; Elbaz and Moisan, 2008), although their effects and the mechanisms involved are still unclear.

While PD symptoms are generally associated with the CNS, the disease also affects PNS tissues, particularly the gastrointestinal tract. The presence of LBs in the gut (Mulak and Bonaz, 2015), and emerging evidence suggest that alterations in the gut microbiota might induce systemic inflammation and contribute to aSyn misfolding that incites PD development (Sampson et al., 2016). This concept aligns with the Braak hypothesis, which proposes that PD pathogenesis begins in the PNS and then progress to the CNS (Braak et al., 2003a). A detailed description of the Braak staging of PD can be found in chapter 1.4.5..

1.5. Genetics of Parkinson's Disease

Only 10% of PD cases are associated with a familiar genetic forms due to alterations in various genes (Crosiers et al., 2011; Singleton et al., 2013). In 1997, the first mutation associated with PD was identified in the *SNCA* gene, which encodes for aSyn

(Polymeropoulos et al., 1997). Since then, studies have shown that aSyn is highly abundant in LBs (Spillantini et al., 1998), and it is associated with both sporadic and familial forms of PD.

To this date, missense mutations in the *SNCA* gene, as well as gene duplications and triplications, have been linked to autosomal dominant forms of PD (Chartier-Harlin et al., 2004; Ibáñez et al., 2004). In contrast, mutations in PTEN-induced putative kinase (PINK1), PARK7, DJ1 (Goldberg et al., 2005), leucine-rich repeat kinase 2 (LRRK2) (Klein and Westenberger, 2012), and ATP13A2, among other genes, are related with autosomal-recessive forms of PD. Although it is not clear whether aSyn loses function or gains cytotoxic function, these findings show that alterations in the levels or structure of aSyn are involved in familial PD. Besides the presence of insoluble, aggregated aSyn in LBs, the contribution of this protein in sporadic forms of the disease was further strengthened by studies of genome-wide association identified a few polymorphisms in non-coding regions of the *SNCA* locus, depicting a genetic risk factor for the sporadic form of PD (Edwards et al., 2010; Satake et al., 2009; Simón-Sánchez et al., 2009). Lately, significant advances in the genetic mechanisms underlying PD pathogeneses have been made. Up to the present, several genetic alterations associated with PD were found in many chromosomal regions, and various proteins were identified in LBs of PD brains (Table 1). Nonetheless, aSyn is thought to be a central player in PD pathology (Goldman et al., 1983; Kuzuhara et al., 1988).

Table 1 | Known genetic loci associated with Parkinson's disease.

Locus	Gene	Protein	Description	Age of onset	Reference
PARK 1/4	SNCA	aSyn	Presynaptic/ nuclear protein	30-40s, fast progression	(Polymeropoulos et al., 1997)
PARK2	Parkin	Parkin	Ubiquitin ligase	20s, slow progression	(Kitada et al., 1998)
PARK3	SPR (?)	Sepiapterin reductase	NADPH-dependent reduction catalyser	60s	(Gasser et al., 1998)
PARK5	UCH-L1	Ubiquitin carboxy-terminal L1	Ubiquitin protease	50s	(Liu et al., 2002)
PARK6	PINK1	Pten-induced kinase 1	Mitochondrial protein kinase	30s	(Hatano et al., 2004)
PARK7	DJ-1	DJ1	Multifunctional protein	30s, slow progression	(Bonifati et al., 2003)
PARK8	LRRK2	Leucine-rich repeat kinase 2	Leucine-rich repeat kinase 2	40s,	(Gasser, 2009)
PARK9	ATP13A2	ATPase type 13A2	Lysosomal ATPase	Juvenile, atypical	(Ramirez et al., 2006)

PARK10	(?)	?	?	50s	(Li et al., 2002b)
PARK11	GIGYF2	GRB10 interacting GYF protein 2	Translation initiation repressor	Late onset	(Lautier et al., 2008)
PARK12	ATP6AP2	ATPase H+ Transporting Accessory Protein 2	ATPase H+ Transporting Accessory Protein 2	Juvenile and early onset	(Pankratz et al., 2002)
PARK13	Omi/ HTRA2	HtrA serine peptidase 2	Serine Protease	50s	(Strauss et al., 2005)
PARK14	PLA2G6	Phospholipase A2, group VI	Phospholipase	Juvenile, atypical	(Paisan-Ruiz et al., 2009)
PARK15	FBXO7	F-box protein 7	F-box protein	Juvenile	(Shojaee et al., 2008)
PARK16	(?)	?	?	?	(Satake et al., 2009)
PARK17	VPS35	Vacuolar protein sorting 35 Homolog	Retromer Complex	Late onset	(Wider et al., 2008)
PARK18	EIF4G1	Eukaryotic translation initiation factor 4 gamma, 1	Translation Initiation Factor	Late onset, mild	(Chartier-Harlin et al., 2011)
PARK19	DNAJC6	HSP40 Auxilin	DNAJ/HSP40 homolog, Subfamily C, Member 6	Juvenile, atypical	(Edvardson et al., 2012)
PARK20	SYNJ1	Synaptojanin 1	Phosphatase	Early onset	(Krebs et al., 2013; Quadri et al., 2013)
PARK21	DNAJC13	DNAJ/Hsp40 Homolog, Subfamily C, Member 13	Co-chaperone	Late onset	(Vilariño-Güell et al., 2014)
PARK22	CHCHD2	Coiled-coil-helix-coiled-coil-helix domain containing 2	Transcription factor	Late onset	(Funayama et al., 2015)
PARK23	VPS13C	Vacuolar protein sorting 13 homolog C	Lipid transfer protein	Early onset	(Lesage et al., 2016)

1.6. Alpha-synuclein

The synuclein protein family was first described and isolated from the *Torpedo californica*, being expressed in the nucleus and the presynaptic terminal (Maroteaux et al., 1988). Thus, due to its cellular localization (SYNapse, NUCLEus, and protEIN), the name synuclein was entitled to this family of proteins (Maroteaux et al., 1988). This family is composed of three members: alpha-, beta-, and gamma-synuclein (aSyn, bSyn, and gSyn, respectively) (Lavedan, 1998). These proteins have in common a strongly

conserved alpha-helical lipid-binding motif (Clayton and George, 1998). Despite the similarity in their sequence, these proteins have different biological properties and various functions in the cells.

Synucleins are ubiquitously expressed and are notably enriched in neurons. In particular, aSyn is mainly expressed in the *neocortex*, *striatum*, *hippocampus*, *thalamus*, and *cerebellum* (Jakes et al., 1994; Nakajo et al., 1990). In the brain, this protein is primarily localized in the soma of immature neurons and then is concentrated in the synapses at presynaptic terminals of mature neurons (Bayer et al., 1999; Hsu et al., 1998; Lavedan, 1998; Withers et al., 1997). During ageing, aSyn changes its localization to the soma and is often interpreted as a marker of developing pathology in PD (Chu and Kordower, 2007; Collier et al., 2011). Interestingly, synuclein knockout animal models did not show major brain morphological changes (Abeliovich et al., 2000; Anwar et al., 2011; Burré et al., 2010; Chandra et al., 2004; Greten-Harrison et al., 2010). However, in the triple knockout model, rations in the synaptic organization and transmission, neuronal dysfunction, and reduced survival rate were observed, suggesting that synucleins contribute to long-term synaptic function. Despite being studied in the context of some diseases, aSyn has appeared as a relevant player in the progression of PD pathology (Brás et al., 2020). aSyn is the main component of LBs and LNs which are involved in the pathogenesis of synucleinopathies (Spillantini et al., 1997, 1998).

aSyn is a small 140 amino acid protein, encoded by the *SNCA* gene located at chromosome 4q22.1, and it is defined as an intrinsically disordered protein (Polymeropoulos et al., 1997). The primary structure of aSyn can be characterized by three main regions: residues 1- 60 comprise the N-terminal domain (amphipathic region); residues 61- 95 delimit the central hydrophobic nonamyloid-beta component (NAC) domain (hydrophobic region); and residues 96- 140 define the hydrophilic glutamate-rich C-terminal region (with the acidic tail) (Longhena et al., 2019) (Figure 2). The N-terminal domain contains seven conserved KTKEGV repeats followed by a variable short hydrophilic tail (Lavedan, 1998). It is unfolded in solution and lipid binding is possible due to the amphipathic structure formed by the repeats. For that reason, aSyn interacts with vesicular structures and it is mostly attached to phospholipid membranes (Chandra et al., 2003; Clayton and George, 1998; Outeiro and Lindquist, 2003). The NAC domain is highly hydrophobic and prone to aggregation (Ueda et al., 1993), while the hydrophilic C-terminal region is in general unstructured and contains mostly 25 charged amino acid residues, such as glutamate and aspartate. The acidic C-terminal is associated with several protein interactions, as with ions and metals (Brown, 2007; Nielsen et al., 2001;

Paik et al., 1999). This domain can reduce the aggregation propensity since the absence of this domain increases aSyn fibrilization (Crowther et al., 1998; Liu et al., 2005; Sang et al., 2002). Rare familial forms of PD are associated with missense mutations in the *SNCA* gene, as well as gene duplications (Chartier-Harlin et al., 2004; Ibáñez et al., 2004) and triplications (Singleton et al., 2003).

In addition, polymorphisms in the regulatory elements of the *SNCA* gene increase the predisposition to develop PD and have an early onset of the disease (Maraganore et al., 2006). aSyn missense mutations are located within the membrane-binding region at the N-terminal and lipid binding is only affected by A30P, G51D, and A53E mutations (Fares et al., 2014; Ghosh et al., 2014; Jo et al., 2002).

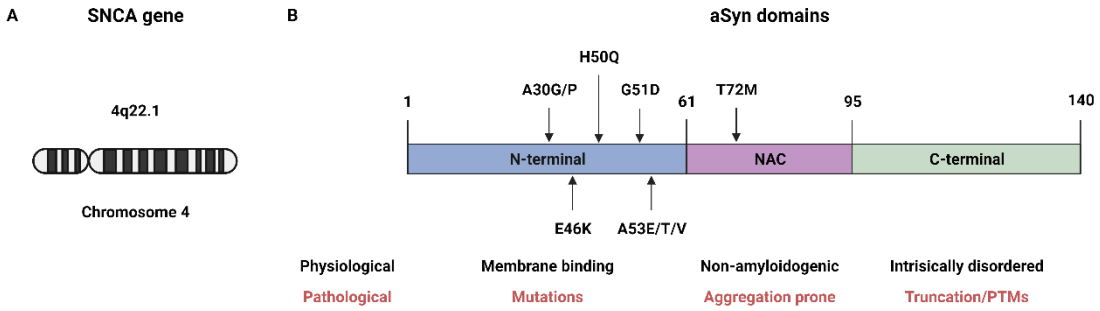


Figure 2 | Schematic representation of alpha-synuclein (aSyn) and familial mutations. (A) *SNCA* gene, located on chromosome 4q22.1 coding for aSyn, a presynaptic neuronal protein. **(B)** aSyn is a 140 amino acid protein composed of three general domains: the N-terminal amphipathic domain (residues 1-60; blue), the non-amyloid-β component (NAC) (residues 61-95; purple), and the C-terminal domain (residues 96–140; green). The N-terminal amphipathic domain has a helical folding propensity and it is responsible for membrane/lipid binding. It contains the amino acid residues affected by the main aSyn gene mutations (A30G, A30P, E46K, H50Q, G51D, A53E, and A53T) associated with familial PD. The NAC domain is highly hydrophobic and is involved in aSyn aggregation. The unstructured C-terminal is highly acidic and the interaction with other proteins and metals is through this domain. Also, many post-translational modifications (PTMs), including phosphorylation at S129, occur in this domain, and truncations in this region are linked with enhanced aggregation.

Various post-translational modifications (PTMs) have been described in the C-terminal domain. This includes truncation, phosphorylation, acetylation, glycation, glycosylation, oxidation, nitration, sumoylation and ubiquitination (Dorval and Fraser, 2006; Zhang et al., 2019). These modifications change the structure and charge of the protein, resulting in alterations in the way it interacts and binds to other proteins and lipids. Notably, aSyn phosphorylation may regulate membrane binding, oligomerization, and neurotoxicity, being serine 87 and 129 (S87 and S129), the major phosphorylation sites (Chen and Feany, 2005; Eun et al., 2006; Ishii et al., 2007; K.E. et al., 2010; Okochi et al., 2000; Pronin et al., 2000). Moreover, LBs are enriched in phosphorylated aSyn at S129, and

this PTM has been considered a PD pathological hallmark (Anderson et al., 2006; Fujiwara et al., 2002; Hasegawa et al., 2002).

Normally, aSyn exists in equilibrium between a membrane-bound and a soluble state. Cytosolic aSyn is natively unfolded and soluble in cells (Burré et al., 2013; Fauvet et al., 2012; Weinreb et al., 1996), while when bound to lipidic membranes assumes an alpha-helical structure (Bussell, 2005; Davidson et al., 1998; Eliezer et al., 2001; Perrin et al., 2000). Also, aSyn oligomerizes into multimers upon membrane binding (Burré et al., 2014; Wang et al., 2014). Under pathological conditions, aSyn adopts conformation rich in beta-sheet amyloid generally related to its aggregation and cell neurotoxicity (Conway et al., 2000a; Ding et al., 2002; El-Agnaf et al., 1998; Lashuel et al., 2002; Uversky, 2007).

The precise function of aSyn is still unknown but it interacts with various proteins, such as actin (Esposito et al., 2007; Sousa et al., 2009), tubulin, synphilin-1, the microtubule-associated protein tau (MAPT), tyrosine hydroxylase (TH), protein kinase c (PKC) and Bcl-2-associated death protein (Huang et al., 2004). aSyn is also involved in multiple cellular processes such as maintaining cell structure and protein trafficking by interacting with diverse cytoskeletal proteins (Alim et al., 2002; Jensen et al., 1999; Prots et al., 2013; Thayanidhi et al., 2010), transmembrane transport and in the formation of synaptic vesicles (SVs) *in vitro* and *in vivo* (Diao et al., 2013; Eisbach and Outeiro, 2013; Sancenon et al., 2012). Additionally, aSyn plays a crucial role in stabilizing the effects of soluble N-ethylmaleimide-sensitive factor attachment protein receptor (SNARE)-complex assembly (Burré et al., 2010; Vekrellis et al., 2011), and modulating synaptic functions (Abeliovich et al., 2000; Burré, 2015; Dikiy and Eliezer, 2012; Nemani et al., 2010).

When overexpressed in certain models, aSyn aggregates and promotes cytotoxicity impairs autophagy and mitochondrial processes due to the generation of H_2O_2, increases sensibility to oxidative stress, compromises vesicular transport, and disrupts the trafficking between the endoplasmic reticulum and Golgi (Cooper et al., 2006; Guardia-Laguarta et al., 2014; Hernández-Vargas et al., 2011; Klucken et al., 2012; Martin, 2006; Nakamura et al., 2011; Outeiro and Lindquist, 2003). All of these impaired mechanisms are likely to contribute to the onset and development of neurodegeneration.

The precise link between the formation of aSyn inclusions and their cytotoxic effects remain unclear, leaving uncertain, whether aSyn is a culprit or a bystander is still unknown. Recent evidence suggests that small oligomeric forms of aSyn play an

important role in neurodegenerative disease, impairing synaptic transmission (Diógenes et al., 2012). These forms are thought to be more toxic (Karpinar et al., 2009; Winner et al., 2011a) (Karpinar et al., 2009; Winner et al., 2011a) than LBs themselves (Outeiro et al., 2008). However, limitations in the ability to correlate some biological effects of neurodegeneration with the accumulation of specific aSyn species have hampered the identification of therapeutic targets.

1.6.1. Alpha-synuclein aggregation and cytotoxic species

The classical view of aSyn as an unfolded, monomeric protein was challenged in 2011 when two studies discussed the possible existence of an aSyn tetrameric conformation, enriched in alpha-helical structure (Bartels et al., 2011; Wang et al., 2011). This tetrameric conformation is still controversial and has not been confirmed in other studies showing mainly a monomeric and disordered state of aSyn (Binolfi et al., 2012; Waudby et al., 2013). However, novel studies suggest that, under physiological conditions, aSyn displays as a tetramer and shifts between tetrameric to monomeric states, as a consequence of mutations in the N-terminal region, leading to a raise in cellular toxicity (Dettmer et al., 2015).

The mechanisms involved in the aggregation pathway of aSyn and the precise link between the formation of aSyn inclusions and cytotoxicity are still a matter of debate. However, a better understanding of these mechanisms is essential for identifying the toxic species that trigger synucleinopathies. These aSyn species can differ in size, morphology, toxicity, binding affinity with other molecules, and seeding potential (Peelaerts and Baekelandt, 2016; Peelaerts et al., 2018). Ribbons and oligomers tend to spread faster than fibrils, but only ribbons can accumulate phosphorylated aSyn (Gribaudo et al., 2019; Rey et al., 2019). Some evidence suggests that small oligomeric forms of aSyn play an important role in neurodegenerative disease, impairing synaptic transmission (Diógenes et al., 2012). Some studies pointing toward these forms are thought to be more toxic (Karpinar et al., 2009; Winner et al., 2011a) than LBs themselves (Outeiro et al., 2008), while other studies suggest the aggregates as the toxic species (El-Agnaf et al., 1998; Tanik et al., 2013). Studies, where aSyn tends to form stable oligomers, are associated with high cytotoxicity (Fusco et al., 2017; Ingelsson, 2016). During oligomerization, dimers form first, followed by soluble and non-fibrillar oligomeric species of varied morphology (annular, chain-like, and spherical). Later, they can be converted into protofilaments, protofibrils, and amyloid fibrils which can be

defined as amorphous or amyloid-like aggregates (Hijaz and Volpicelli-Daley, 2020). The spherical oligomers can be converted into ring-like structures that can permeabilize membranes by forming pores-like structures and altering membrane potential and ion distribution (Kim et al., 2009; Lashuel et al., 2002).

According to a generally accepted hypothesis, different aSyn species are involved in the process (Peelaerts et al., 2015). Misfolded, monomeric aSyn can lead to the formation of oligomers, intermediate species, that may evolve into larger insoluble aggregates. Additionally, more recent findings have also shown that aSyn oligomerizes into multimers on membrane binding (Burré et al., 2010, 2014; Wang et al., 2014), indicating that aSyn might be constantly shifting from a monomeric to a functional oligomeric state (Figure 3). These new findings reinforce the importance of distinguishing between a functional and a toxic oligomer, and determining whether aSyn is a culprit or a bystander protein in the numerous processes that contribute to synucleinopathies. It is crucial to make this distinction in order to better understand the underlying mechanisms of these diseases and develop effective therapeutic interventions.

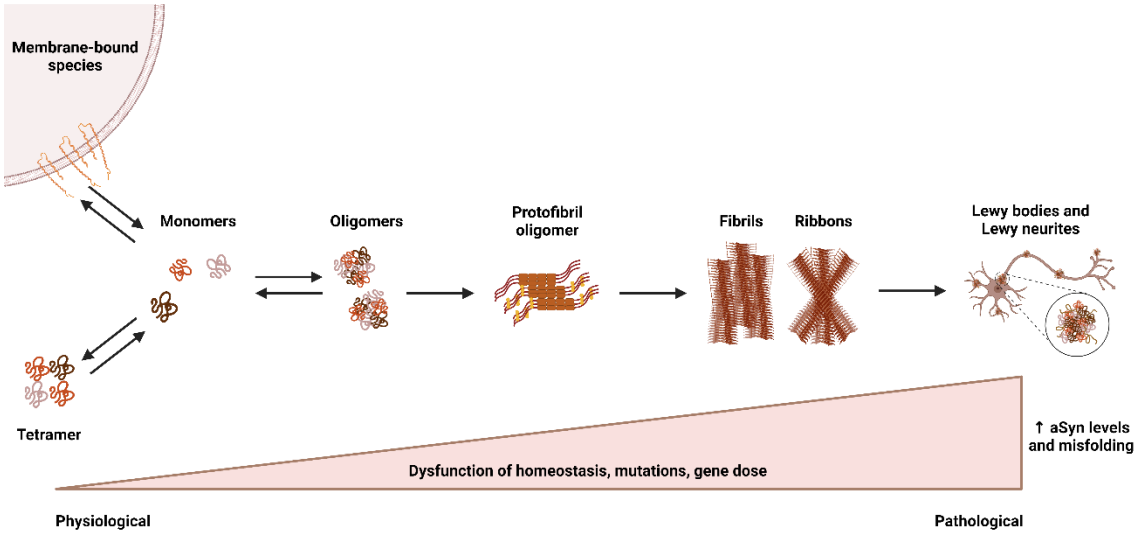


Figure 3 | Proposed model of the process of aSyn aggregation. In physiological conditions, aSyn exists as a highly disordered monomer in a dynamic equilibrium with membrane-bound species that promote SNARE-complex assembly and tetramers that can resist pathological aggregation. When the balance between aSyn synthesis and clearance is disrupted due to genetic mutations or environmental factors, the monomers tend to aggregate to form oligomers in a nucleation-dependent process. Some of these oligomers continue to aggregate and can give rise to protofibrils and other species as fibrils or ribbons, which further aggregate into cytoplasmic and neuronal protein inclusions, known as Lewy bodies and Lewy neurites, respectively. Which of these species of aSyn are responsible for cytotoxicity and neurodegeneration remains unclear. The image was created using BioRender.com.

Critically, once aSyn acquires a state of “toxicity”, it can also become “infectious”. Based on the available evidence, the toxic forms of aggregated protein may confer toxicity to

naïve recipient cells and trigger the spread of the pathology associated with the phenotypic presentation via “permissive templating” or “prion-like transmission” (Luk and Lee, 2014). These observations endorsed the conclusions of linear progression that Dr Braak and his colleagues proposed (described in more detail in chapter 1.4.5. Braak staging of Parkinson’s disease).

The concept of a misfolded, native protein turning toxic inside a cell, and also becoming “infectious” to neighbouring cells, was reminiscent of rare prion disorders, such as Creutzfeldt-Jakob disease. As a result, PD is now often referred to as a prion-like disorder and the respective proteins related to the disease as prionoid proteins (Olanow, 2014; Olanow and Prusiner, 2009; Scheckel and Aguzzi, 2018; Tamgüney and Korczyn, 2018).

Prions are aggregations of infectious proteins capable of transmitting and spreading disease throughout the brain and can be transmitted between individuals of the same or different species (Prusiner, 1982). Both aSyn and the cellular form of the prion protein (PrP^{C}) adopt, under physiological conditions, an alpha-helical-rich conformation, and both can refold into a β-sheet-rich conformation, leading to aggregation into oligomers and amyloid fibrils. Moreover, aggregates formed from each of these misfolded proteins can lead to the misfolding of the additional native protein, acting as prion conformers (Ferreon et al., 2009; Jao et al., 2008; Prusiner, 1998). Beta-structure-rich insoluble conformer (PrP^{Sc}) results from the aberrant folding of the PrP^{C}, or repetitions, mutations, or truncations in the *PRNP* gene (Hansen et al., 2011; Luk et al., 2012a). This was the first protein described as having infectious properties and exhibited ability to propagate neurodegeneration (Luk et al., 2012b). In the CNS, PrP^{Sc} accumulate and cause to spongiform degeneration and neuronal death (Masuda-Suzukake et al., 2014). The continuous accumulation of misfolded proteins challenges the lysosomes and proteasomes to remove them up, promoting their further accumulation and spreading and ultimately leading to neurodegeneration.

In this way, PD may be defined as a prion-like disorder resulting from increased production and/or impaired clearance of aSyn, leading to misfolding of this protein, the formation of toxic species, and consequent cell death. In addition, aSyn may be considered a prion protein that can self-aggregate and be transmitted to unaffected cells, continuing the disease process (Olanow and Prusiner, 2009). Classifying PD as prion-like proteinopathy has driven efforts in identifying and developing a biomarker for the treatment of PD based on assumed toxic protein forms and designing treatments that

reduce the formation and/or facilitate the clearance of misfolded aSyn, to block or reverse the propagation process of the proteins from one cell to another one.

1.6.2. Mutations in alpha-synuclein

Missense mutations in the *SNCA* gene (A18T, A29S (Hoffman-Zacharska et al., 2013), A30P (Krüger et al., 1998), A30G (Liu et al., 2021), E46K (Zarranz et al., 2004), A53T (Polymeropoulos et al., 1997), A53E (Pasanen et al., 2014), A53V (Yoshino et al., 2017), H50Q (Appel-Cresswell et al., 2013), G51D (Lesage et al., 2013), T72M (Fevga et al., 2021)) (Figure 2B), as well as gene duplications (Chartier-Harlin et al., 2004; Ibáñez et al., 2004) and triplications (Singleton et al., 2003), are involved in autosomal dominant forms of PD (Chartier-Harlin et al., 2004; Ibáñez et al., 2004). In contrast, mutations in PTEN-induced putative kinase (PINK1), PARK7, DJ1 (Goldberg et al., 2005), leucine-rich repeat kinase 2 (LRRK2) (Klein and Westenberger, 2012), and ATP13A2, among other genes, are associated with autosomal-recessive forms of PD. Furthermore, multiplications of the *SNCA* locus are also associated with the onset of the pathology, being more frequent than the carrying point mutations (Wales et al., 2013). Cellular and animal model studies showed that overexpression of wildtype (WT) aSyn and the presence of *SNCA* mutations promoted toxicity (Giasson et al., 2002; Outeiro and Lindquist, 2003; Van Der Putten et al., 2000; Sisk et al., 2000). Various studies reported that *SNCA* gene mutations influence the kinetics of aSyn aggregation due to their different propensities rates of oligomer and fibrillary inclusions formation (Conway et al., 1998, 2000b; Li et al., 2002a). Thus, investigating and understanding the familial mutations of aSyn could help to better comprehend the mechanisms of aSyn aggregation underlying the pathology and develop better models and therapies.

The A53T missense point mutation was the first aSyn mutation associated with PD, and was identified in families of Italian and Greek descent with an autosomal dominant inheritance of PD (Bozi et al., 2014; Polymeropoulos et al., 1997). This mutation is caused by a change of guanine to adenine at position 209 of the *SNCA* gene (G209A), leading to a change from alanine to threonine in the amino acid residue 53. The substitution of this amino acid causes long-range interactions between the NAC domain and the N- or C-terminal domains of aSyn to disappear, leading to an increase and stabilization of β-sheet structures, involved in oligomerization and fibrillization, enhancing the aggregation propensity (Bussell and Eliezer, 2001; Coskuner and Wise-Scira, 2013). A53T aSyn fibrillizes in solution faster than WT aSyn (Conway et al., 1998). However,

the mutation has a greater effect on aSyn protofibril formation rate than on the fibril elongation rate (Flagmeier et al., 2016). This mutation results in an earlier age of onset and a shorter pathology duration (Polymeropoulos et al., 1997). It also increases the membrane binding propensity and affects mitochondria homeostasis (Conway et al., 1998; Lücking and Brice, 2000; Tsigelny et al., 2012; Xie and Chung, 2012).

Next, another aSyn mutation, A30P, was identified in a German family. In this mutation, the substitution of an alanine for proline at amino acid residue 30 has been described as a rare autosomal dominant trait (Krüger et al., 1998). This aSyn mutant shows a decreased affinity for membrane and vesicle binding and, as a consequence, a decline in fibrillation kinetics, promoting the accumulation of protofibrillar and oligomeric structures within the cell (Conway et al., 2000a, 2000b; Jo et al., 2002). Additionally, A30P aSyn overexpression has been shown to affect TH synthesis, impairing neurite and axonal regeneration in dopaminergic neurons (Tönges et al., 2014). The fact that these two mutations, A53T and A30P, are both linked to early-onset PD and promote protofibril and oligomeric formation suggests that protofibril structures could have a crucial role and contribute to the neurodegeneration seen in PD (Lashuel et al., 2002).

Later, other *SNCA* gene mutations linked to familial forms of PD were discovered. The E46K mutation was first identified in a Spanish-origin family with hereditary PD (Ono et al., 2011; Zarranz et al., 2004). The replacement of glutamic acid for an alanine at position 46 increases the aSyn fibril formation, but the polymers formed can have different structures, indicating that this residue position has a key effect on the assembly process (Ono et al., 2011). E46K has also been shown to increase the pathogenicity of aSyn fibrils compared to WT, A53T, and A30P aSyn (Pandey et al., 2006). It has also been reported that the E46K mutation increased the number of cells with inclusions in human neuroglioma cells (Lázaro et al., 2014). Moreover, transgenic mice expressing E46K aSyn showed phosphorylated S129 aSyn positive inclusions and severe motor impairment (Emmer et al., 2011). Some *in vitro* studies have shown that E46K mutation results in an increase in aSyn phospholipid binding ability (Choi et al., 2004).

Later, two other aSyn mutations were discovered and characterized, the H50Q and G51D mutations (Appel-Cresswell et al., 2013; Lesage et al., 2013; Proukakis et al., 2013). The H50Q mutation was associated with late-onset PD and increased aSyn aggregation and amyloid formation, leading to increased aSyn secretion and cellular toxicity (Ghosh et al., 2013; Khalaf et al., 2014). The G51D mutation was found in a French family with parkinsonian-pyramidal syndrome and it was associated with an early onset of the disease, showing rapid progression (Lesage et al., 2013). In *in vitro* studies,

this mutation showed less propensity for aggregation, increased mitochondrial fragmentation, and reduced membrane binding propensity (Fares et al., 2014).

The A53E mutation is another aSyn familial mutation, that was discovered in a 36 years old Finish patient with atypical PD. The patient showed aSyn inclusions in the striatum and severe cortical pathology (Pasanen et al., 2014). In addition, this mutation was shown to decrease the aggregation of aSyn and increase the accumulation of oligomers (Ghosh et al., 2014; Lázaro et al., 2016). A53E mutation was also reported to have lower membrane binding affinity compared to the WT aSyn (Ghosh et al., 2014), as well as an impact on mitochondrial dysfunction and Golgi fragmentation (Lázaro et al., 2016; Rutherford and Giasson, 2015).

A30G is another aSyn familial mutation associated with PD identified in individuals of Greek families (Liu et al., 2021). The substitution of an alanine for glycine at the amino acid residue 30 shows an increase in fibril formation or differences in fibril structure. This mutation altered the α-helical structure of the protein, leading to alterations in the intrinsically disordered structure of aSyn, perturbations on the membrane binding, and instigating the fibril formation/changing fibril structure (Liu et al., 2021). The finding that the A30G mutation slightly perturbed membrane binding and promoted fibril formation/changed fibril structure is also unique among the known *SNCA* missense mutations (Liu et al., 2021). This replacement of amino acids in the *SNCA* gene might cause a double-hit effect, in which the equilibrium between soluble protein and membrane-bound protein is affected, increasing the levels of soluble protein, which leads to an increase of aggregated protein in the cytosol when compared to WT aSyn.

The T72M mutation, a recent aSyn familial mutation, was identified in two Turkish families. The substitution at position 72 of threonine for a methionine, there is a larger residue, can potentially destabilize the tetramer. This mutation is positioned in the central hydrophobic region of the aSyn, the NAC domain, that is required for polymerization into amyloid filaments (Waxman et al., 2009). The T72M mutation began to aggregate robustly and much earlier than the WT aSyn, which can be explained in part by the replacement of threonine, a small hydrophilic residue, with methionine which is a larger and hydrophobic residue, which could result in significant structural alterations and thereby in changes in aggregation propensity.

1.6.3. Alpha-synuclein post-translational modifications

The aSyn composition is prone to be affected by several post-translational modifications (PTMs) that may play important roles in its biological behaviour, aggregation propensity, and toxicity.

Of all PTMs, phosphorylation is one of the most studied in aSyn, and it can occur at both serines (S129 and S87) and on tyrosines (Y125, Y133, and Y135) (Wales et al., 2013). pS129 is considered the main PD pathological modification of aSyn (Anderson et al., 2006). It is estimated that about 90% of aSyn found in LBs is phosphorylated on S129 (Fujiwara et al., 2002), suggesting a close interaction between aSyn pS129 and its aggregation, and the disease. S129 phosphorylation has an impact on aSyn fibrillation, but conflicting studies suggest both an enhancement and inhibition of this process (Basso et al., 2013; Chen and Feany, 2005; Fujiwara et al., 2002; Tenreiro et al., 2014a). In addition, some studies also report a protective and a toxic effect of S129 phosphorylation on aSyn (Basso et al., 2013; Oueslati, 2016; Tenreiro et al., 2014a, 2014b)., making the effects of the phosphorylation on this residue still controversial. In addition to modulating its aggregation, pS129 may also interfere with protein function, as it modulates aSyn-protein interactions (McFarland et al., 2008) and its subcellular localization (Gonçalves and Outeiro, 2013; Oueslati et al., 2010; Wakamatsu et al., 2007). The phosphorylation at other residues is largely unclear, although phosphorylation of tyrosine Y125 is usually associated with inhibition of aSyn oligomerization, while the effect of phosphorylation at S87 yields contradictory results (Tenreiro et al., 2014b).

Other aSyn PTMs are truncation, acetylation, glycation, glycosylation, oxidation, nitration, SUMOylating, and ubiquitination (Dorval and Fraser, 2006; Zhang et al., 2019). Glycation is the covalent binding of sugar to protein or lipids, targeting the N-terminal residues, mainly arginines, and lysines. aSyn can be glycated, promoting the formation of oligomers but not fibrils, increasing aSyn toxicity (Konig et al., 2018; Vicente Miranda et al., 2017). Nitrated aSyn is also present in LBs in high concentrations (Giasson et al., 2000). Nitration can occur at the residue Y39, Y125, Y133, and Y136, and it has been generally associated with increased aSyn toxicity (Liu et al., 2011; Yu et al., 2010). Similar to phosphorylated and nitrated aSyn, ubiquitinated aSyn is also present in LBs (Tofaris et al., 2003). Ubiquitination occurs essentially at the lysine residues on the N-terminus of aSyn (Nonaka et al., 2005) and seems to affect its degradation via the endolysosomal and autophagy pathways (Engelender, 2008; Tofaris et al., 2011). Lysine residues 96 and 102 are particularly susceptible to SUMOylating (Krumova et al., 2011).

Like pS129, this PTM is thought to interfere with the aSyn function by modulating its secretion through vesicles, as exosomes (Kunadt et al., 2015). Both SUMOylating (Kim et al., 2011; Krumova et al., 2011) and acetylation of the N-terminal of aSyn (Bartels et al., 2014; Dikiy and Eliezer, 2014) are correlated with decreased aggregation propensity. Truncated aSyn may also have a role in pathology induced by aSyn, as truncated aSyn is also present in LBs (Baba et al., 1998) and these PTMs are increased in the presence of PD-related familial mutations (Li et al., 2005). Nevertheless, its impact on aSyn aggregation may depend on the domain where it takes place. While, *in vitro*, truncation of aSyn at the C-terminus increases its fibrilization (Murray et al., 2003; Ulusoy et al., 2010), truncation of the NAC domain is associated with a diminishing of fibrillation (Kasai et al., 2008; Mishizen-Eberz et al., 2005).

The functional understanding of aSyn PTMs in their physiological and pathological roles is still limited. Moreover, PTMs can create a complex network and modulate each other, as shown in previous study (Kleinknecht et al., 2016). Although studying the presence of multiple PTMs, currently poses technical challenges, understanding the impact of these modifications on aSyn will likely require addressing the full picture of these interrelationships.

1.6.4. Putative functions of alpha-synuclein

aSyn was first identified as a nuclear and synaptic protein. While its synaptic function has been extensively studied due to its implication in neurodegenerative diseases, its nuclear function remains poorly understood (Gonçalves and Outeiro, 2013; Maroteaux et al., 1988).

aSyn mutant forms, such as, A30P, G51D, and A53T, have been found to increase aSyn levels in the nucleus, which in turn can lead to an increase in phosphorylated aSyn in S129 (Kontopoulos et al., 2006; Pinho et al., 2019). In addition, aSyn affects directly the gene expression, interacting with histones and inhibiting histone acetylation under physiological conditions (Goers et al., 2003; Kontopoulos et al., 2006). In accordance with this, various epigenetic processes have been studied and may be involved and lead to the pathogenesis of synucleinopathies (Pavlou et al., 2017; Sturm and Stefanova, 2014).

Due to its high solubility, aSyn is also abundant in the cytosol and interacts with a wide range of pathways and cellular mechanisms due to the lipophilic alpha-helix (Snead and

Eliezer, 2014). In the cytosol, aSyn can interact with mitochondria via a specific domain at its N-terminus, being able to be imported to the mitochondria, interact with its inner membranes and disrupt its protein import mechanisms (Devi and Anandatheerthavarada, 2010; Ludtmann et al., 2016; Zigoneanu et al., 2012).

The accumulation of aSyn inside the mitochondrion can lead to membrane disruption and alterations in the physiological function, affecting synaptic mitochondria before the somatic mitochondria (Chinta et al., 2010; Nakamura et al., 2011; Szegő et al., 2019). Studies in *C.elegans* models showed that aSyn overexpression leads to disruption in mitochondrial fusion, culminating in mitochondrial fragmentation (Kamp et al., 2010). Furthermore, studies in isolated mitochondria from mice showed that soluble prefibrillar aSyn oligomers could promote complex I dysfunction (Luth et al., 2014). Also, mitochondrial A53T aSyn accumulation in dopaminergic neurons impairs complex I function and results in increased mitophagy *in vivo* (Chinta et al., 2010). Moreover, aSyn overexpressing mice are more susceptible to MPTP neurotoxicity and show extensive mitochondrial alterations (Song et al., 2004), while the aSyn knockout mice show resistance to the same toxin (Dauer et al., 2002).

aSyn can also affect endoplasmic reticulum (ER) related processes (Gómez-Suaga et al., 2018; Guardia-Laguarta et al., 2015; Lázaro et al., 2016; Wang and Hay, 2015) by interacting with ER membranes, several chaperones and/or ER proteins that can accumulate inside the ER and inhibiting its physiological function (Bellucci et al., 2011; Lázaro et al., 2014; Thayanidhi et al., 2010; Winslow et al., 2010). Moreover, it was also observed that aSyn expression increases ER stress and activates the unfolded protein response (UPR) (Smith et al., 2005). However, it is still unclear, how aSyn can increase ER stress stimuli. One hypothesis would be the inhibition ER-to-Golgi transport and/or dysfunction of the secretory pathway, leading to an overload of the ER (Gitler et al., 2008; Yin et al., 2014). Another hypothesis would be the aggregation of aSyn with chaperones into the ER lumen, resulting in ER stress (Colla et al., 2012). Also, aSyn can trigger ER stress by inhibiting ER-associated degradation (ERAD), resulting in the accumulation of unfolded proteins. Curiously, Homocysteine-induced ER protein (Herp) plays a crucial role in ERAD and it is overexpressed in PD patients (Slodzinski et al., 2009).

Dysfunctions in the Golgi apparatus are also observed in PD (Bexiga and Simpson, 2013; Gonatas et al., 2006; Paiva et al., 2018). Notably, alterations in Golgi morphology are generally associated with neurodegenerative diseases (Gosavi et al., 2002). According to previous studies, aSyn expression led to Golgi fragmentation in neurons and aSyn prefibrillar aggregates disrupted Golgi (Gosavi et al., 2002). Other studies have

shown that aSyn accumulation leads to the mislocalization of Golgi markers and secretory vesicle aggregation (Soper et al., 2008). There have been several mechanisms proposed to explain how aSyn affects Golgi morphology, but further investigation needs to be conducted. aSyn can be recognized by the lysosomes, via the VKKDQ motif, and internalized for degradation, for that reason an increase of aSyn inside lysosomes could lead to an impairment of autophagy processes. Similar consequences happen with the proteasome complex, where aSyn mutation or oligomerization, could lead to dysfunctions of this complex (Tofaris et al., 2001; Vekrellis et al., 2011). aSyn has also been described as an interacting partner of cytoskeletal proteins, like actin and tubulin. The interaction of aSyn with these proteins can inhibit their proper polymerization process thus leading to cytoskeletal impairments (Chen et al., 2007; Sousa et al., 2009).

As mentioned before aSyn has been implicated in the maintenance of the pre-synaptic vesicle pool and participates in the neurotransmitter release process (Abeliovich et al., 2000; Burré, 2015; Nemani et al., 2010; Spillantini and Goedert, 2000). The balance of aSyn levels is essential for proper synaptic function, as evidenced by various studies that manipulate aSyn levels in different models to observe the effects on synaptic phenotypes. An equilibrium of protein levels seems essential to keep a proper synaptic function. aSyn is important for the SNARE-complex assembly through its interaction with synaptobrevin-2 (Burré et al., 2010). Also, it has been shown that aSyn interacts with synaptic SNARES, facilitating vesicle fusion (Burré et al., 2014). Removal of aSyn leads to neurotransmission acceleration and SNARE complex formation inhibition, therefore, disturbing exocytosis (Abeliovich et al., 2000; Burré et al., 2010). Furthermore, aSyn knockout mice are found to have altered synaptic vesicle dynamics and reduced striatal dopamine (Abeliovich et al., 2000; Cabin et al., 2002). On the other hand, aSyn overexpression can rescue SNARE disturbances occurred by removing chaperons like cysteine-string protein-α (CSPα) (Chandra et al., 2005) and it can facilitate synaptic vesicle (SV) interactions and regulates their assembly (Burré et al., 2010, 2014). However, aSyn overexpression has also shown dubious results showing a reduction in the reuptake of SVs and a potential mislocalization of SNARE proteins like SNAP-25, syntaxin-1, and synaptobrevin-2 (Garcia-Reitböck et al., 2010; Nemani et al., 2010). aSyn aggregates could also similarly interact with synaptobrevin-2 to the monomeric form and interfere with vesicle docking (Choi et al., 2013). Although we are unsure what role aSyn plays in the different stages of SVs' life cycle, its important interaction with SVs is unquestionable. Studies on cell models overexpressing aSyn showed a reduction in the reserve pool of SVs and inhibition of the vesicle priming, leading to an inhibition of the neurotransmitter release (Larsen et al., 2006; Nemani et al., 2010) (Figure 4). Other

studies on aSyn transgenic animals have demonstrated a redistribution of SVs further from the active zone, leading to longer but less dense post-synaptic density (Janezic et al., 2013). Also, Scott and colleagues reported that overexpression of aSyn in hippocampal neurons seemed to result in various synaptic deficits mediated by loss of critical synaptic proteins and enlarged vesicles (Scott et al., 2010). The mechanism behind the abnormalities in SVs priming, docking, and fusion remains unclear.

The increase of aSyn levels seems to disrupt dopaminergic transmission by modulating the activity of TH in dopamine production (Kirik et al., 2002; Perez et al., 2002). Moreover, aSyn can bind the dopamine active transporter (DAT) and inhibit the reuptake of dopamine (Paxinou et al., 2001; Wersinger and Sidhu, 2003). Yet, oligomeric aSyn was found to interact with vesicles in a way that introduces pores in their surface culminating in neurotransmitter leakage in the synapse. In the case of dopamine leakage, aSyn oligomerization and also oxidative stress are expected (Mosharov et al., 2006; Volles et al., 2001). aSyn was proposed as a synaptopathology towards neurodegeneration (Iwai et al., 1995). In synapses, aSyn aggregates are present before the formation of LBs, indicating that the pathology progresses in dying back-like neurodegeneration, from synapses to the cell bodies (Kramer and Schulz-Schaeffer, 2007; Marui et al., 2002). According to Volpicelli-Daley and coworkers, cortical neurons exposed to aSyn fibrils developed into LB-like aggregates in the soma similar to LNs pathology (Volpicelli-Daley et al., 2011). The results from mouse models of synucleinopathies corroborate the idea that synaptic dysfunction may be considered a prodromal stage followed by neuronal death (Schirinzi et al., 2016). Lastly, it was found that synapses decline gradually alongside LB formation (Mahul-Mellier et al., 2020).

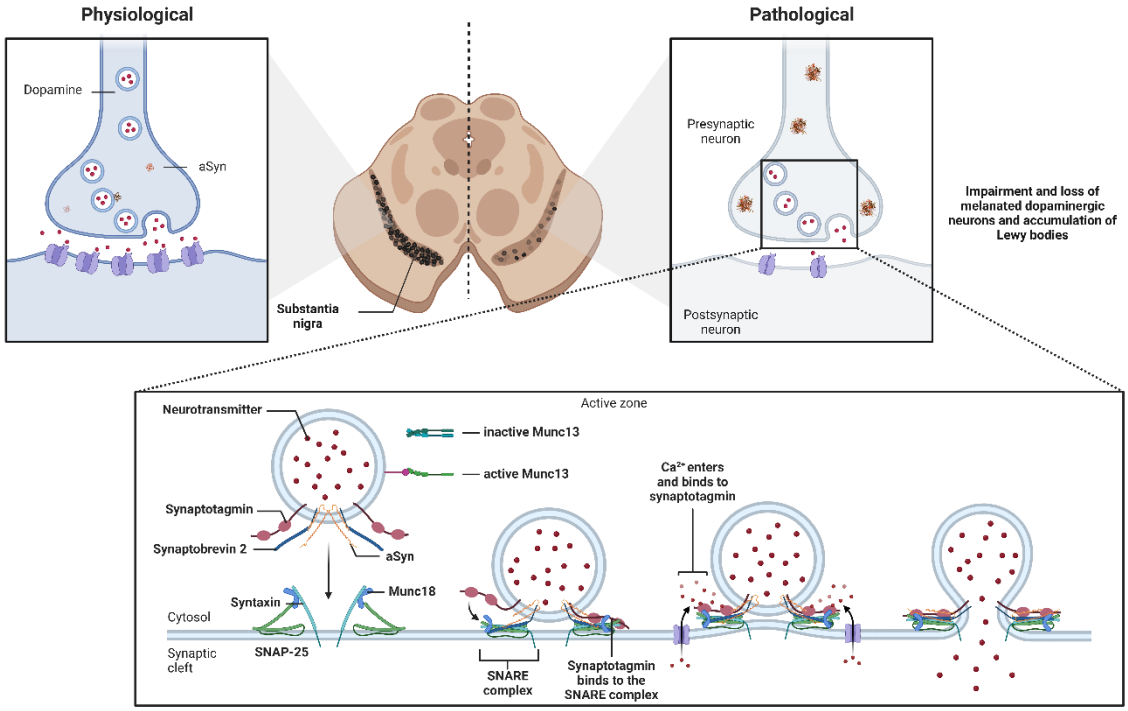


Figure 4 | aSyn under physiological and pathogenic conditions. Compared to physiological conditions (left), in PD (right) there is an accumulation of Lewy bodies and Lewy neurites, and a progressive impairment and profound loss of dopaminergic neurons in the SNpc resulting in the depletion and ultimate loss of the neurotransmitter dopamine on synaptic terminals of striatal neurons. Monomeric aSyn, under physiological conditions, promotes SVs clustering, drives the docking of the SVs to the active zone, and regulates SNARE-complex assembly via interaction with SNARE protein synaptobrevin-2 and with SVs membrane. Under pathological conditions, aSyn aggregates into oligomers and fibrils that have a stronger binding affinity for synaptobrevin-2. The reduced availability of unbound synaptobrevin-2 molecules inhibits the SNARE complex formation, reduces the number of vesicles in the active zone, and impairs vesicle docking. The image was created using BioRender.com.

1.7. Braak staging of Parkinson's disease

PD is a very complex and widespread neuropathology. The idea that cell-to-cell spreading of aSyn oligomers or aggregates would be responsible for the progression of aSyn pathology in synucleinopathies has emerged as a compelling model for explaining how aSyn-related neurotoxicity and neurodegeneration could affect neuroanatomically connected regions of the brain. Braak and colleagues suggested that in the early stages of the disease, aSyn-positive LBs and LNs start in the dorsal motor nucleus of the vagus nerve (dmX) in the lower brain stem and olfactory bulb and then progressively and stereotypically spread throughout the brain to involve eventually large parts of the CNS, as the neocortex affecting primary sensory functions (Braak et al., 2002, 2003b, 2004; Tyson et al., 2016) (Figure 5). This proposal can be compared with a falling row of dominos, where misfolded aSyn spreads from an infected neuron to a yet healthy

neuron. This theory proposes that misfolded aSyn can be taken up by neurons, transported along axons, and later released into the extracellular space, being transferred between connected neurons and acting as a template for endogenous protein aggregation in healthy neurons as well as recipient neurons (Brundin and Melki, 2017; Brundin et al., 2008, 2010; Tyson et al., 2016). In general, cellular stress, mitochondrial and proteasomal dysfunction (Desplats et al., 2009) as well as overexpression and cytosolic accumulation seem to lead to neuronal aSyn secretion, contributing to the progression of the disease. This theory was supported by several observations from autopsies that were performed on PD patients in the 1980-1990s (Kordower et al., 2008a; Li et al., 2008). In these patients, LB-like inclusions were not only present throughout the brain but also in the previously grafted neurons (Kordower et al., 2008a, 2008b, 2017; Kurowska et al., 2011; Li et al., 2008, 2010, 2016; Olanow et al., 2003). These findings support the idea that the grafted embryonic dopaminergic neurons were infected by the host neurons because the grafted neurons were relatively young (10-15 years beyond the embryonic stage) it seems very unlikely that they have developed aggregates through an independent cell-autonomous process (Kordower et al., 2008a; Li et al., 2008). This complex series of events has been examined in numerous *in vitro* and *in vivo* studies (Brundin and Melki, 2017; Danzer et al., 2009; Desplats et al., 2009; Freundt et al., 2012; Hansen et al., 2011; Lee et al., 2008; Lesage et al., 2013; Luk et al., 2009, 2012b, 2012a; Reyes et al., 2015; Volpicelli-Daley et al., 2011).

It has been demonstrated that exogenously added aSyn oligomers and aggregates can bind to cultured cells, either free or associated with extracellular vesicles, as a result of interactions with membrane proteins (Mao et al., 2016; Shrivastava et al., 2015), or lipid components of the membranes (Holmes et al., 2013). Several studies are consistent with the use of prion-like spreading mechanisms for the transmission of aSyn pathology, some of which appear to depend on the assembly state (Brás and Outeiro, 2021; Brundin and Melki, 2017). These mechanisms include passive diffusion (Ahn et al., 2006; Auluck et al., 2010; Lee et al., 2008), EVs as ectosomes and exosomes (Alvarez-Erviti et al., 2011; Danzer et al., 2012; Emmanouilidou et al., 2010; Jang et al., 2010; Lee et al., 2005; Paillusson et al., 2013), misfolding-associated protein secretion pathway (Lee et al., 2016), membrane pores (Lashuel and Lansbury, 2006), tunnelling nanotubes (Abounit et al., 2016; Dieriks et al., 2017), membrane carrier proteins (Yang et al., 2017), receptor-mediated endocytosis (Mao et al., 2016), and ER-Golgi dependent exocytosis (Lee et al., 2005) (Figure 5A).

As a result of the interaction between aSyn assemblies and lipid membranes, an annular pore-like structure can be formed in the plasma membrane (Hoogerheide et al., 2017; Lashuel and Lansbury, 2006; Lashuel et al., 2002; van Rooijen et al., 2009). However, it remains unclear whether these structures exist *in vivo*. In addition to interacting with membrane receptors, pathological seeds may also induce internalization by triggering downstream signalling pathways. Despite extensive research, the factors that modulate the mechanisms involved in protein transmission remain poorly understood.

In addition, aSyn can also be found in the media of cell lines and primary neuronal cultures expressing this protein (Lee et al., 2005; Sung et al., 2005), as well as in cerebrospinal fluid (CSF) and plasma in PD patients (El-Agnaf et al., 2003; El-Agnaf et al., 2006; Mollenhauer et al., 2008). Also, in multiple experiments in rodent and nonhuman primates, using intracerebral injection of brain homogenates from PD and DLB patients, brain tissues from transgenic animals with aSyn pathology, or recombinant aSyn seeds was shown the propagation of misfolded aSyn and the spreading of the related neuropathology in different and distant brain regions (Ayers et al., 2017; Luk et al., 2012b; Masuda-Suzukake et al., 2014; Mougenot et al., 2012; Peelaerts et al., 2015; Recasens et al., 2014; Sacino et al., 2014).

Prion-like mechanisms like these are also found in other neurodegeneration diseases for instance AD (Frost et al., 2009). Interestingly, PD shares other similarities with AD as well. AD follows a stereotypic pattern in all patients, where the distribution of tau-containing neurofibrillary tangles is linked to the clinical disease stage (Braak et al., 2003b). Braak and colleagues suggested that this is also the case in PD, where the distribution of LBs and LNs progresses in a largely caudo-rostral direction over time. They proposed a six-stage neuropathological progression of PD with an increasing number of brain regions showing aSyn pathology, representing pre-symptomatic and symptomatic phases, suggesting a prion-like process (Braak et al., 2003b, 2003a; Hawkes et al., 2007).

In stage 1, Lewy pathology appears at two sites: the olfactory bulb, in the anterior olfactory nucleus and in the dmX, which is connected to the enteric nervous system (ENS). Due to the fact that the pathology in anterior olfactory structures makes fewer incursions than in the lower brain stem, it is postulated that the dmX is presumably the starting point of the disease process, which takes an ascending path (Braak et al., 2002, 2003b) (Figure 5B and C). Moreover, further observations lead to the suggestion that PD could be initiated via the gastric route, and move upwards to the dmX and affect the CNS

(Braak et al., 2006a). This theory was also supported by the observation of Lewy pathology in the ENS and PNS (Braak et al., 2006b; Shannon et al., 2012).

In stage 2, Lewy pathology is more widespread within the medulla, including the lower raphe nuclei, the locus coeruleus, and the gigantocellular reticular nucleus (Figure 5B and C). These nuclei work as constituents of the gain-setting system (Braak and Braak, 2000) and they receive major input from components of the limbic and motor systems, such as the central subnucleus of the amygdala. It is capable of limiting the conduction of incoming pain signals in stressful situations and ensures that the motor neurons are in a heightened state of preparedness for action (Randich and Gebhart, 1992).

In the third stage, the Lewy pathology progresses from the brain stem to the midbrain. In the central subnucleus of the amygdala, there is massive neuronal destruction. The central subnucleus of the amygdala projects to the gain system and dmX. The central subnucleus in its turn receives projections from the amygdala basolateral complex, which is received from the nuclei of the basal forebrain (Braak et al., 1994) (Figure 5B and C). The connection between these different parts of the brain and the fact that all these parts are infected by one after the other supports the hypothesis that the pathology develops in a non-random manner. During stages 1, 2 and 3, the individuals do not have visible motor symptoms.

During stage 4, individuals start to show for the first-time symptoms. At this point, the pathology progress in the temporal mesocortex, which projects all signals from the neocortex to the limbic loop (including amygdala, hippocampal formation, entorhinal region) and prefrontal cortex. Notably, the temporal mesocortex is the site of the most pathology throughout the following stages (Braak et al., 2003b) (Figure 5B and C).

In the last stages, the neocortex is affected and the motor symptoms are severe. Also, cognitive dysfunction becomes apparent. Moreover, LBs and LNs develop in the high-order sensory association and prefrontal areas of the neocortex (Braak et al., 2003b) (Figure 5B and C).

The heterogeneity observed in synucleinopathies may be explained by the spread of different types of aSyn strains between cells (Peelaerts et al., 2018). Nevertheless, the prion-like hypothesis faces some contradictions. For example, some PD cases do not present typical patterns of Lewy pathology as staged by Braak (Halliday et al., 2011; James Surmeier et al., 2017; Jellinger, 2009a). Also, aSyn aggregate distribution does not correlate with clinical progress in patients and with Lewy pathology (Berg et al., 2014; Dijkstra et al., 2014; Jellinger, 2009b). Moreover, there is still no evidence that aSyn can

spread between individuals, and various studies report that grafted cells do not show pathology (Hallett et al., 2014; Mendez et al., 2008). The aSyn pathology in transplanted cells may also be caused by stress conditions or alterations in the cellular milieu around the graft (Surmeier et al., 2017).

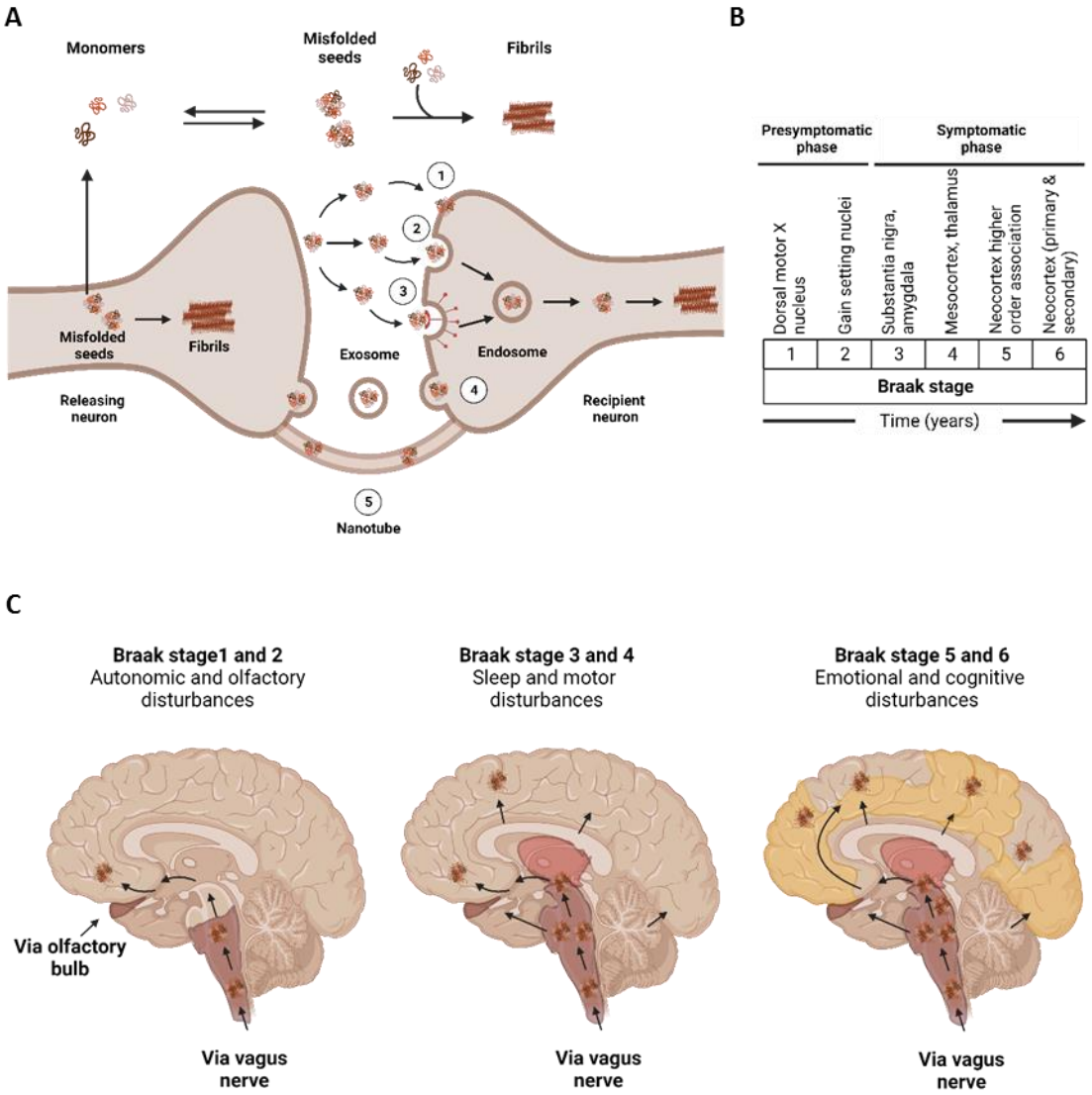


Figure 5 | Proposed mechanisms mediating cell-to-cell aSyn transmission in PD pathology according to Braak´s staging hypothesis. A) Misfolded protein seeds such as oligomers and protofibrils are primarily formed in the cytoplasm of the donor neuron. The misfolded seeds can be created through recruitment of soluble monomers or fragmentation of the protofibrils or fibrils. aSyn aggregates can be released into the extracellular space via: (1) passive diffusion, (2) fluid-phase endocytosis, (3) endocytosis mediated by receptor, (4) exosomes, or (5) nanotubes. In the recipient neuron, internalized seeds then recruit native monomers, repeat the aggregation process and spread the pathology. **B)** In PD, there are presymptomatic and postsymptomatic stages, and these stages can be further classified into six stages. **C)** Schematic summarizing the disease progression and increasing aSyn pathology severity as proposed by Braak and colleagues. The first inclusions are present in the olfactory bulb and in the dmX (stages 1 and 2). Then, the pathology spreads from the brain stem to the midbrain and basal forebrain (stages 3 and 4), affecting later the neocortex (stages 5 and 6). The image was created using BioRender.com.

1.8. Therapeutic strategies in Parkinson's disease

Although great accomplishments in the PD field have been made over the last years (Li and Le, 2017), the clinical diagnosis of this pathology is still based on the identification of motor features, which occur years after the neurodegenerative process has started and irreversible loss of dopaminergic neurons in the *substantia nigra* is already observed (Kalia and Lang, 2015). Moreover, even when the diagnostic criteria are correctly applied, the misdiagnosis rate is still high (16%–20%) due to considerable clinical overlap among other Parkinsonian disorders, making aSyn pathology detection in the postmortem brain the primary means of reaching a conclusive diagnosis (Rizzo et al., 2016).

Late diagnosis and misdiagnosis delay the therapeutic benefits of therapies. Unfortunately, there are currently no therapeutic interventions available to prevent or stop the progression of the disease. One of the major challenges to early detection of the disease is the lack of a deep understanding of the underlying mechanisms and a shortage of reliable diagnostic biomarkers (Hinz and Geschwind, 2017). Therefore, the discovery of PD-specific biomarkers that would allow a differential diagnosis between PD and other synucleinopathies is a priority for the development of novel and effective therapies that allow early-stage diagnosis of the disease and early initiation of neuroprotective therapy (Magdalinou et al., 2014). PD is characterized by a long prodromal phase, which creates an interval between the formation of the first protein inclusions and the appearance of disease symptoms. This interval provides a good therapeutic window for the development of sensitive diagnostic techniques that can enable early detection and intervention.

1.9. Biomarkers in Parkinson's disease

Biomarkers are quantitative indicators of physiological or pathological processes, or pharmacological responses to clinical interventions (Strimbu and Tavel, 2010). Based on their functional characteristics, biomarkers can be classified into three categories: (i) susceptibility risk biomarkers used to identify the potential for developing PD (Parnetti et al., 2019); (ii) diagnostic biomarkers used to confirm the presence of PD; and (iii) prognostic biomarkers used to indicate the disease progression, treatment-associated changes, or disease recurrence (Perlis, 2011). Biomarkers for PD can also be categorized as clinical, genetic, imaging and biochemical biomarkers (Emamzadeh and

Surguchov, 2018; He et al., 2018). The potential benefits of PD biomarkers are limited when only one of these categories is considered, but when considered collectively, they provide a more comprehensive and robust assessment for measuring disease progression and modification. Despite several studies that have been looking for potential PD biomarkers, only a few have been translated into clinical use (Chen-Plotkin et al., 2018).

Clinical biomarkers such as rapid eye movement sleep behaviour disorder (RBD), constipation, hyposmia, and mood disorders are promising prodromal PD biomarkers (Fullard et al., 2017). RBD, in particular, is strongly linked to PD with a 45% risk of developing neurodegeneration within five years and a 76% chance within ten years, according to a 7-year follow-up study, (Lotankar et al., 2017). Olfactory dysfunction is also prevalent in PD in a range from 45% to 90% (Haehner et al., 2009) and most PD patients develop olfactory dysfunction four to six years before they develop motor impairment (Reichmann, 2017). Nevertheless, olfactory dysfunction is less specific to PD than RBD and motor markers (Le et al., 2017), as it is also present in other synucleinopathies. Consequently, a combination of assessments may be necessary to improve the accuracy of early PD diagnosis, given the limited specificity of non-motor symptoms (Poewe and Mahlknecht, 2012).

The identification of familial PD-related genes has helped understand the mechanisms underlying PD pathogenesis. Although only a small proportion of PD patients have a positive family history or present with early onset of the disease, or complex phenotype that can benefit from identifying mutations associated with monogenic PD. In the case of the vast majority of sporadic PD cases, identifying susceptibility or heterozygous mutations in familial PD genes could lead to the development of a genetic biomarker in the future, but large-scale statistically powered GWAS or other types of analyses would be required (Ganguly et al., 2021).

Multiple imaging techniques such as magnetic resonance imaging (MRI), single-photon emission computed tomography (SPECT), and positron emission tomography (PET) have proven useful in detecting early changes in PD patients and tracking disease progression. Unlike subjective measures that can be influenced by medication or placebo effects, these imaging techniques provide unbiased measurements (Guan et al., 2017). These techniques can estimate the levels of dopamine transporter (DAT) or vesicular monoamine (VMAT), abnormal accumulation of proteins like aSyn, post-synaptic dopamine receptors, or iron in the midbrain or other areas (Ganguly et al., 2021). By estimating levels of dopamine transporter (DAT) or vesicular monoamine (VMAT), abnormal

accumulation of proteins like aSyn, post-synaptic dopamine receptors, or iron in the midbrain or other areas (Ganguly et al., 2021), these techniques have deepened our understanding of PD pathogenesis and the effects of drugs on the disease (Niethammer et al., 2012; Wang et al., 2012a).

MRI techniques have shown hints at early PD diagnosis and monitoring disease progression (Heim et al., 2017; Jonkman et al., 2019). A unique cluster of dopaminergic neurons in SN called nigrosome-1, was identified by high-resolution MRI and the 'swallow-tail' appearance of healthy nigrosome-1 was lost in PD patients (Schwarz et al., 2014). Neuromelanin MRI is a novel technique that as the name indicates measures the content of neuromelanin. A study performed by Miyoshi observed a loss of neurons containing neuromelanin and a reduced signal intensity of the SN in PD patients (Miyoshi et al., 2013).

The imaging of DAT with SPECT or PET scan had been proposed as supportive evidence in PD diagnosis and has been used to detect changes in the dopamine system (Brooks, 2016; Suwijn et al., 2015). Many studies on DAT-SPECT imaging have shown a high accuracy of diagnostic performance with a 79% to 100% sensitivity and an 80% to 100% specificity (Bajaj et al., 2013; Suwijn et al., 2015). Several SPECT radiotracers for DAT imaging have been used to access the severity of the disease and to differentiate PD from other types of parkinsonism (Ba and Martin, 2015). Interestingly in a 4-year clinical follow-up study, the combination of hyposmia and DAT deficit was highly predictive of the PD onset, where a 5% reduction in DAT binding annually was observed (Jennings et al., 2017). Studies with PET imaging with 6-[18F] Fluoro-L-DOPA (F-DOPA), similar to studies with DAT-SPECT, observed a decrease in F-DOPA uptake in the caudate and putamen of PD patients (Wing et al., 2015). Additionally, decreased F-DOPA uptake was also reported to occur contralaterally to hypokinesia-rigidity symptoms and correlated with their severity (Ganguly et al., 2021). Then, F-DOPA PET could become an important diagnostic tool for the differential diagnosis of PD (Calabria et al., 2016). Further, PET scans with 18F-labeled 2-deoxy-2-fluoro-D-glucose (18F-FDG) are being accepted as a reliable method for differentiating PD from other Parkinsonian disorders or assessing cognitive impairment in this disease (Meyer et al., 2017). Although, the results obtained from such studies are not indisputable to suggest any particular imaging biomarker for PD diagnosis.

There is a wide range of biochemical biomarkers for PD that have been studied in CSF, blood, and other bodily fluids. Some of them include dopamine and its catabolites, neuropeptides, purine catabolite like uric acid, oxidative damage markers, neurotrophic factors like brain-derived neurotrophic factor (BDNF), inflammatory markers,

microRNAs, and various proteins known to be associated with PD pathogenesis (Emamzadeh and Surguchov, 2018; He et al., 2018; Jiang et al., 2019; Jiménez-Jiménez et al., 2014; Katayama et al., 2020; Wei et al., 2018). For a long time, dopamine, as well as its degradation products like dihydroxyphenylacetic acid (DOPAC) and homovanillic acid (HVA), have been studied in CSF to assess the loss of central dopaminergic function in PD and to correlate it with PD motor impairments. The levels of these catabolites were reported to be increased at the early stages of PD patients, and the values rose further with the degree of motor impairment (Stefani et al., 2017). Alternatively, a recent follow-up study showed that low baseline CSF dopamine and DOPAC levels were associated with PD in subjects with several PD risk factors (Goldstein et al., 2018). In another study, the levels of HVA in CSF could not differentiate PD patients from the controls, contrary to the xanthine/HVA ratio (Lewitt et al., 2011). Nevertheless, other studies reported a decrease in HVA and serotonin catabolite levels in CSF of PD patients (Jiménez-Jiménez et al., 2014). Uric acid, a purine derivate and a potent antioxidant have been explored as a risk factor and a prognostic and diagnostic biomarker for PD (Cipriani et al., 2010; Paganoni and Schwarzschild, 2017). A meta-analysis of eligible studies on serum has shown that this compound is present in lower amounts in the serum of PD patients (Wen et al., 2017). BDNF is a relevant regulator of differentiation, proliferation, and survival of neurons, and its neuroprotective role was previously described in different PD experimental models (Palasz et al., 2020). Although, the BDNF levels alterations in CSF and blood of PD patients are complex and inconsistent, and its potential as a biomarker is not yet established (Jiang et al., 2019; Nagatsu et al., 2000; Salehi and Mashayekhi, 2009; Ventriglia et al., 2013). Inflammatory and oxidative stress markers are not specific to PD and are involved in the pathogenesis of multiple neurological diseases that exhibit inflammation and oxidative damage.

In the past few years, aSyn has gained extensive attention as a biomarker in PD due to its involvement in PD pathogenesis and its presence in different body fluids. The distribution of aSyn species in biofluids may provide unique opportunities to develop non-invasive diagnostic and prognostic tools based on measuring individual or multiple aSyn species levels that reflect the pathology or progression of the disease (Fayyad et al., 2019; Ganguly et al., 2021; Parnetti et al., 2019). However, aSyn is promptly secreted into extracellular spaces and can be found in different forms in body fluids such as CSF (Eusebi et al., 2017; Foulds et al., 2012; Hong et al., 2010; Mollenhauer et al., 2011, 2013; Shi et al., 2010; Tokuda et al., 2006, 2010; Wang et al., 2012b), blood components (Abd-Elhadi et al., 2015; Barbour et al., 2008; Bryk and Wiśniewski, 2017; Cariulo et al., 2019; Foulds et al., 2011, 2013; Gorostidi et al., 2012; Ishii et al., 2015; Klatt et al., 2020;

Koehler et al., 2015; Shi et al., 2014), saliva (Devic et al., 2011; Kang et al., 2016; Shaheen et al., 2020; Vivacqua et al., 2019) and tears (Hamm-Alvarez et al., 2018; Maass et al., 2020) as well as in peripheral tissues (e.g., skin, oesophagus, colon) (Chung et al., 2016; Fenyi et al., 2019; Tanei et al., 2021; Wang et al., 2021), and it is transported bi-directionally between PNS and CNS (Sui et al., 2014). This makes it even more difficult to interpret and correlate the alterations of aSyn levels in body fluids with brain PD pathology. Moreover, since this protein can also exist in different conformations and can be translationally modified, the complexity of the measurement of this protein is increased and there is not yet a consensus on which form or forms constitute reliable biomarker(s) for early diagnosis or following disease progression. Nevertheless, further research is needed to determine the most reliable aSyn biomarker(s) and their potential applications in PD diagnosis and management.

1.9.1. Biochemical biomarkers: alpha-synuclein in CSF

As mentioned above, the CSF is an ideal body fluid to look for PD biomarkers as it provides a window to biochemical and neuropathological changes in the brain and it provides the metabolic-pathological profile of the CNS. Several studies have investigated the aSyn levels alterations in the CSF, but the results are not consistent. Some studies report a low level of total aSyn in CSF of PD patients while other studies do not find any significant difference between PD and control groups (van Dijk et al., 2013; Hall et al., 2012; Hansson et al., 2014; Hong et al., 2010; Mollenhauer et al., 2008, 2011, 2013; Öhrfelt et al., 2009; Park et al., 2011; Parnetti et al., 2011; Shi et al., 2011; Tokuda et al., 2006; Toledo et al., 2013; Wennström et al., 2013). Most studies which examined the validity of CSF aSyn as a putative biomarker for PD emphasized that a decreased level of aSyn reliably separated PD from control subjects, but the specificity of this measurement was low (Aerts et al., 2012; Chahine et al., 2020; L. et al., 2015; Mollenhauer et al., 2011, 2013; Zhou et al., 2015). Although aSyn is frequently detected in the CSF, there was no association between total aSyn levels and the disease progression or severity, nor changes in this protein levels provide a reliable marker (Aerts et al., 2012; Eusebi et al., 2017; Foulds et al., 2012; Hong et al., 2010; L. et al., 2015; Mollenhauer et al., 2011, 2013; Shi et al., 2010; Tokuda et al., 2006, 2010; Wang et al., 2012b; Xiong et al., 2015). However, two studies showed a correlation between CSF aSyn and PD severity/progression (Kang et al., 2013; Majbour et al., 2016a). In several studies, the total aSyn levels were reported to be in the range of 67–68,900 pg/ml in controls and 61.5–55,000 pg/ml in PD patients (Hong et al., 2010; Mollenhauer et al.,

2008, 2011, 2013; Parnetti et al., 2011; Reesink et al., 2010; Tokuda et al., 2006, 2010). However, most of these studies had small sample sizes. On another side, a study by Foulds and colleagues reported levels of total aSyn in PD patients in the range of µg/ml (1.85 ± 2.40 µg/ml (average)) instead of the previously observed pg/ml (Foulds et al., 2012). These disparities have been attributed to diverse preanalytical and analytical confounding factors, demographic and clinical heterogeneity, comorbidities and potential medical treatments (Mollenhauer et al., 2017, 2019; Parnetti et al., 2019). In addition to the confounding factors mentioned above, different levels or distribution of modified aSyn forms could also play a role in the variations in aSyn levels, particularly since the majority of the antibodies used in most aSyn immunoassays target the C-terminal domain of aSyn (residues 110–130), which contains the most pathology-associated aSyn modifications such as phosphorylation and C-terminal truncations (Magalhães and Lashuel, 2022).

Because of these limitations, aggregated forms of aSyn (Hansson et al., 2014; Majbour et al., 2016a; Park et al., 2011) or modified aSyn species such as pS129 (Cariulo et al., 2019; Foulds et al., 2012; Majbour et al., 2016a, 2016b; Stewart et al., 2015; Wang et al., 2012c) have been investigated as potential biomarkers. Therefore, increased oligomeric aSyn levels in CSF have been reported in PD with dementia compared to age-matched controls. Other studies have reported decreased levels of aSyn, increased levels of oligomeric aSyn and also increased oligomeric aSyn/total aSyn ratio in the CSF of PD patients (Hansson et al., 2014; Park et al., 2011; Parnetti et al., 2014a, 2014b; van Steenoven et al., 2018; Tokuda et al., 2010). Combining the measurement of oligomeric aSyn and total aSyn improved the specificity of PD diagnosis (Parnetti et al., 2014a, 2014b). A recent study observed an increased concentration of aSyn protofibrils in the CSF of PD patients (Von Euler Chelpin et al., 2020). The increased levels of aSyn aggregates in CSF have been reported in a follow-up study as a risk factor for dementia in PD (Ning et al., 2019). Several studies reported that these alterations in aSyn levels and forms in PD patients could result from changes in the secretion, solubility or aggregation of this protein, affecting its overall turnover.

The search for aSyn PTMs in CSF, specially pS129, is because increasing evidence indicates that pS129 is the main modified form of aSyn in LBs and correlates with the increased brain pathology formation (Cariulo et al., 2019; Foulds et al., 2012; Majbour et al., 2016a, 2016b; Stewart et al., 2015; Wang et al., 2012b). Several studies have suggested that CSF pS129 levels may be able to distinguish not only between PD patients and control subjects (Eusebi et al., 2017; Majbour et al., 2016b; Stewart et al., 2015; Wang et al., 2012b), but also between different synucleinopathies (Constantinides

et al., 2021; Foulds et al., 2012; van Steenoven et al., 2018). However, the results of these studies showed a wide range of pS129 levels in the CSF, ranging from no detectable levels to 7.14 ± 9.19 µg/ml (Cariulo et al., 2019; Foulds et al., 2012). Diverse studies reported higher pS129 levels in PD patients than in healthy controls (Eusebi et al., 2017; Majbour et al., 2016b; Oosterveld et al., 2020; Stewart et al., 2015; Wang et al., 2012b). Nevertheless, other studies did not observe any significant change in the pS129 levels between healthy controls and PD patients (Foulds et al., 2012; Majbour et al., 2020; Schulz et al., 2021; van Steenoven et al., 2018). The correlation between pS129 levels and disease severity was also assessed in different studies, which culminated in inconsistent results. Wang et al. showed that the levels of pS129 significantly correlate, although weakly, with PD severity (Wang et al., 2012b). This study also reported a higher ratio of pS129/total aSyn in PD patients than in healthy controls. These results were further corroborated by other groups (Majbour et al., 2016a; Stewart et al., 2015). However, one study did not show any association between pS129 levels and PD severity and progression (Majbour et al., 2016b). It has been proposed that the ratios of pS129/total aSyn and oligomeric aSyn/total aSyn, contrarily to pS129 levels alone, may be a better marker and could differentiate between PD and other synucleinopathies (Hansson et al., 2014; Majbour et al., 2020; Parnetti et al., 2014a, 2014b; Stewart et al., 2015; Wang et al., 2012b).

Unfortunately, replicating and validating many of these studies is still challenging. One major obstacle is the lack of robust assays that capture the diverse range of aSyn species, including the modified and aggregated forms, thereby making it difficult to evaluate and validate their diagnostic value.

However, more recently, seed amplification assays (SAAs) have been shown as promising diagnostic tools for PD and other synucleinopathies. They have been employed to detect or classify synucleinopathies based on the presence of misfolded aSyn forms. Initially, SAAs were developed for antemortem detection of prions (Saborio et al., 2001), and recently they have been adapted to also detect aSyn seeds. SAAs are capable of detecting aSyn seeds from several biospecimens, such as the brain (Bargar et al., 2021; Groveman et al., 2018; Manne et al., 2019), CSF (Bongianni et al., 2019; Concha-Marambio et al., 2019, 2021; Fairfoul et al., 2016; Groveman et al., 2018; Kakuda et al., 2019; van Rumund et al., 2019; Shahnawaz et al., 2017), submandibular gland (Manne et al., 2020a), olfactory mucosa (De Luca et al., 2019; Perra et al., 2021), skin (Donadio et al., 2021; Manne et al., 2020b; Wang et al., 2021), and gut (Fenyi et al., 2019). aSyn-SAAs have shown high diagnostic accuracy in detecting aSyn seeds in

validated cohorts of PD patients (Concha-Marambio et al., 2019, 2021; Fairfoul et al., 2016; Groveman et al., 2018; Manne et al., 2020b; Rossi et al., 2020; Shahnawaz et al., 2017; Wang et al., 2021) and distinguishing aSyn aggregates from different synucleinopathies, such as PD and MSA (Shahnawaz et al., 2020; Singer et al., 2020). Moreover, another study showed that aSyn-SAAs had the potential to differentiate PD and DLBs from control subjects, even in the prodromal stages (Bellomo et al., 2022).

The largest analysis of aSyn-SAAs in CSF to date has demonstrated high sensitivity for PD (87.7%), and high specificity for healthy controls (96.3%). They also observed a sensitivity of 98.6% of the aSyn-SAA in sporadic PD with the typical olfactory deficit. On the other hand, patients with the LRRK2 variant showed a lower aSyn-SAA positivity rate (78.3%). Patients with the LRRK2 variant and normal olfaction showed an even lower aSyn-SAA positivity rate (34.7%) (Siderowf et al., 2023).

These findings suggest a crucial role for the aSyn-SAA in therapeutic development to identify PD and other synucleinopathies patient cohorts and to establish risk cohorts. These assays have also provided important insights into the mechanisms underlying synucleinopathies and may have implications for the development of new therapeutic strategies for these diseases.

Although CSF appears to be an ideal biofluid to check for biomarkers, the process of obtaining CSF involves an invasive and painful procedure, and there may be additional technical and ethical issues related to the collection procedure. For that reason, researchers have begun to explore alternative biofluids and investigate potential PD biomarkers in these biofluids.

1.9.2. Biochemical biomarkers: alpha-synuclein in blood

The easy accessibility of blood in clinical circumstances makes it a good alternative to the conventional use of CSF as a biofluid to monitor biomarker levels of disease for diagnostic or prognostic evaluation (Fayyad et al., 2019). aSyn is largely expressed outside the CNS, and it can be measured in the blood (El-Agnaf et al., 2003). aSyn levels have been examined in serum, plasma, erythrocytes, and whole blood in PD. Nevertheless, erythrocyte contamination and hemolysis strongly affect it quantifies in the blood, even more than in the CSF. Since erythrocytes account for more than 99% of aSyn in blood, even low levels of erythrocyte contamination could significantly increase aSyn in serum or plasma (Barbour et al., 2008). In familial PD patients with *SNCA*

multiplications, high levels of aSyn in blood were reported (Miller et al., 2004). In one case-control study increased levels of aSyn were reported in PD plasma and serum, and the serum levels were correlated with disease severity (Chang et al., 2020). The increase in plasma levels of aSyn in PD patients was also observed in other studies, although the correlation between this parameter and motor disability varied (Ding et al., 2017; Lee et al., 2006; Lin et al., 2017; Wang et al., 2020). Nevertheless, several other studies observed the opposite, a decrease in the levels of aSyn in PD patients' plasma or failed to observe any significant difference in this parameter (Li et al., 2007; Shi et al., 2010). In another study, where patients were followed for up to 20 years after initial presentations, plasma total aSyn and phosphorylated aSyn or pS129 were determined repeatedly (Foulds et al., 2013). It was observed that plasma levels of pS129 but not total aSyn were higher in PD patients on the first presentation, but over time, the levels of total aSyn increased while the pS129 levels remained unaltered (Foulds et al., 2013). These inconsistent results for the changes in the aSyn level in the blood of PD patients could be due to technical reasons such as contamination from erythrocyte hemolysis, different approaches to sample collection, use of different assay techniques, and the differential ability of antibodies to bind to different forms of aSyn (Lee et al., 2006; Li et al., 2007; Lin et al., 2017). As opposed to such studies, efforts have been made to validate erythrocyte aSyn as a PD biomarker. In a recent study, total and aggregated levels of aSyn were increased in the membrane fraction while pS129 levels were increased in the cytosolic fraction in PD subjects (Tian et al., 2019). Similarly, in another study erythrocyte aSyn oligomers or oligomeric aSyn were found to be higher in PD patients compared to the healthy controls and MSA patients, and this measurement could differentiate, with good sensitivity and specificity, PD patients from control or MSA patients (Li et al., 2019). Later this study showed that an increase in erythrocyte oligomeric aSyn reflected increased aSyn levels in the brain of rat models. In another study, it was reported an increase in the levels of erythrocyte aSyn in PD patients, however, it was also showed that higher plasma levels of aSyn in PD had a better positive predictive value and also a stronger correlation with disease severity than erythrocyte aSyn levels (Wang et al., 2020). The levels of erythrocyte aSyn dimers were higher in idiopathic forms of PD and PD patients with GBA mutations (Papagiannakis et al., 2018). Likewise, a decrease in the ratio of total aSyn to proteinase K-resistant aSyn (phospholipid-bound aSyn) in erythrocytes allowed to discriminate PD patients from healthy controls (Abd-Elhadi et al., 2015). In a later study from the same group, it was shown that total aSyn, proteinase-K resistant aSyn and pS129 levels were higher in PD patients with motor symptoms and without dementia and it was possible to differentiate with a high degree of accuracy between this group and healthy controls (Abd Elhadi et

al., 2019). On the other hand, a recent study observed a decrease in total aSyn levels and an increase in oligomeric aSyn and aSyn-amyloid β42 complex levels in PD patients (Daniele et al., 2018). This study also suggested that the level of aSyn-amyloid β42 complex in erythrocytes correlates with the degree of PD severity. Overall, the potential of aSyn as a blood biomarker for PD remains unclear and it is still not clear if it can be used in clinical practice. Although the studies conducted so far have shown promising results, further research in this area is necessary.

1.9.3. Biochemical biomarkers: alpha-synuclein in saliva

Studies have detected aSyn in the saliva of PD patients, which reports a decrease in the total levels of aSyn in the saliva of PD patients (Al-Nimer et al., 2014; Devic et al., 2011). Vivacqua and colleagues found a significant increase in oligomeric aSyn and oligomeric aSyn/total aSyn ratio, along with a decrease in total aSyn levels in the saliva of PD patients, suggesting that this decrease could partly be attributed to the oligomerization of monomeric aSyn (Vivacqua et al., 2016).

Similar results were reported in another study, which showed that oligomeric aSyn levels were positively correlated with disease duration, with higher levels observed in bradykinesia and rigidity-dominant phenotypes compared to tremor-dominant ones (Shaheen et al., 2020). Another study measured the levels of total aSyn and oligomer aSyn in the saliva of PD patients and healthy controls and looked for single nucleotide polymorphisms or SNP variants of SNCA in the PD group (Kang et al., 2016). No significate difference was observed in the total aSyn levels between both groups, in contrast with a significant increase in oligomeric aSyn levels in PD patients. It was also observed that the total aSyn levels in saliva decreased with age without any correlation with disease duration or severity but depended upon the SNP variant of SNCA (Kang et al., 2016). There have been inconsistent findings regarding the association between salivary alpha-synuclein (aSyn) levels and PD. A meta-analysis study reported a significant association between the increased levels of salivary oligomeric aSyn with PD, but no association between salivary total aSyn and the disease (Bougea et al., 2019). Due to these inconsistent results, there has been a growing interest in investigating aSyn in other tissues, such as the enteric nervous system, retina, and skin to understand various non-motor features of PD and overall pathology of the disease. Currently, the implications of aSyn as a biomarker for PD are not clear and research in this area is still

in the early stages. Nevertheless, the non-invasive nature of the saliva collection and the potential of aSyn as a biomarker make it a valuable area for further research.

1.9.4. Biochemical biomarkers: MicroRNAs

MicroRNAs (miRNA) are small, single-stranded, non-coding RNA (ncRNA) molecules containing 19-22 nucleotides. They regulate gene expression by complementary binding to messenger RNA, resulting in RNA silencing or post-translational inhibition (Bartel, 2004; Jonas and Izaurralde, 2015; Krol et al., 2010). These ncRNAs are broadly studied due to their unique size and specialized protein machinery (Jonas and Izaurralde, 2015). In recent studies, miRNAs have been found to regulate PD-related genes and alterations in the expression levels of specific miRNAs may play a role in either disease onset or progression (Leggio et al., 2017; Yang et al., 2019a). MiRNAs present in certain biofluids are known to be tissue-specific, abundant, highly stable and quantifiable. These characteristics have made the circulating miRNAs potential biomarkers for the early detection of PD and for monitoring its progression over time (Mushtaq et al., 2016; Wang et al., 2017).

MiR-7, miR-153 and miR-34b/c by binding the 3'UTR can downregulate *SNCA*, suggesting a strong regulatory role of these miRNAs in synucleinopathies (Doxakis, 2010; Miñones-Moyano et al., 2011). Of those, miR-7 is proposed for miRNA replacement therapy in synucleinopathies to reduce the production of aSyn and the consequent aggregation of the protein (Titze-de-Almeida and Titze-de-Almeida, 2018). Apart from miRNAs targeting *SNCA* directly, several other miRNAs have been identified in synucleinopathies in human samples and animal or cellular models (Heman-Ackah et al., 2013). Moreover, miRNAs are identified in CSF as well as in the blood (Fyfe, 2020; Marques et al., 2017). To develop novel therapeutic approaches and diagnose PD and other synucleinopathies, it is crucial to target PD miRNA signatures and identify their functions and correlate them with disease initiation and progression.

The first miRNA discovered, let-7, and the cluster miRNAs let-7a to let-7-k, miR-98 and miR-202, consist of one family upregulated in PD brain and CSF of PD patients (Briggs et al., 2015; Gui et al., 2015). This miRNA family is linked with apoptosis, negative regulation of axon guidance and regeneration, and immune response leading to neurodegeneration (Lehmann et al., 2012; Li et al., 2017; Wang et al., 2019).

The miR-30 family of microRNAs has been shown to be upregulated in the *substantia nigra* and *cingulate gyri* of PD patients, and has been associated with neuroinflammation and disease progression, making them promising biomarkers (Briggs et al., 2015; Li et al., 2018a; Tatura et al., 2016). Additionally, miR-30a was increased in peripheral blood of PD patients treated with L-DOPA (Margis et al., 2011). In contrast, Martins and collaborators identified 18 miRNAs that were under-expressed in peripheral blood mononuclear cells (PBMCs) from PD patients, and identified miR-30b and miR-30c, as key modulators of two pathways relevant to PD (i.e., the glycosphingolipid biosynthesis and the protein ubiquitination), which may contribute to PD susceptibility (Martins et al., 2011).

A group of miRNAs, miR-29a, miR-29b-1, miR-29b-2, and miR-29c, are upregulated in the cingulate gyri of PD brain, and these alterations can be modulated by L-DOPA treatment, which can be monitored over time to track both the effectiveness and outcome of treatment.

On the other hand, in 2011, a study conducted by Margis and her group showed that miR-29a, miR-1, and miR-22 were downregulated in peripheral blood from L-DOPA-treated PD patients (Margis et al., 2011). This miR-29 family regulates important processes such as epigenetic and apoptosis, and neuronal survival (Roshan et al., 2014; Schwienbacher et al., 2017; Tatura et al., 2016).

Another important family of miRNA, miR-26a-1, miR-26a-2 and miR-26b, was found upregulated in the *substantia nigra* of the PD brain and the striatum of a rodent PD model (Briggs et al., 2015; Horst et al., 2018). In addition, miR-26a was also found upregulated in the peripheral blood from L-DOPA-treated PD patients (Margis et al., 2011). This miR-26a was also identified as a main modulator of glycosphingolipid biosynthesis and the protein ubiquitination pathways (Martins et al., 2011). It is indirectly linked with GWAS-classified PINK1 suggesting a correlation with familial PD (Huse et al., 2009; Pickrell and Youle, 2015). Furthermore, miR-26 is linked to long-term potentiation (LTP) induced gene expression in response to neuronal activity, indicating that impaired neuronal function may occur prior to neuronal loss (Gu et al., 2015).

The levels of miR-4639-5p levels were found significantly upregulated in PD patients, suggesting its potential biomarker for early diagnosis of PD. Interestingly, this upregulation was uncorrelated with age of disease onset, gender, L-DOPA treatment, or severity of PD motor symptoms (Chen et al., 2017). A recent study showed that hsa-miR-29c-3p, hsa-miR-214- 3p, and hsa-miR-221-3p were differentially expressed in the

blood of PD patients (Schulz et al., 2019). Additionally, Yang and colleagues reported elevated levels of plasma hsa-miR-105-5p in PD patients (Yang et al., 2019b). These findings suggest that different miRNAs may serve as potential biomarkers for PD diagnosis and monitoring.

In fact, several microRNAs have been found to be associated with the onset and progression of PD, and their combination may offer a promising avenue for accurate diagnosis. However, inconsistent results have been observed in different types of specimens, such as post-mortem brain specimens, blood, and CSF samples. Thus, further research is needed to evaluate the potential of microRNAs and other small molecules as potential biomarkers before they can be applied in clinical practice.

1.9.5. Biochemical biomarkers: extracellular vesicles

Extracellular vesicles (EVs) are a subset of small membranous vesicles that originated from endosomes and are released into biofluids by different kinds of tissues, including the CNS (Shi et al., 2019). EVs can be distinguished into exosomes (50nm-150nm), microvesicles (100nm-1000nm), and apoptotic bodies (1000nm) (Croese and Furlan, 2018). In the CNS, EVs are likely to carry proteins and nucleic acids that suggest pathogenic processes. EVs have been shown to transfer and transport toxic aSyn between cells, suggesting that they may play an important role in the spreading of aSyn aggregates and accelerating of PD (Vella et al., 2016). It has been reported that the level of aSyn in plasma from CNS-derived exosomes is higher in PD patients, however, their performance is not very effective (Shi et al., 2014). Recent studies reported consistent results with the ones described previously and a lack of adequate diagnostic performance (Cerri et al., 2018; Si et al., 2019). Changes in other proteins or nucleic acids levels, such as microRNAs have also been shown (Fraser et al., 2016; Vella et al., 2016; Zhao et al., 2019). In order to enhance diagnostic accuracy, it would be beneficial to investigate a panel of combined biomarkers in EVs using a larger clinical PD cohort.

1.9.6. Inflammation-related biomarkers

Inflammation is widely considered to be a contributing factor to pathology in PD. In PD patients, elevated levels of inflammatory mediators and abnormally activated microglia are associated with neuroinflammation. Many PD-causing mutations in *SNCA*, *PRKN*,

LRRK2, *PINK1*, and *DJ-1* are also associated with neuroinflammation. Multiple anti-inflammatory medications have shown neuroprotective properties in *in vivo* and *in vitro* models of PD, these include nonsteroidal anti-inflammatory drugs (NSAID), agonists of nuclear factor erythroid 2-related factor 2 (NRF2), peroxisome proliferator-activated receptor gamma (PPAR-γ), inhibitors of tumour necrosis factor (TNF)-α and NLR family pyrin domain containing 3 (NLRP3), and steroids (Liu et al., 2022).

Inflammatory factors can be used as potential biomarkers to reflect the neuroinflammatory pathogenesis of PD (Parnetti et al., 2013; Qin et al., 2016; Reale et al., 2009). Studies have shown that changes in the CSF and peripheral blood can be used to detect molecular changes underlying neurodegenerative diseases. Additionally, the alterations in inflammatory biomarkers in the blood of PD patients suggest the involvement gut-brain axis in the pathogenesis of PD.

Researchers have shown that PD patients have significantly higher levels of inflammatory cytokines, such as interleukin (IL)-1β, IL-4, and IL-6, and TNF-α, in their CSF and plasma (Le et al., 1999; Reale et al., 2009). Moreover, four biomarkers, CXCL12, CX3CL1, IL-8 and CCL15, were significantly higher in PD than in healthy controls. Additionally, in the follow-up cohort, the levels of CX3CL1 were associated with PD development and motor progression (Li et al., 2022). The expression levels of inflammatory cytokines may not be specifically elevated in PD, for that reason a combination of inflammatory cytokines with other candidate biomarkers has been studied to help detect and diagnose PD development and progression (Eidson et al., 2017; Li et al., 2018b). Several inflammatory factors with aSyn in serum and CSF were measured with stable results regardless of sample collection time, and they were able to distinguish between PD and healthy controls with high sensitivity and specificity, and monitor inflammation as well as disease progression (Eidson et al., 2017).

Moreover, different proteins related to T cell-mediated immunity have been identified as potential biomarkers of PD in the peripheral blood. Serum lymphocyte activation gene-3 (sLAG-3), a helper T cell marker, was able to differentiate PD from healthy controls, suggesting that it could be a novel candidate biomarker for PD (Cui et al., 2019). As some PD patients had T-cells with specific epitopes to aSyn, other studies have also looked at the aSyn-specific T-cell activation. One study observed elevated levels of aSyn-specific T-cell responses in the periphery of PD patients with declined over time, and found a correlation between the T-cell responses and the age of the patients and an inverse correlation between the first one and the levodopa dosage (Abdi et al., 2022; Lindestam Arlehamn et al., 2020).

There is no doubt that inflammation plays a crucial role in the pathogenesis of PD, however, its potential value in the diagnosis or monitoring of the progress of PD is still limited due to discordant results and a lack of information regarding its reproducibility. Thus, overall, more longitudinal and cross-sectional studies are needed to investigate the association between numerous immune biomarkers and the development and progression of PD.

1.10. Models for studying alpha-synuclein-mediated toxicity

PD is predominantly characterized by the presence of motor symptomatology, such as rest tremor, rigidity, bradykinesia, and postural instability (Jankovic, 2008). Along with these motor signs, there are a multiplicity of non-motor manifestations such as hyposmia, constipation, fatigue, depression, anxiety, speech and swallowing difficulties, and cognitive and behavioural problems (Pandya et al., 2008) which, together, have a tremendous impact on the quality of life. Motor symptoms may appear much earlier than the clinical diagnosis of PD (Schapira et al., 2017; Tibar et al., 2018). Unfortunately, there is no cure for PD, although medicines, surgical treatments, and other therapies can often relieve some symptoms. A common treatment strategy involves medications that deplete dopamine or mimic its effect on dopamine receptors. For the moment, levodopa (L-DOPA) combined with carbidopa, a peripheral inhibitor of its breakdown, is our most effective medication for reducing side effects and maximizing therapeutic results (Milligan, 2019). Other alternatives include the use of dopamine agonists (pramipexole and ropinirole), monoamine oxidase B (MAO-B) inhibitors (eg. selegiline, rasagiline, and safinamide), Catechol-O-methyl transferase inhibitors (eg. entacapone) and in certain cases anticholinergics (eg. trihexyphenidyl and benztropine). Some interventional therapies for PD include deep brain stimulation (DBS) and carbidopa/L-DOPA enteral suspension (Duopa) (Hayes, 2019; Lang and Espay, 2018). However, current therapies are not capable of slowing or stopping the progression of neurodegenerative diseases (disease course modifiers), or replacing lost neurons (neuro-restorative). Then, as new immunomodulatory and oligonucleotide acid-based therapies emerge, aSyn-targeting therapies generate significant interest (Lang and Espay, 2018).

One of the major reason for these shortfalls for PD treatment is the lack of a comprehensive knowledge of its pathophysiology, pathogenesis, aetiology, and molecular mechanisms (Dauer and Przedborski, 2003; Duda and Dickson, 2012). For that reason, employing an effective experimental model system to gain a deeper

understanding of this disorder and identify new potential therapeutic targets is crucial. Nowadays, there are many experimental models, with different levels of complexity that have contributed towards the understanding of PD, such as cell models, human post-mortem analysis, animal disease models of PD, iPSC-derived brain organoids, organ-chips (e.g., brain-chip, substantia nigra brain-chip, neurovascular unit-on-a-chip, gut-brain-liver-on-a-chip, etc.), omics and multi-omics approaches (e.g., genomics, transcriptomics, proteomics, metabolomics, exposomics), computational models (e.g., PBPK, PDPK, QSP) (Cassotta et al., 2022; Wichmann, 2018). However, this thesis focuses only on two different approaches: a cell-based model that uses primary hippocampal neuronal cultures derived from rats, and a transgenic mouse model (WT and A30P).

1.10.1. Cell-based models

As previously described, PD is a complex disease with several pathological events involved. PD cellular models have been fundamental for the understanding of the disease molecular mechanisms behind crucial biological processes, such as protein degradation, trafficking, and cell division. Human embryonic kidney 293 (HEK293), human neuroglioma (H4), human neuroblastoma (SH-SY5Y), and immortalized Lund Human Mesencephalic (LUHMES) cells (Lázaro et al., 2017) are some of these cellular models. They allow gene manipulation and can reduce some ethical issues related to mammalian models. Other advantages of these types of models are: being easy to culture and transfect, presenting a rapid proliferation, that enables large-scale studies, minimum variability between cultures, rapid screening of investigational drugs, and the possibility to study aSyn aggregation and release/secretion (Astashkina et al., 2012; Falkenburger and Schulz, 2006; Hansen et al., 2011; Lázaro et al., 2017; Xicoy et al., 2017). In contrast to cell lines, primary cultures are isolated from tissues and they have a finite lifespan and limited expansion capacity. Moreover, primary cells maintain many of the relevant markers and functions that are seen *in vivo*, being more representative of the tissue of origin (Alge et al., 2006; Pan et al., 2009).

Although, cellular models have been useful in studying PD and aSyn-mediated toxicity, they have limitations and cannot fully replicate all aspects of the disease, especially the ageing process (Lázaro et al., 2017; Miller et al., 2013; Sánchez-Danés et al., 2012). However, these models simplify the experimental features of the disease and allows unique opportunities to study cell-specific pathways and help to interpret a single pathway and to identify possible therapeutic targets (Miller et al., 2013; Sánchez-Danés et al., 2012). Albeit, more efforts are needed to develop cellular-based models able to

fully mimic the pathobiology associated with aSyn, including its aggregation and toxicity (Lázaro et al., 2017), the currently models have provided valuable insights into the molecular basis of PD, as well, as other synucleinopathies.

1.10.2. Transgenic mouse models

Our understanding of PD has been improved through the use of animal models (Blesa et al., 2012) and has contributed to the development of novel pharmacological agents and new treatment strategies for PD (Buhidma et al., 2020). Animal models commonly used for modelling PD include rodents, non-human primates (NHP), and non-mammalian species, with various available with each group such as pharmacological, toxin, genetic, and aSyn models (Lama et al., 2021). However, it is essential to consider the advantages and limitations of each model and choose the most suitable model based on the specific purpose and aim of the study. For that reason, understanding the specific characteristics of each model facilitates the experimental design as well as the interpretation of pathophysiology.

The pharmacological models of PD could be developed by introducing drugs such as L-DOPA (Carlsson et al., 1957), reserpine, an inhibitor of the vesicular monoamine transporters (VMATs) (Colpaert, 1987), and haloperidol, an antagonist of dopamine D2 and, to a lesser extent, D1 receptors (Sanberg, 1980). The administration of these drugs to rodents and rabbits led to short-term Parkinsonian-like symptoms (Duty and Jenner, 2011).

For more enduring effects, toxin models have been developed. These models can mimic sporadic PD, by inducing rapid degeneration of the dopaminergic neuronal population. These toxins can be largely subcategorized into neurotoxins such as 6-hydroxydopamine (6-OHDA) and 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine (MPTP), pesticides such as rotenone, paraquat, and permethrin, and endotoxins as lipopolysaccharide (LPS) (Duty and Jenner, 2011; Lama et al., 2021; Polinski et al., 2018). However, these models fail in the formation of Lewy bodies, the main pathology hallmark of PD. Despite some limitations, these types of models are relevant to discover disease mechanisms and potential therapeutic targets in PD (Zeng et al., 2018).

PD genetic models try to mimic relevant features of familial forms of the disease (Figure 6). These models are created through transgenic overexpression of aSyn and LRKK2 or knockout/knockdown of genes such as DJ-1, Parkin, and PINK1 (Dawson et al., 2010;

Gasser, 2009). However, just a few of these models can reproduce the complete features of PD, and quite often the most common motor features of the disease are not observed (Vingill et al., 2018). For example, mice carrying a deletion of the *SNCA* gene only show minor phenotypes, such as synaptic dynamics changes, and no behavioural changes (Abeliovich et al., 2000; Cabin et al., 2002). Several aSyn transgenic models have been developed, which express aSyn under the control of promoters such as PDGFs, Thy1, or PrP (Giasson et al., 2002; Lee et al., 2002; Masliah et al., 2000). In the majority of these models, behavioural phenotype and neuropathological features, are present, including loss of striatal dopamine (Ono et al., 2009; Richfield et al., 2002). Among those, the Thy1 promoter model is the one more extensively used since it mimics many PD features, such as the activation of glia (Watson et al., 2012). Curiously, this model also shows aggregates with phosphorylated aSyn that are resistant to proteinase K and develop mitochondrial dysfunction (Chesselet et al., 2012; Nakamura et al., 2011). Furthermore, non-motor phenotypes are also observed in this model, including impaired olfactory dysfunction, cognitive disturbances, and disrupted circadian rhythms (Chesselet et al., 2012). In addition, mutant aSyn models such as A30P and A53T have also been generated (Giasson et al., 2002; Kahle et al., 2001). These models present neuroinflammation, motor dysfunction, and pathological inclusions, (Giasson et al., 2002; Kahle et al., 2001; Lee et al., 2002). Most of aSyn transgenic mice do not show dopaminergic neuronal loss within the SN, however, models based on aSyn mutations, like the doubly mutated (A30P/A53T) or truncated aSyn (Vingill et al., 2018), show mild loss of these neurons. Moreover, WT aSyn Thy1 mice displayed stronger motor phenotype when compared to the other models, including A30P aSyn Thy1 transgenic mice. The reason why these transgenic mice fail to mimic certain symptoms of PD is still unclear. As ageing is the major risk factor for PD (Niccoli and Partridge, 2012), it is possible that the mice do not live long enough to develop all the symptoms of the disease. In addition, we are only modulating aSyn expression in these mice, while LBs from PD patients contain a wide range of other proteins that might also play a role in the onset and progression of the disease. Thus, it is crucial to develop and characterize new animal models of PD for a better understanding of how the disease works, and to pave the way for novel therapeutic strategies.

The aSyn models take advantage of its spreading to promote the synucleinopathy observed in PD (Luk et al., 2012a). Some of the models include adeno-associated viral vectors (AAVs)-mediated overexpression (Kirik et al., 2003; Oliveras-Salvá et al., 2013), pre-formed fibrils (PFF), or a combination of the previous ones (Thakur et al., 2017). Despite the AAV model giving relevant information about the pathophysiology of aSyn

and the importance of its expression in the severity and development of PD, it is difficult to standardize this model due to the differences in the AAV's production and purification methods between laboratories. aSyn PFFs can drive the aggregation of endogenous aSyn into LB-like inclusions over 3–6 months (Luk et al., 2012a; Paumier et al., 2015), replicating several features of PD. Moreover, the combination of AAV and PFFs injection in both SN and ventral tegmental rat areas was able to speed up the aggregation formation process and progressive neurodegeneration, when compared with either AAV or PFF delivery alone (Thakur et al., 2017).

It is important to consider that species, the genetic background of a strain, and the environment in which an animal model is raised affect the symptoms and neurodegenerative hallmarks displayed by it. For that reason, these models can be combined to study the interaction between genetics and environment unravel the heterogeneity and mechanisms involved in PD, and discover new therapeutic strategies.

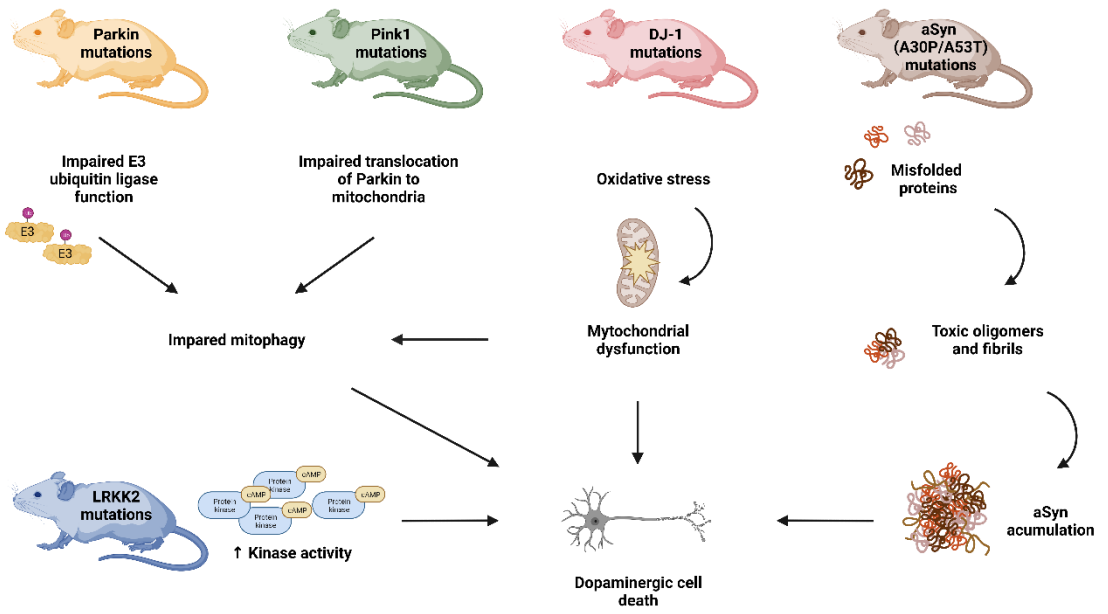


Figure 6 | Transgenic mouse models of PD. Transgenic overexpression of aSyn (A30P and A53T model) and LRKK2 or knockout/knockdown of Parkin, PINK1 and DJ-1 are some of the mouse models used to mimic relevant features of the disease and to understand PD pathogenesis. The image was created using BioRender.com.

CHAPTER 2

AIMS OF THE STUDY

In this study, we focused on the study of the molecular mechanisms underlying PD. In particular, since we still have a limited understanding of the role of aSyn in synaptotoxicity at the single-cell and molecular levels, we aimed to provide novel insight into the effects of aSyn exposure on synapses and to investigate potential diagnostic approaches using cell-based and transgenic mouse models of PD. Hence, the specific aims of this study were to:

Aim 1. Characterize the forms of aSyn which accumulate predominantly in the synaptic compartment;

For the first aim, the presence of aSyn in the synaptic compartment was investigated and characterized in primary hippocampal cultures that were treated with different types of recombinantly-produced aSyn species or transduced with lentiviral vectors encoding for wild-type or mutant forms of aSyn.

Aim 2. Define the morphological, molecular and functional changes induced by aSyn-increased levels;

To understand the effects of deregulated aSyn levels and investigate their role in PD pathogenesis, we examined whether morphological, molecular and functional alterations on the hippocampal neurons at different time points were correlated or dependent on increased levels of different species of aSyn.

Aim 3. Identify specific interacting partners of aSyn in isolated synaptic vesicles;

In order to identify the interacting partners of aSyn, isolation, biochemical and physical characterization of synaptic vesicles from wild-type mice and transgenic mice overexpressing the human wild-type aSyn were carried out.

Aim 4. Identify the occurrence of synaptic proteins in biofluids from transgenic mice;

In this last aim, we investigated potential biomarkers in different biofluids, such as cerebrospinal fluid (CSF), blood, and saliva from wild-type mice and also the transgenic mice overexpressing the human wild-type, or A30P mutant aSyn at 3, 6, and 9 months old.

CHAPTER 3

MATERIALS AND METHODS

3.1. Materials

3.1.1. Mouse models and ethical statement

In this study, we used mice over-expressing full-length, human, WT aSyn (Thy1-WT aSyn mice) or A30P aSyn (Thy1-A30P aSyn mice) under the murine Thy-1 or Thy1.2 promoter, respectively. WT B6D2F1 (BDF1) and WT C57BL/6J were used as controls.

The Thy1-WT aSyn mice, also known as Line 61, were developed by crossing a female C57BL/6 with a male DBA/2F1 mice, which influences some aspects of their phenotype (Figure 7) (Chesselet et al., 2012; Rockenstein et al., 2002). Compared to other promoters often used in other models, the Thy-1 promoter has distinct advantages, such as broad expression and over-expression of aSyn throughout the brain (Chesselet et al., 2012; Rockenstein et al., 2002). In this model, many of the features of sporadic PD are replicated, including progressive changes in dopamine release and striatal content, aSyn pathology, impairments in motor and nonmotor functions, inflammation, and biochemical and molecular changes that are similar to those observed in PD (Chesselet et al., 2012). The use of this model to mimic sporadic PD is validated by genetic studies that have shown that the multiplication of the *SNCA* causes familial forms of PD, and polymorphisms in noncoding regions of the gene increase the risk of PD and lead to early onset and a faster progression (Edwards et al., 2010; Gatto et al., 2010; Huang et al., 2011).

The Thy1-A30P aSyn mice were backcrossed into the C57BL/6 mouse strain (Kahle et al., 2000a). Similarly to the previous model, the expression of A30PaSyn was driven by the brain neuron-specific Thy1 promotor (Figure 7) (Kollias et al., 1987; Lüthi et al., 1997; Moechars et al., 1996; Vidal et al., 1990). In this model, A30PaSyn expression was upregulated since the first postnatal month and remained high until later stages (Kahle et al., 2000a). This time course of expression is similar to the one of endogenous aSyn in WT mice (Kahle et al., 2000a). This model was used to mimic a familial form of PD and to study the *in vivo* consequences of expression of A30P aSyn in the brain since patients heterozygous for the A30P mutation in the *SNCA* develop an aggressive and early onset form of PD (Kahle et al., 2000a).

All animal procedures were performed in accordance with the European Community (Directive 2010/63/EU), and in compliance with protocols approved by institutional and national ethical committees (Landesamtes für Verbraucherschutz und

Lebensmittelsicherheit (LAVES), Braunschweig, Lower Saxony, Germany, license number: T20.7 and 19.3213).

All mice were housed at a controlled temperature and 12:12 h light/dark cycle. The number of animals included in the analysis was estimated by power analysis (R software packages, Vienna, Austria), and the selection of animals was randomized. Young (3 months old), middle-aged (6 months old) and old (9 months old) mice were used in the study. Male and female mice mixed groups were used in all the experiments.

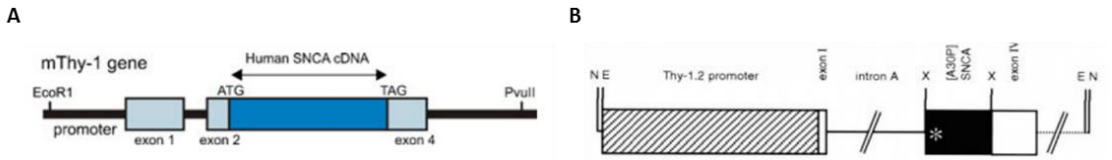


Figure 7 | Tg mice expressing haSyn. Schematic diagram of the Thy1-WT aSyn construct over-expressing the full length, human, WTaSyn under the murine Thy-1 promoter **(A)** and the Thy1-A30P aSyn construct over-expressing the full length, human, A30PaSyn driven by the murine Thy-1.2 promoter **(B)**. Adapted from (Chesselet et al., 2012; Kahle et al., 2000b).

3.2. Methods

3.2.1. Primary Hippocampal Neuronal Cultures

Primary hippocampal neuronal cultures from E18 Wistar rat embryos were prepared as previously described with slight modifications (Tönges et al., 2014; Villar-Piqué et al., 2016). In detail, pregnant rats were sacrificed by carbon dioxide inhalation and the embryos were extracted from the uterus. The meninges were removed, and the hippocampi were dissected and transferred to ice-cold 1x Hanks balanced salt solution ($CaCl_2$ and $MgCl_2$ free; HBSS) (Gibco Invitrogen, CA, USA) supplemented with 0.5% sodium bicarbonate solution (Sigma-Aldrich, MO, USA). Enzymatic digestion of the tissue in 1mL of 0.25% trypsin (Gibco Invitrogen, CA, USA) was performed at 37°C for 15 minutes (min). A mixture of 100µL DNase I (0.5mg/mL, Roche, Basel, Switzerland) and 100µL fetal bovine serum (FBS) (Anprotec, Bruckberg, Germany) was added to stop the reaction. The tissue was gently shaken and centrifugated for 5 minutes at 300xg. 1 mL of FBS was added to the tissue and this was gently dissociated using a glass Pasteur pipette. Then, the cell suspension was centrifugate again at 300xg for 5 min, and cells were resuspended in pre-warmed neurobasal medium (Gibco Invitrogen, CA, USA) supplemented with 1% penicillin-streptomycin (Pan Biotech, Aidenbach, Germany), 0.5% GlutaMax and 2% B27 (Gibco Invitrogen, CA, USA). Primary cells were seeded in 24-well microplates (100 000 cells/well), 12-well microplates (400 000 cells/well) and/or in multi-electrode arrays (MEAs) (250 000 cells/array) for immunocytochemistry (ICC), immunoblotting, and neuronal activity recordings respectively, all coated with poly-L-ornithine (0.1 mg/mL in borate buffer) (PLO; Sigma-Aldrich, MO, USA). Cells were maintained at 37°C with 5% CO_2, and one-third of the medium was changed every 3-4 days. Cells were infected with lentivirus coding GFP, WTaSyn, A30PaSyn or A53TaSyn on day 3 (MOI 1), or treated with 100nM recombinantly-produced aSyn species (monomers (aSyn Mono), oligomers (aSyn Oligo), and pre-formed fibrils, (aSyn PFF)). To limit the growth of glial cells, 4 µM cytosine arabinoside (Sigma) were added once to the cultures. Cultures were kept for further 1-4 weeks. For immunocytochemistry (ICC), cells were fixed with 4 % PFA and then quenched (0.5% hydrogen peroxide) (Szegő et al., 2017). For protein quantification, cells were lysed in RIPA buffer (50 mM Tris, pH 8.0, 0.15 M NaCl, 0.1% SDS, 1.0% NP-40, 0.5% Na-Deoxycholate, 2mM EDTA, supplemented with protease and phosphatase inhibitors cocktail (completeTM protease inhibitor and PhosSTOPTM phosphatase inhibitor; Roche, Basel, Switzerland), sonicated and centrifuged (20 min, 4 °C, 16 000xg).

3.2.2. Lentivirus production

Full-length human WTaSyn, A30P or A53T mutant aSyn were subcloned into the pWPI, bicistronic lentiviral vector of second generation (Tronolab, Switzerland), under the cytomegalovirus (CMV) early enhancer/chicken β-actin (CBA) promoter (CAG promoter), as previously described with slight modifications (Follenzi and Naldini, 2002; Tiscornia et al., 2006). The vector pWPI containing IRES-GFP cassette without any type of aSyn was used as infection control in the experiments. HEK 293FT cells were seeded in T175 cm^2 flasks (Corning, Merck, Darmstadt, Germany) and grew until they reach 90-100% confluency in DMEM (PAN Biotech, Aidenbach, Germany) supplemented with 10% FBS (Anprotec, Bruckberg, Germany), 1% penicillin-streptomycin (PAN Biotech, Aidenbach, Germany), 250mg G418 (Geneticin) (Anprotec, Bruckberg, Germany) and 1% Minimal Essential Medium (MEM) (Opti-MEM™ Reduced Serum Medium, Invitrogen, CA, USA) and maintained at 37°C with 5% CO_2. Then, cells were detached using trypsin (Gibco Invitrogen, CA, USA) at 37°C for 5 min, and the reaction was stopped with the addition of fetal bovine serum (FBS) (Anprotec, Bruckberg, Germany). The cells were centrifuged at 800xg for 5 min, and the cell pellet was resuspended in medium and seeded with fresh medium on 0.1% gelatine (Merck, Darmstadt, Germany) pre-coated plates (Corning, Merck, Darmstadt, Germany) and kept overnight at 37°C with 5% CO_2. Then, 3 hours (h) before transfection, the medium was removed and replaced by DMEM with 2% FBS (Anprotec, Bruckberg, Germany). The cells were then co-transfected with 144µg of pCMV-delta 8.9 (Trono lab, EPFL, Switzerland) packaging virus, 57.9µg vesicular stomatitis virus glycoprotein (VSV-G) packing virus and 160µg of the plasmid of interest, according to the calcium phosphate ($CaPO_4$) precipitation method. Then, the DNA mixture was added to 6 mL of 1x BBS (50 mM BES, 1.5 mM Na2HPO_4, 280 mM NaCl) and in a vortex shaker, 0.36 mL $CaCl_2$ (2.5M $CaCl_2$) was added to this mixture. Before adding it to the cells, the solution was incubated 20min in the dark. On the following day, the medium was changed to Panserin (PAN Biotech, Aidenbach, Germany) supplemented with 1% penicillin-streptomycin (PAN Biotech, Aidenbach, Germany) and 1% MEM. 48h post-transfection, viruses were harvested and centrifuged at 3 000xg for 15 min at 4°C. The supernatant was filtered through a 0.45µm filter (Corning, Merck, Darmstadt, Germany), mixed with 1x PEG solution (SBI System Bioscience, CA, USA) to pellet the virus, and then incubated for one day at 4°C. Next, the viruses were centrifugated at 1 500xg for 30min at 4°C and the pellet was resuspended in Panserin 401 (PAN Biotech, Aidenbach, Germany). The viruses were tested for transduction efficacy and viral titers were determined using qPCR.

3.2.3. Cell treatments

3.2.3.1. Recombinant aSyn treatment

Primary hippocampal neuronal cultures were treated at day *in vitro* (DIV) 4, 7, 14 or 21 with 100nM recombinantly-produced aSyn species (aSyn Mono, aSyn Oligo, and aSyn PFF) and PBS, as a negative control, and kept in culture until DIV 5, 7, 8, 14, 15, 21, 22, 26, 28 or 30 (Figure 8). Recombinant aSyn was prepared as previously described (Dominguez-Meijide et al., 2020; Szegő et al., 2019). Culturing conditions were the same as specified above in the section "3.2.1. Primary Hippocampal Neuronal Culture".

3.2.3.2. Viral transduction

Primary hippocampal neuronal cultures were transduced at DIV 3 with IRES lentivirus (pWPI-CAG-aSynWT-IRES-GFP, pWPI-CAG-aSynA30P-IRES-GFP, and pWPI-CAG-aSynA53T-IRES-GFP (MOI 1)) and pWPI-CAG-IRES-GFP, as a negative control, and kept in culture until DIV 4, 7, 14 or 21 (Figure 8). Lentiviral production was prepared as previously described (in "3.2.2. Lentivirus production protocol" section). Culturing conditions were the same as specified above in the section "3.2.1. Primary Hippocampal Neuronal Culture".

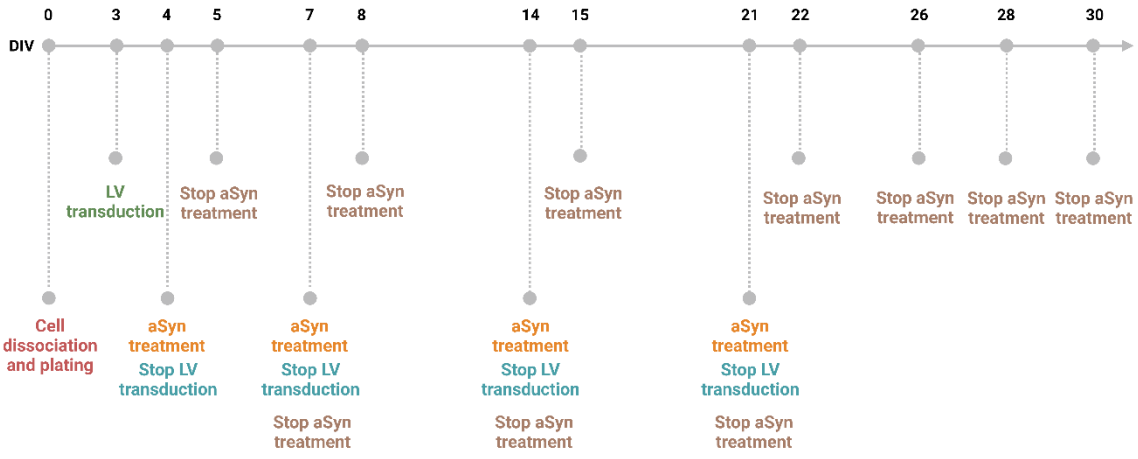


Figure 8 | Schematic workflow for recombinant aSyn-treatment and -transduction protocol. Primary hippocampal neuronal cultures from E18 Wistar rat embryos were prepared at DIV 0. At DIV 3, some cultures were transduced with IRES lentivirus encoding for wild type (WT) or mutant forms (A30P or A53T) of aSyn, or GFP. The transduction was kept for 1, 4, 11 or 18 days. In parallel, other cultures were treated with different species of aSyn recombinant protein at DIV 4, 7, 14 or 21 and kept for 1, 3, 10, 17, 22, 24 or 26 days.

3.2.4. Immunocytochemistry

After treatment, primary cultures were washed with 1x PBS (PAN Biotech, Aidenbach, Germany) and fixed with 4% of paraformaldehyde solution (PFA) for 30min at room temperature (RT). Then, the cells were washed three times with 1x PBS. To quench PFA autofluorescence, samples were incubated with 50mM of ammonium chloride (NH4Cl) and 50mM glycine solution for 30min. Afterwards, cells were washed with 1x PBS three times for 5min and permeabilized with 0.1% Triton X-100 (Sigma-Aldrich, MO, USA) for 10min. Following permeabilization, cells were washed three times for 5min with 1xPBS with 0.1% Triton X-100. Then, cells were blocked with 3% bovine serum albumin (BSA) (Sigma-Aldrich, MO, USA) and 0.1% Triton X-100 in 1x PBS for 1h at RT and later incubated with the primary antibodies overnight at 4°C [alpha-synuclein (1:1000, 610787, BD Transduction Laboratories), phosphorylated alpha-synuclein (1:1000, 015-25191, Wako), MAP2 (1:1000, 1790-1-AP, Proteintech), MAP 2 (1:1000, AB11267, Abcam), GFAP (1:1000, AB4674, Abcam), Iba 1 (1:1000, AB5076, Abcam), O4 (1:1000, MAB345, Millipore), homer I (1:1000, 16006, Synaptic Systems), PSD95 (1:1000, 124011, Synaptic Systems), SNAP25 (1:1000, 111002, Synaptic Systems), synapsin 1 (1:1000, 106011, Synaptic Systems), synaptophysin (1:1000, S5768, Sigma-Aldrich), synaptotagmin (1:1000, 105008, Synaptic Systems)]. Afterwards, the cells were washed three times with 1x PBS with 3% BSA and 0.1% Triton X-100 and then incubated with fluorescence-conjugated secondary antibodies for 2h at RT [Alexa Fluor 488 donkey anti-mouse (1:1000, A21202, Invitrogen), Alexa Fluor 488 donkey anti-rabbit (1:1000, A21206, Invitrogen), Alexa Fluor 555 donkey anti-rabbit (1:1000, A31572, Invitrogen), Alexa Fluor 555 donkey anti-mouse (1:1000, A31570, Invitrogen), Alexa Fluor 568 goat anti-mouse (1:1000, A11031, Invitrogen), Alexa Fluor 568 goat anti-rabbit (1:1000, A11036, Invitrogen)]. Later, nuclei were counterstained with DAPI (Carl Roth, Karlsruhe, Germany) and cells were washed three times with 1x PBS with 3% BSA and three times with 350mM NaCl in 1xPBS solution. Finally, cells were washed with 1xPBS and mounted with mowiol® for microscopy.

3.2.5. Biofluids and tissue collection

All mice were injected intraperitoneally with a mixture of 80mg/kg body weight Ketamine (CuraMed Pharma GmbH, Karlsruhe, Germany) and 5mg/kg body weight of Xylazine (Nycomed GmbH, Austria) and additionally with Pilocarpine (5mg/kg body weight; Sigma) to stimulate the salivation.

Saliva was collected by aspiration with a micropipette from all the mice. Saliva samples were then vortexed for 1 min, centrifuged at 16 000xg for 5 min at 4°C to remove particulate matter and salivary proteins, transferred to new tubes, and then stored at −80°C for further analyses.

Cerebrospinal fluid (CSF) was obtained by a *cisterna magna* puncture, an opening between the cerebellum and dorsal surface of the *medulla oblongata*, and collected in Eppendorf tubes. CSF samples were centrifuged at 16 000xg for 5 min at 4°C, aliquoted and stored at −80°C until required for further analyses.

Whole blood was collected directly after CSF collection, centrifuged at 3 000 r.p.m for 10 min at 4°C, aliquoted and stored at −80°C.

To extract serum, whole blood was collected and incubated undisturbed at RT for 20 min. Then, the samples were centrifugated at 3 000 r.p.m for 10 min at 4°C, aliquoted and stored at −80°C.

For plasma extraction, whole blood was collected in Eppendorf tubes coated with 10% 0.5M sterile EDTA of the expected blood volume, an anti-coagulant used to prevent clotting. Immediately after collecting the plasma, the tubes were centrifuged at 3 000 r.p.m for 10 min at 4°C, aliquoted and stored as previously described.

After the biofluids collection, the rest of the mice were perfused and/or dissected for brain collection. Some of the brain samples were lysed in RIPA buffer (50 mM Tris, pH 8.0, 0.15 M NaCl, 0.1% SDS, 1.0% NP-40, 0.5% Na-Deoxycholate, 2mM EDTA) supplemented with protease and phosphatase inhibitors cocktail (cOmplete™ protease inhibitor and PhosSTOP™ phosphatase inhibitor; Roche, Basel, Switzerland) using a mechanic homogenizer (Precellys24, Peqlab, Erlangen, Germany). Lysates were cleared by centrifugation at 10 000x*g* for 10 min at 4°C. Samples were resolved by SDS-PAGE and analysed as described in “section 3.2.11. Western blotting”. Other brain samples were perfused with 1x PBS (PAN Biotech, Aidenbach, Germany) and followed by 4% PFA fixation for further routine histological procedures.
The concentration of aSyn in biofluid samples was measured using enzyme-linked immunosorbent assay (ELISA), as described below. Due to technical reasons, not all the samples were measured in all the mice.

3.2.6. ELISA

aSyn concentrations in the biofluids (saliva, CSF, whole blood, serum, and plasma) were determined using an ECL-based detection system essentially as previously described with slight modifications (Kruse et al., 2012). We used the antibody hSA4 (polyclonal goat-anti human aSyn antibody provided by M. Schlossmacher) at 2 µg/ml to capture aSyn from the biofluids. The CSF samples were diluted 1:20, 1:4 or 1:20 in the case of saliva samples, 1:8 in the serum and plasma samples while the whole blood samples were 1:10 000 in 1% BSA/PBS + 0.05% Tween-20 (PBS-T). Detection was performed using 0.5µg/ml SulfoTAG-labeled Syn1 antibody (610787, BD Transduction Laboratories) dissolved in 1% BSA/PBS-T supplemented with 0.1% mouse IgG (Rock-land Immunochemicals, PA, USA) and 0.1% goat IgG (Equitech-Bio, TX, USA) to quench background signals. Experiments were performed in 96-well standard assay plates and the aSyn concentration was measured in MESO QuickPlex SQ 120 from Meso Scale Discovery.

3.2.7. Protein quantification

Protein concentration was determined by the bicinchoninic acid (BCA) (Thermo Fisher Scientific, MA, USA) or Bradford protein assay (Bio-Rad, CA, USA). In both methods, BSA was used as a protein to draw a standard curve. Dilutions of BSA standards (according to the manufacturer's manual) and samples (5x, 10x, 15x, and 30x) were prepared in triplicates with either 20mM Tris pH 8.0 or H2O as diluent. BCA or Bradford reagent was added to each well of the 96-well plate, incubated at 37°C for 30 min, and the absorbance read at 570 nm in Infinite M200 fluorescence plate reader (TECAN) or incubated at RT for 5 min, and the absorbance read at 595nm, respectively. The standard curve was then used to calculate the protein concentration in µg/mL of the different samples.

3.2.8. Isolation of synaptic vesicles

Synaptic vesicle (SV) isolation from mice brain homogenates was performed as previously described (Hell et al., 1988; Von Mollard et al., 1991; Nagy et al., 1976) (Figure 9). Briefly, 10 twelve-week-old mice were sacrificed and the cortices were dissected. Samples were divided in two and each half was homogenized in 50mL homogenization

buffer (320mM sucrose, 5mM HEPES, pH 7.4) supplemented with PMSF (0.2mM) and pepstatin (1µg/µL) in a 60 mL glass-Teflon homogenizer with 9 strokes at 900 r.p.m to reduce fragmentation and vesiculation of large membranes. The homogenizer was washed with 10mL of homogenization buffer. Homogenate (BH) was cleared from cell debris and nuclei by centrifugation at 2 700 r.p.m for 10 min at 4°C in a Beckmann SS34 rotor. The supernatant (S1) was recentrifuged at 1 000 r.p.m for 15 min at 4°C in the same rotor to obtain a cytosolic fraction (S2) and a crude synaptosomal fraction (P2). The resulting supernatant, S2 fraction, was discarded since it is contaminated with cell fragments such as microsomes, small myelin fragments, and also soluble proteins. Synaptosomes, present in the P2 fraction, were carefully resuspended with homogenization buffer in a total volume of 36 mL using pre-cut 1mL tips and avoiding the dark patch of the pellet, which represents mitochondrial contamination. Crude P2 was diluted for washing with 35mL of fresh homogenization buffer supplemented with protease inhibitors and centrifugated at 11 000 rpm for 15min at 4°C in the same rotor as previously. Pellets were resuspended in a final volume of 20 mL sucrose buffer, 180 mL of ice-cold water was added and synaptosomes were osmotically lyzed with 3 strokes of max speed in a glass-Teflon homogenize to release synaptic vesicles, followed by immediate addition of protease inhibitors (120µL of PMSF (1:1 000 stock solution) and 120µL pepstatin (1:1,000 stock solution)) and 600µL of 1 M HEPES pH 7.4 (1:200 stock solution). The pellet after lysis was centrifugated at 16 500 r.p.m for 20 min at 4°C (SS34 rotor) to remove all large membranous particles (mostly mitochondria, junctional complexes, and myelin). The pellet obtained (LP1 fraction), contains mainly active zone (AZ)-enriched presynaptic membranes with docked vesicles attached to a portion of PSD. The supernatant (LS1) was collected and centrifuged at 50 000 r.p.m for 2 hours at 4°C in a 50.2Ti rotor. The pellet (LP2) was resuspended in a total volume of 2 mL 40mM sucrose and transferred into a tight-fitting glass-Teflon homogenizer. Since SVs tend to aggregate during the isolation procedure; the LP2 fraction was homogenized using 3 strokes at 900 r.p.m. Then to ensure proper disruption of any remaining SV clusters, the supernatant was drawn through a 20-gauge hypodermic needle attached to a 5-mL syringe and then changed to a 27-gauge needle and expelled. The protein concentration was determined using BCA assay (Thermo Fisher Scientific, MA, USA).

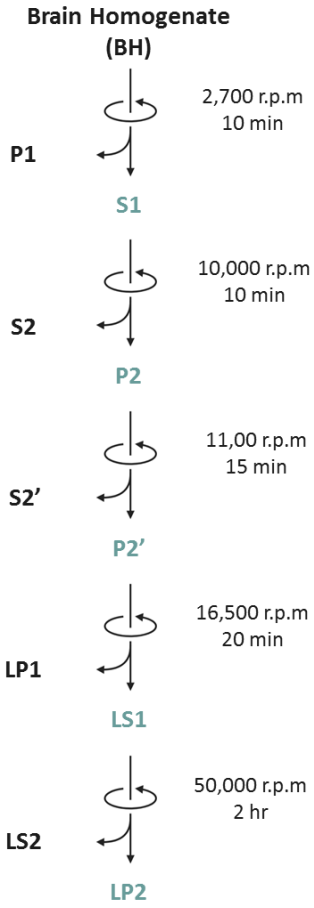


Figure 9 | Flowchart of the workflow procedure for synaptic vesicles (SVs) isolation from mouse brain. The procedure of SVs isolation consists of steps of differential centrifugations of homogenate of mice brain (BH), followed by hypo-osmotic lysis of synaptosomes resulting in the release of soluble protein contents and SVs (LP2) as well as AZ-enriched presynaptic membranes (LP1).

3.2.9. Proteomic analyses of synaptic vesicles

Samples were reconstituted in LDS Sample Buffer (Invitrogen) and run 4-12 % SDS-PAGE (Invitrogen) gels. The gels were stained with Coomassie Blue, each sample was cut out in one piece. After washing, gel slices were reduced with 10 mM DTT for 30 min at 37 °C and alkylated with 40 mM 2-iodoacetamide for 30 min at 25 °C. Protein digestion was performed with trypsin. The mixture with peptides was then extracted, dried, and resuspended in 2% acetonitrile and 0.1% formic acid and prepared for nanoscale liquid chromatography coupled to tandem mass spectrometry (nano LC-MS/MS) as described previously (Atanassov and Urlaub, 2013).

Mass nano LC-MS/MS analysis samples were purified on an in-house reversed phase-C18 precolumn (Reprosil-Pur120 C18-AQ 5 µm, 150µm inner diameter and 20 cm in length, Dr Maisch GmbH, Ammerbuch-Entringen, Germany) and separated on an

analytical reversed phase-C18 column (ReproSil-Pur 120 C18-AQ, 75µm inner diameter and 20 cm in length, Dr Maisch GmbH, Ammerbuch-Entringen, Germany) separated on a 37-min linear gradient of 5-35% acetonitrile and 0.1% formic acid at a flow rate of 300nl/min). In order to analyze the eluent, a Q Exactive hybrid quadrupole/orbitrap mass spectrometer (ThermoFisher Scientific, Dreieich, Germany) equipped with a FlexIon nanoSpray source was used (Q Exactive HF-X). The data was operated under Excalibur 2.5 software with a data-independent acquisition (DIA) method using 11 different window sizes (Muntel et al., 2019) with a range of 350 to 1 250 m/z (Muntel et al., 2019). Fragments were produced using staggered NCE settings (26/28/30%) for charge state 3+ with 60.000 FWHM of resolution setting, an AGC target of 1 000 000 and a maximum fill time of 54ms. Three technical replicates were acquired for each sample.

Protein identification, construction of a spectral library and DIA peak integration were achieved Spectronaut Software v16.0.220606.53000 (Biognosys, Schlieren, Switzerland) (Bruderer et al., 2015). Peptides and proteins were identified using the in-built Pulsar algorithm at default settings. The combined qualitative analyses were searched against the UniProtKB mouse reference proteome (reviewed on 01-2021) augmented with a set of 52 known common laboratory contaminants to identify peptide-to-sequence matches, peptides and proteins at false discovery rates (FDR) of 1%, respectively. Peptide identifications were transcribed into an annotated hybrid MS/MS spectral library using all DDA and DIA runs. DIA peak integration was performed using in Spectronaut using default parameters. Following dynamic retention time correction using endogenous peptides, peak areas were extracted using information from the spectral library at a permutation-based FDR of 0.1 for multiple testing correction. The resulting peak areas were then summed to peptide and finally protein area values per replicate, which were statistically evaluated. Quantitative values were averaged across three technical replicates.

3.2.10. Western blotting

Cultured cells were collected in lysis RIPA buffer (50 mM Tris, pH 8.0, 0.15 M NaCl, 0.1% SDS, 1.0% NP-40, 0.5% Na-Deoxycholate, 2mM EDTA, supplemented with protease and phosphatase inhibitors cocktail (completeTM protease inhibitor and PhosSTOPTM phosphatase inhibitor; Roche, Basel, Switzerland), sonicated, and centrifuged (20 min, 4 °C, 16000xg). Total protein concentration was determined by the Bradford protein assay (Bio-Rad, CA, USA). The lysates (30µg of protein) were denatured for 5min at

95°C in protein sample buffer (PSB, 50mM Tris–HCl pH 6.8; 2% SDS; 10% glycerol; 1% β-mercaptoethanol; 0.02% bromophenol blue), loaded into 11% SDS-PAGE gels and transferred during 7min to nitrocellulose membranes using iBlot2 (Invitrogen, CA, USA). Membranes were blocked for 1 hour in 5% BSA (Sigma-Aldrich, MO, USA) in Tris-buffered saline (pH 8) with 0.05% Tween 20 (TBS-T) and then incubated overnight at 4°C with primary antibodies in 5% BSA in TBS-T [alpha-synuclein (1:1,000, 610787, BD Transduction Laboratories), phosphorylated alpha-synuclein (1:1000, 015-25191, Wako), GFP (1:1000, sc-9996, Santa Cruz), homer I (1:1000, 13185, Cell Signaling), PSD95 (1:1000, 124011, Synaptic Systems), SNAP25 (1:1000, 111002, Synaptic Systems), synapsin 1 (1:1000, 106011, Synaptic Systems), synaptobrevin2 (1:1000, 104202, Synaptic Systems), synaptotagmin (1:1000, 105008, Synaptic Systems), tyrosine hydroxylase (1:1000, AB152, Millipore)]. Afterwards, the cells were washed three times with TBS-T, and then incubated with horseradish peroxidase (HRP) conjugated secondary antibodies [ECL™ Mouse IgG (1:10000, NXA931, Amersham), ECL™ Rabbit IgG (1:10000, NA934V, Amersham)] or IRDye 800CW or 680LT (Li-Cor, Bad Hamburg, Germany) secondary antibodies for 2h at RT. After incubation with the secondary antibody, membranes were washed three times with TBS-T, and immunoreactivity was visualized by chemiluminescence system (Fusion FX Vilber Lourmat, Vilber, France) using chemiluminescent HRP substrate (Millipore, MA, USA) or by fluorescence system (Li-Cor Odyssey® CLx imaging system). The intensity of each band was normalized to beta-actin (1:5000, A5441, Sigma-Aldrich), a protein loading control, and quantified using Fiji software (National Institutes of Health).

3.2.11. Toxilight Assay

Using a ToxiLight™ bioassay kit non-destructive bioluminescent cytotoxicity assay (Lonza, Rockland), the adenylate kinase (AK) released from damaged cells into culture supernatants was measured following the manufacturer's instructions. The reaction involves two steps. The first involves the addition of ADP as a substrate for AK, which will be converted to ATP. Then, the enzyme luciferase catalyzes the formation of light from ATP and luciferin. This combination of reactions leads to a linear relationship between the emitted light intensity and AK concentration. As a result of cell integrity being compromised, AK, as well as other factors, leak into the surrounding medium from *in vitro*-cultured cells. Therefore, measuring the amount of AK released from cells provides accurate and sensitive measurements of cytotoxicity and cytolysis. Briefly, lyophilized adenylate kinase (AK) detection reagent (AKDR) was reconstituted in 20ml assay buffer

and left at RT for 15 minutes to ensure complete rehydration. In a luminescence-compatible 96-well plate, 20 µl of supernatant was transferred, and 100 µl of AKDR was added to each well, and after 5 minutes, the plate was measured using an Infinite M200 fluorescence plate reader (TECAN).

3.2.12. Microelectrode array

Microelectrode array (MEA) experiments were performed following standard protocols, as previously described (Brás et al., 2021; Khani and Gollisch, 2021; Wagenaar et al., 2004). Primary hippocampal neuronal cultures were directly plated on 60MEA200/30iR-Ti-gr planar arrays (60 electrodes, 200µm electrode spacing and 30µm electrode diameter), 60-3DMEA100/12/40iR-Ti-gr planar arrays (60 electrodes, 100µm electrode spacing and 12µm electrode diameter), and 60HexaMea40/10iR-ITO-gr planar arrays (60 electrodes, 40µm electrode spacing and 10µm electrode diameter) (MultiChannel Systems, Reutlingen, Germany). MEAs were coated with polyornithine solution (PLO) (500µg/mL in sterile double distilled H2O (ddH2O); Sigma-Aldrich, MO, USA) overnight at 4°C and the next day rinsed three times with distilled water before coating with laminin (5µg/mL in ddH2O; Sigma-Aldrich, MO, USA) for at least 1h at RT. The laminin solution was removed, and the neural cells were plated on top of the electrodes. Neurons were treated with 100nM of recombinantly-produced aSyn species (aSyn Mono, aSyn Oligo, and aSyn PFF) at DIV4 and recorded at DIV14, DIV21, DIV26, DIV28 and DIV30. The extracellular electrical neuronal activity was recorded using the MultiChannel MEA2100 system (MultiChannel Systems, Reutlingen, Germany) with temperature maintained at approximately 37°C during recordings. Recordings started 10 min after placing the MEAS in the headstage, and the spontaneous activity was recorded for 30 min at each time point. The electrode signals were amplified, band-pass filtered (200 Hz to 3 kHz) and recorded digitally at 25 kHz, using the MultiChannel Experimenter software (version 2.18.0, MultiChannel Systems, Reutlingen, Germany).

Spike sorting was carried out using a modified version of the Kilosort automatic sorting software, as previously described (Brás et al., 2021; Pachitariu et al., 2016a, 2016b), followed by visualization and manual curation of the electrophysiological data sorting with the “Phy 2” software (https://github.com/cortex-lab/phy). Only clusters of spikes with a specific shape (waveform) and a clear refractory period were included in the final analysis as individual neuronal cells. The spike clusters were pre-processed and analysed using custom-made MATLAB scripts (R2019b Update 2 (9.7.0.1247435); MathWorks Inc., MA, USA). The bursts/minute, inter-burst-intervals, intra-burst spiking

frequency, spikes in burst, burst duration, the total number of spikes, firing rate, average inter-spike-intervals, spike amplitude and prestimulus time histogram (PSTH) were measured from the spontaneous activity of each recorded neuron. To detect bursts, the population firing rate was computed as a histogram (100ms bin size) of array-wide spiking activity. The peaks of the firing rate histogram were used to detect synchronous, array-wide network bursts with at least 500ms time window between two consecutive single-channel burst. Peaks smaller than 20% of the largest peak were excluded since they do not represent array-wide synchronous activity. A burst window of 650ms around each peak, with an onset of 150ms and offset of 500ms, was defined. The spikes in bursts in each recorded cell were measured during the defined burst windows, and cells with less than six spikes were excluded. The following parameters were calculated from the detected bursts: (i) burst rate as the number of bursts per time within the recording time frame; (ii) burst duration as the time between the first and last spike of a neuron during the burst window; (iii) inter-burst-interval as the time between the offset of a burst and the onset of the following burst, calculated for all successive bursts in a recording; (iv) intra-burst-frequency as the spike's rate occurring within a burst, averaged over all the detected bursts for each neuron; and (v) percentage of spikes in bursts as the ratio of burst spikes relative to the total number of spikes for each neuron.

3.2.13. Confocal microscopy

Images from neuronal hippocampal cultures were acquired using the Zeiss LSM 800 – Airyscan, Carl Zeiss Microscopy GmbH, with 20x, 63x and/or 100x magnification objectives. Samples were excited using 405, 488, and 561 laser lines, pinhole = 1, 0.250 µm thickness Z stacks, step size of 1 µm (7-10 slices per neuron) and 2 averaging line-by-line. The acquisition settings were optimized to avoid underexposure and oversaturation effects and kept equal throughout the image acquisition of the samples. For quantification of axonal length 10-12 images were randomly taken out of three independent experiments, and analysed by ImageJ software.

3.2.14. Electron microscopy

Electron microscopy images from purified SVs were performed following a protocol previously described (Tracz et al., 1997). Samples were bound to a glow discharged carbon foil-coated copper grids (400 mesh). Then, samples were stained with NanoVan

(methylamine vanadate, Nanoprobes. Inc), and evaluated at RT using a Talos L120C transmission electron microscope (Thermo Fisher Scientific, Eindhoven, The Netherlands).

3.2.15. Statistical data analysis

The statistical analysis was carried out in GraphPad Prism version 6.01 (GraphPad Software Inc., CA, USA). All data are expressed as mean ± standard deviation (SD) for n experiments. Each n represents one independent experiment, and data are presented with at least three independent experiments. For multiple comparisons, a one-way analysis of variance (ANOVA) followed by Tukey's post-hoc Tests, or a two-way ANOVA with Dunnet´s post-hoc test was used. Differences were considered significant with $p < 0.05$ (* p-value ≤ 0.05, ** p-value ≤ 0.01, ***p-value ≤ 0.001, ****p-value ≤ 0.0001). For mass spectrometry, the spectral count differences were considered significant for values of FDR < 0.1. Statistical tests, the number of independent experiments, and significant differences were indicated in each figure's caption.

CHAPTER 4

RESULTS

4.1. Effects of aSyn overexpression and of treatment with extracellular aSyn *in vitro*

In order to assess whether aSyn has an effect on synapses we tested the effect of aSyn overexpression in cultured hippocampal rat neurons.

The cultures were analysed at different stages of neuronal development: at day *in vitro* (DIV) 4, since this is the timepoint when axon and dendrites are generated from minor neurites and it is the beginning of synaptogenesis, at DIV 7, a dynamic phase, where there is axon-dendrite commitment and branches instability with fast expansion and regression; DIV 14, a maturation phase, where the axon and dendrites develop further and dendritic protrusions and spines appear, and organized patterns of spontaneous action potential firing are detectable; DIV 21, matured neurons where the formation of LB-like inclusions was reported, and DIV 26, DIV 28 and DIV 30 to extend the characterization of the neuronal seeding model to mimic biochemical, morphological, and structural features of late stages of PD (Baj et al., 2014; Dotti et al., 1988; Mahul-Mellier et al., 2020) (Figure 10). Our cultures had a high percentage of neurons (92%), which was confirmed by immunostaining against MAP2. Only a low number of astrocytes (7%) and no presence of microglia or oligodendrocytes was detected.

Neurons were infected with viral particles to induce the expression of full-length human aSyn (wild-type aSyn [WTaSyn]), human mutant A30P or A53T aSyn (A30PaSyn and A53TaSyn, respectively), or were treated with the same volume of medium as a negative control. Since, all viruses expressed green fluorescent protein (GFP) as a reporter for gene expression, a virus expressing only GFP was used as infection control. In parallel, other neurons were treated with recombinantly-produced aSyn species (aSyn Mono, aSyn Oligo, and aSyn PFF) or PBS, as a negative control. After the increase in the aSyn levels, the effects of aSyn on biochemical, morphological and functional levels were analysed.

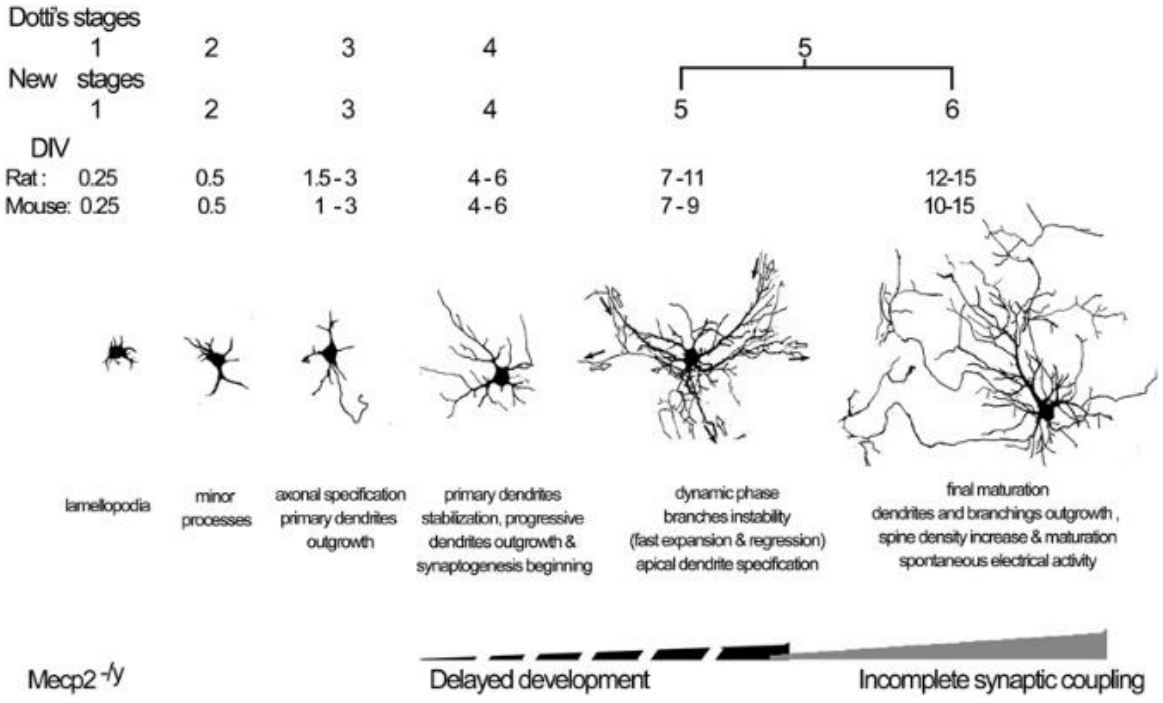


Figure 10 | The generic stages of development of rat and mouse hippocampal neurons in culture. The approximate times at which neurons enter each of the stages are expressed in Days *In Vitro* (DIV) and indicated in the figure (Baj et al., 2014).

4.1.1. aSyn transduction results in increased total aSyn expression

aSyn expression was induced on DIV 3 and neurons were allowed to grow until DIV 4, DIV 7 (immature neurons) or DIV14, DIV21, DIV 26 or DIV28 (mature neurons). In order to verify the transduction efficiency, the percentage of infected neurons in the cultures and the expressed levels of GFP per condition were calculated.

Fluorescent images of the infected cultures were acquired and the GFP fluorescence intensity was determined (Figure 11A and B). The GFP mean fluorescence intensity of neurons was consistent among all the conditions (control 94539.0 ± 9423.0, GFP 116840.0 ± 11369.0, WTaSyn 92513.0 ± 3864.0, A30PaSyn 82825.0 ± 3509.0 and A53TaSyn 126165.0 ± 16383.0) indicating that similar numbers of cells are infected in each condition.

Next, to verify if all viruses were expressed in similar levels, the GFP expressed levels were quantified by immunoblotting. GFP showed no significant changes among the four conditions (GFP (734.6 ± 287.7), aSynWT (380.9 ± 257.3), aSynA30P (638.8 ± 845.6), and aSynA53T (993.3 ± 431.3)) (Figure 11C). Then, to confirm the viral induction of

aSyn, the levels of aSyn were also quantified. An increase in the aSyn levels was observed in cells that were inducted with virus encoding for aSyn (control 100.0 ± 0.0, GFP 106.3 ± 37.5, WTaSyn 187.6 ± 101.9, A30PaSyn 193.1 ± 95.1 and A53TaSyn 184.9 ± 59.7) (Figure 11C). An increase in the phosphorylated aSyn levels were also observed in the neurons infected with WTaSyn and A53TaSyn (Figure 11D). In conclusion, these data verify that WTaSyn, A30PaSyn and A53TaSyn are expressed in hippocampal neuronal cultures in similar levels as the GFP control and that transduction with these viruses lead to an increase in the levels of total aSyn and phosphorylated aSyn.

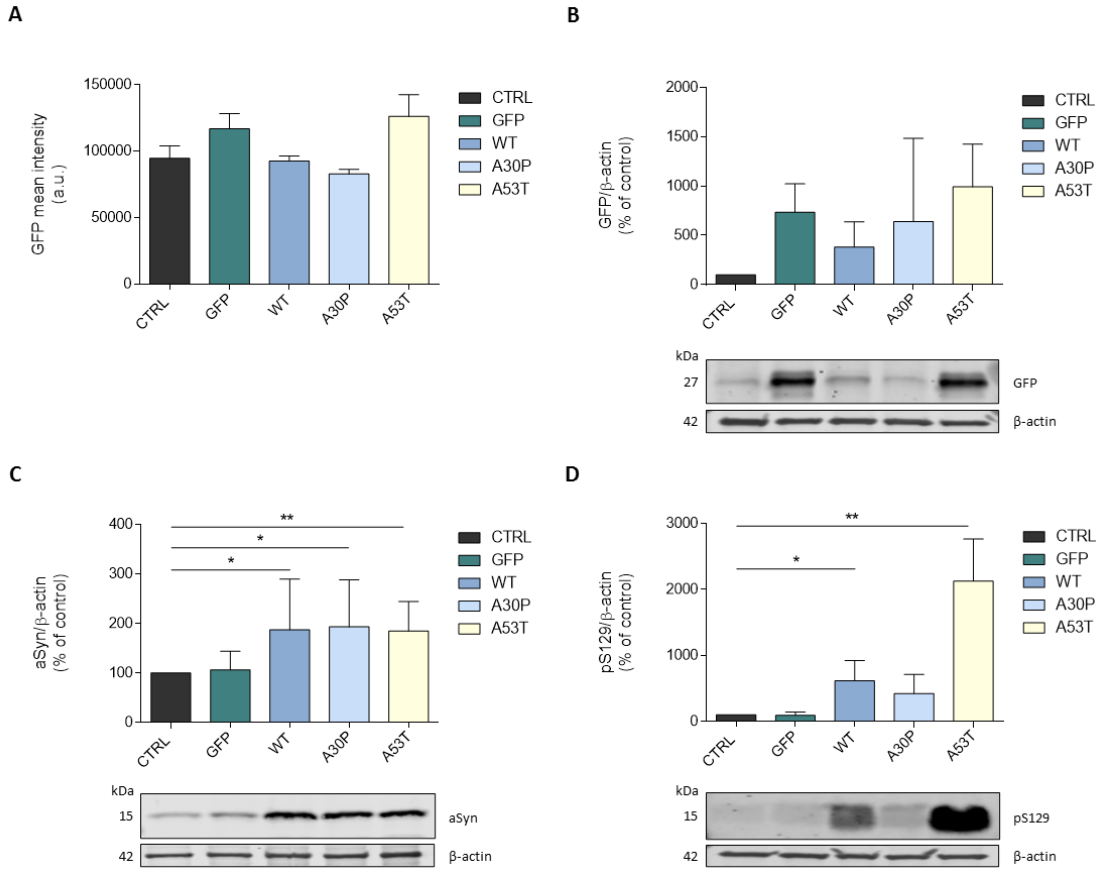


Figure 11 | Viral induction of aSyn in hippocampal primary cultures. A) Quantification of GFP fluorescence mean intensity in infected neurons (n=10-12). Quantification of GFP (B), aSyn (C) and phosphorylated aSyn (pS129; D) levels by immunoblotting analysis and a representative immunoblot (n=3, n=7, n=3, respectively). All data are expressed as mean ± SD; Student's t-test; * p-value ≤ 0.05, ** p-value ≤ 0.01.

4.1.2. aSyn treatment results in increased total aSyn levels

Cultured neurons were exposed to recombinant aSyn Mono, aSyn Oligo, and aSyn PFF species or PBS as a negative control and incubated for 1, 3, 10, 17, 22, 24 or 26 days according to a recently published protocol (Mahul-Mellier et al. 2020).

To confirm the internalization of different aSyn species, western blots of neurons treated with aSyn were performed and quantified. An increase in the levels of aSyn and pS129aSyn was observed in cells that were treated with aSyn Mono (aSyn: 134.9 ± 44.6; pS129aSyn: 184.5 ± 45.0), aSyn Oligo (aSyn: 237.5 ± 125.7; pS129aSyn: 125.6 ± 21.1), and aSyn PFF (aSyn: 290.5 ± 174.7; pS129aSyn: 1105.0 ± 175.2) (Figure 12). In conclusion, these data verify that aSyn species exposure in hippocampal neuronal cultures lead to their internalization and consequently to an increase in the levels of aSyn and pS129aSyn.

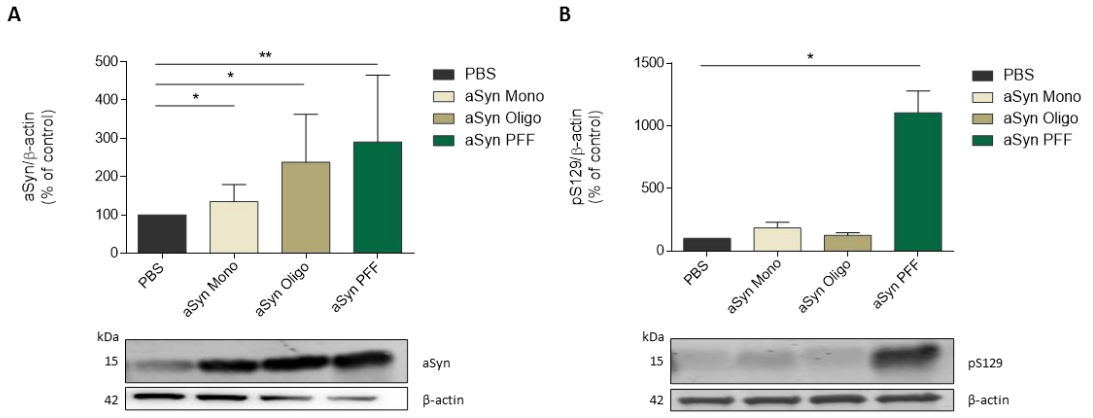


Figure 12 | Recombinant aSyn species exposure in hippocampal primary cultures. Quantification of aSyn (C) and phosphorylated aSyn (pS129; D) levels by immunoblotting analysis and a representative immunoblot (n=10-12, n=3, respectively). All data are expressed as mean ± SD; Student's t-test; * p-value ≤ 0.05, ** p-value ≤ 0.01.

4.1.3. Increased levels of aSyn do not induce neurotoxicity

Next, we sought to evaluate the effect of aSyn increased levels on cytotoxicity, as neurodegeneration is considered to be associated with aSyn aggregation. To assess the cytotoxicity, we used Toxilight bioassay, a bioluminescent cytotoxicity assay that measures the enzyme adenylate kinase (AK) released from cells. The leakage of AK from cells cultured *in vitro* into the surrounding medium, is a consequence of loss of cell

integrity, through damage to the plasma membrane. Therefore, measuring the amount of AK released from cells, which is linearly related to the emitted light intensity, provides accurate and sensitive measurements of cytotoxicity and cytolysis.

First, we determined the cytotoxicity of lentiviral vectors encoding WTaSyn, A30PaSyn and A53TaSyn or GFP as a control. We did not observe any evidence for acute neurotoxicity in infected neurons when compared to non-infected neurons or GFP-infected neurons (GFP (113.1 ± 10.7), WTaSyn (128.0 ± 30.0), A30PaSyn (130.3 ± 24.9), and A53TaSyn (131.5 ± 29.5)) (Figure 13A). We also assessed the cytotoxicity of exogenous recombinant aSyn species added to hippocampal neurons. We observed that the addition of recombinant monomeric, oligomeric and PFFs aSyn to primary neurons does not induce cytotoxicity when compared to PBS-treated neurons in a time-dependent manner (monomers (117.2 ± 17.0), oligomers (117.3 ± 21.5), and PFFs (112.6 ± 14.6)) (Figure 13B). These results show that increasing the levels of aSyn intracellularly using lentivirus encoding for aSyn or extracellularly using recombinant aSyn species does not show toxicity and does not induce apoptotic cell death for hippocampal neurons in the conditions analysed.

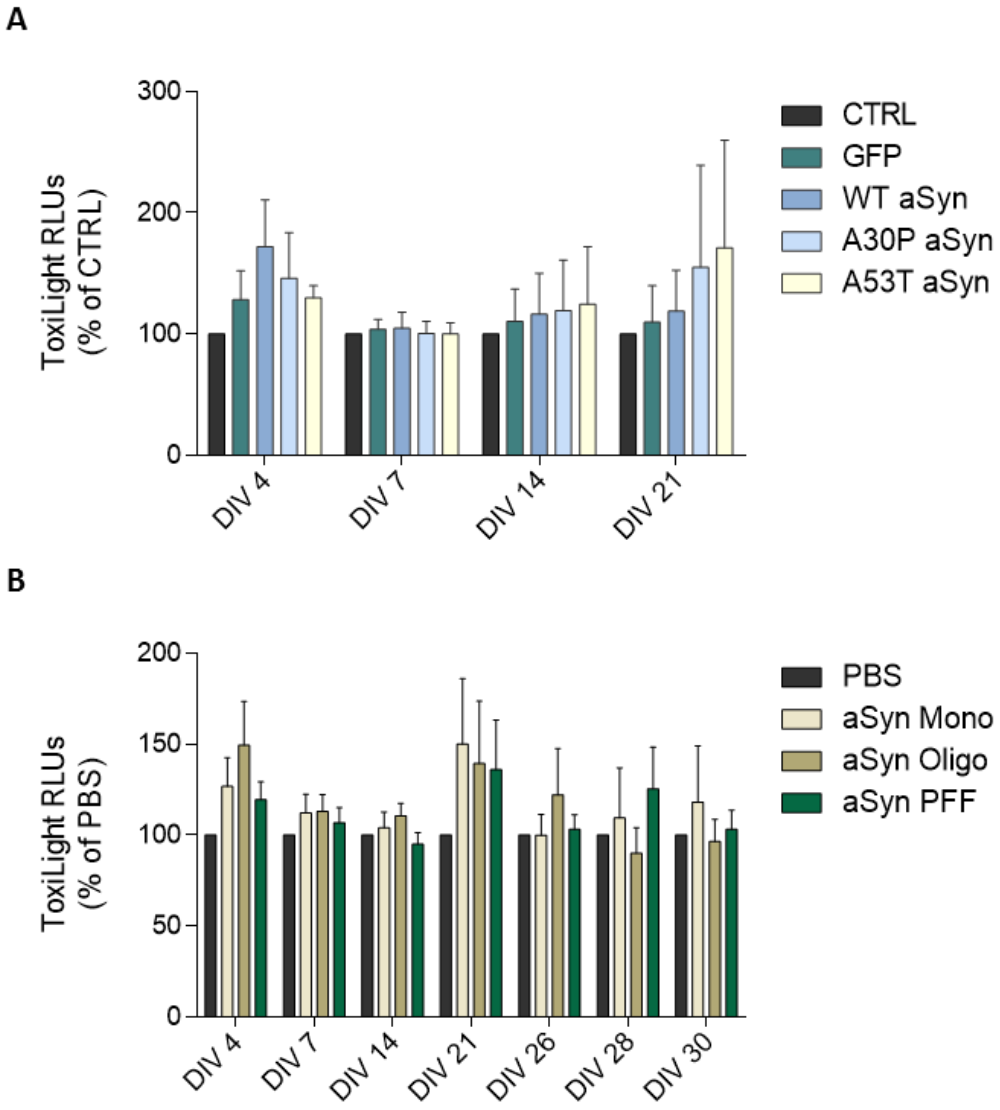


Figure 13 | Transduction of aSyn or addition of aSyn species to primary neurons does not induce cell death. A) Cell death was assessed in primary neurons transduced with lentiviral encoding for human WTaSyn or human mutant aSyn (A30PaSyn and A53TaSyn) for up to DIV21 using Toxilight bioassay (n=4). **B)** Cell death was assessed in primary neurons treated with 100 nM of aSyn species (monomers (aSyn Mono), oligomers (aSyn Oligo), and pre-formed fibrils (aSyn PFFs)) for up to DIV30 using Toxilight bioassay (n=7). All data are expressed as mean ± SD; Student's t-test.

4.1.4. Increased levels of aSyn do not alter the levels of synaptic proteins

In PD, it has been suggested that aSyn may acquire abnormal properties and form toxic species that accumulate at presynaptic terminals, disrupt neurotransmitter release and impair synaptic transmission, and, ultimately, lead to synaptic dysfunction and loss of neuronal connections and subsequent neuronal death (Bae and Kim, 2017; Calo et al., 2016; Fang et al., 2017; Grosch et al., 2016; Kouroupi et al., 2017; Lu et al., 2014; Morales et al., 2015; Roy, 2017; Schulz-Schaeffer, 2015; Scott et al., 2010; Tagliaferro

and Burke, 2016). To investigate whether the accumulation of toxic aSyn species could lead to synaptic alterations or the trapping of synaptic proteins by aSyn we assessed the protein levels of a pre- and postsynaptic markers (Synaptosomal associated protein 25 [SNAP25], postsynaptic density 95 [PSD95]) by Western blot analysis. However, we did not observe any significant difference of these synaptic markers in aSyn-transduced neurons (Figure 14A and B) and aSyn-treated neurons (Figure 14C and D).

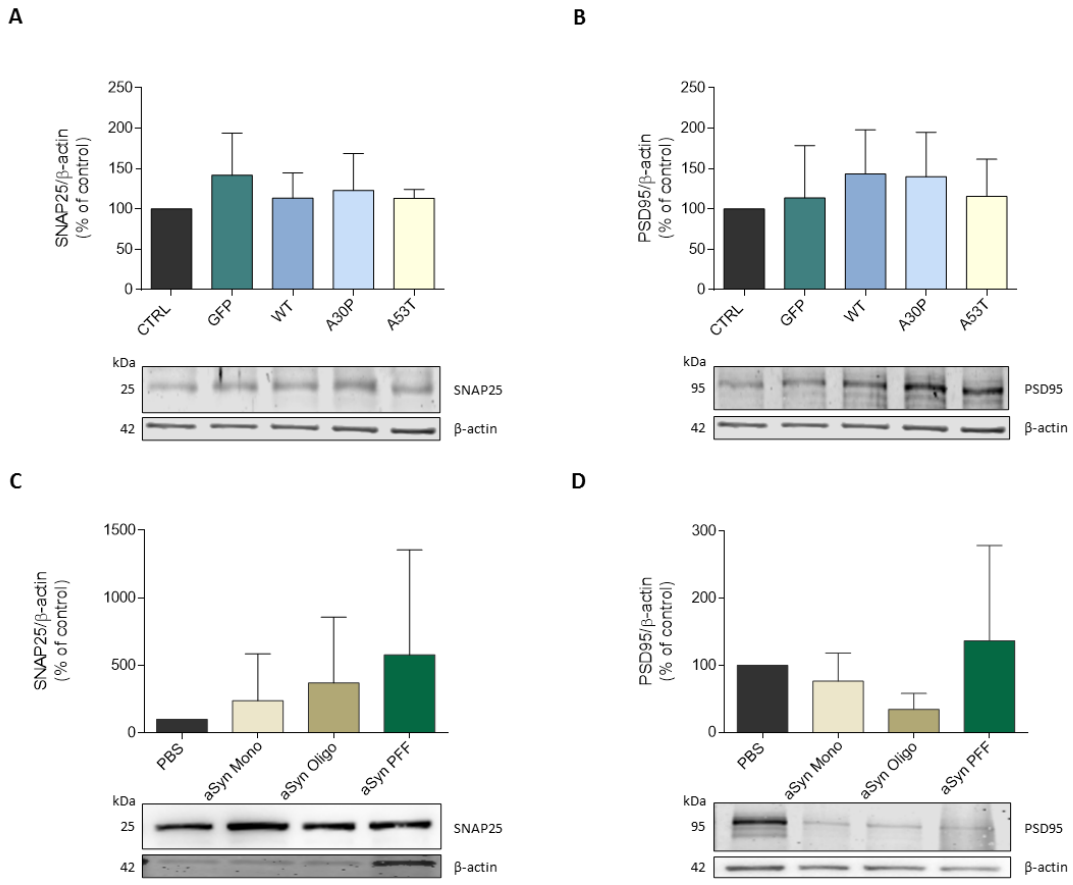


Figure 14 | Transduction of aSyn or addition of aSyn species to primary neurons does not change the levels of pre- and postsynaptic markers. Quantification and a representative immunoblot of SNAP25 **(A)** and PSD95 **(B)** levels were assessed in primary neurons transduced with lentivirus encoding for human WTaSyn or human mutant aSyn (A30PaSyn and A53TaSyn) for up to DIV21 (n=4-5). Quantification of SNAP25 **(A)** and PSD95 **(B)** levels in primary neurons treated with 100 nM of aSyn species (monomers (aSyn Mono), oligomers (aSyn Oligo), and pre-formed fibrils (aSyn PFFs)) for up to DIV21 (n=4-7). All data are expressed as mean ± SD; Student's t-test.

4.2. Alterations in dendrite length

4.2.1. aSyn-transduced neurons do not display alterations in dendrite length

Dendrite morphogenesis is a complex and well-orchestrated process, that involves the formation of characteristic dendrite arbors and dendritic spines, which facilitate communication among neurons. Dendritic arbors are highly dynamic structures that

continuously branch and retract in response to stimulus, allowing neurons to respond to changes in their environment (Cline, 2001; Dailey and Smith, 1996; Niell et al., 2004). Several neurological and neurodevelopmental disorders are associated with altered dendrite morphology or defects in neuronal development, including changes in patterns of dendrite branching, fragmentation of dendrites, retraction or loss of dendrite branching, abnormal spine density and morphology, and loss of synapses (Calabresi et al., 2006; Kaufmann and Moser, 2000; Selkoe, 2002; Stephan et al., 2009).

To assess if there were dendrite branching effects related to aSyn induction in *in vitro* neurons, the dendritic length was measured. For this, neurons were stained with MAP2 marker and observed under fluorescent microscopy. Then, they were traced and the successive branching levels were measured. Our data shows no significant changes in the apical (CTRL (108.5 ± 73.8), GFP (164.3 ± 51.9), WTaSyn (128.0 ± 79.2), A30PaSyn (169.1 ± 118.2, and A53TaSyn (100.7 ± 63.8)) , basal (CTRL (66.6 ± 40.0), GFP (90.4 ± 52.8), WTaSyn (68.1 ± 47.3), A30PaSyn (96.4 ± 58.0, and A53TaSyn (53.98 ± 37.7)) and total dendritic length or arborization (CTRL (78.4 ± 55.1), GFP (105.7 ± 60.5), WTaSyn (84.9 ± 63.8), A30PaSyn (118.9 ± 87.9, and A53TaSyn (73.6 ± 55.3)) of aSyn-transduced neurons compared to non-transduced neurons (Figure 15A, B and C).

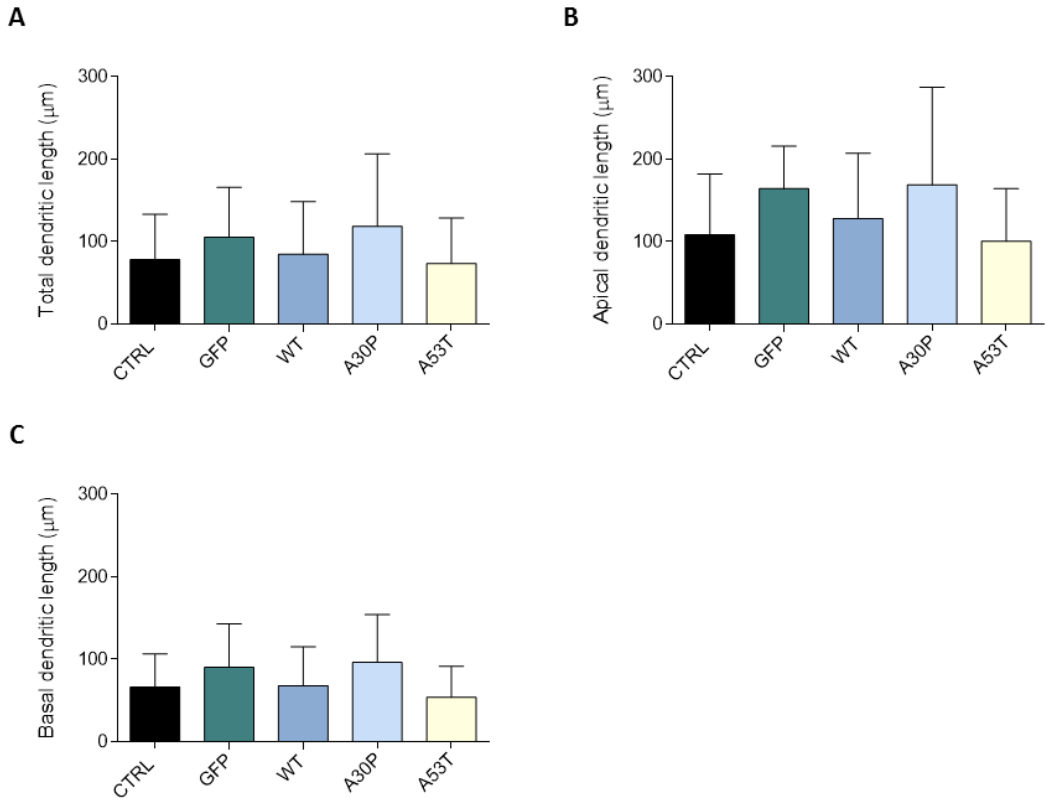


Figure 15 | Transduction of aSyn does not influence the dendritic length of primary neurons. aSyn transduction did not alter the total dendritic length (µm) (A), the apical dendritic length (µm) (B), or the basal dendritic length (µm) (C) at DIV 21 (n=5 neurons from 3 independent experiments per condition). All data are expressed as mean ± SD; One-way ANOVA, post-hoc Tukey's test.

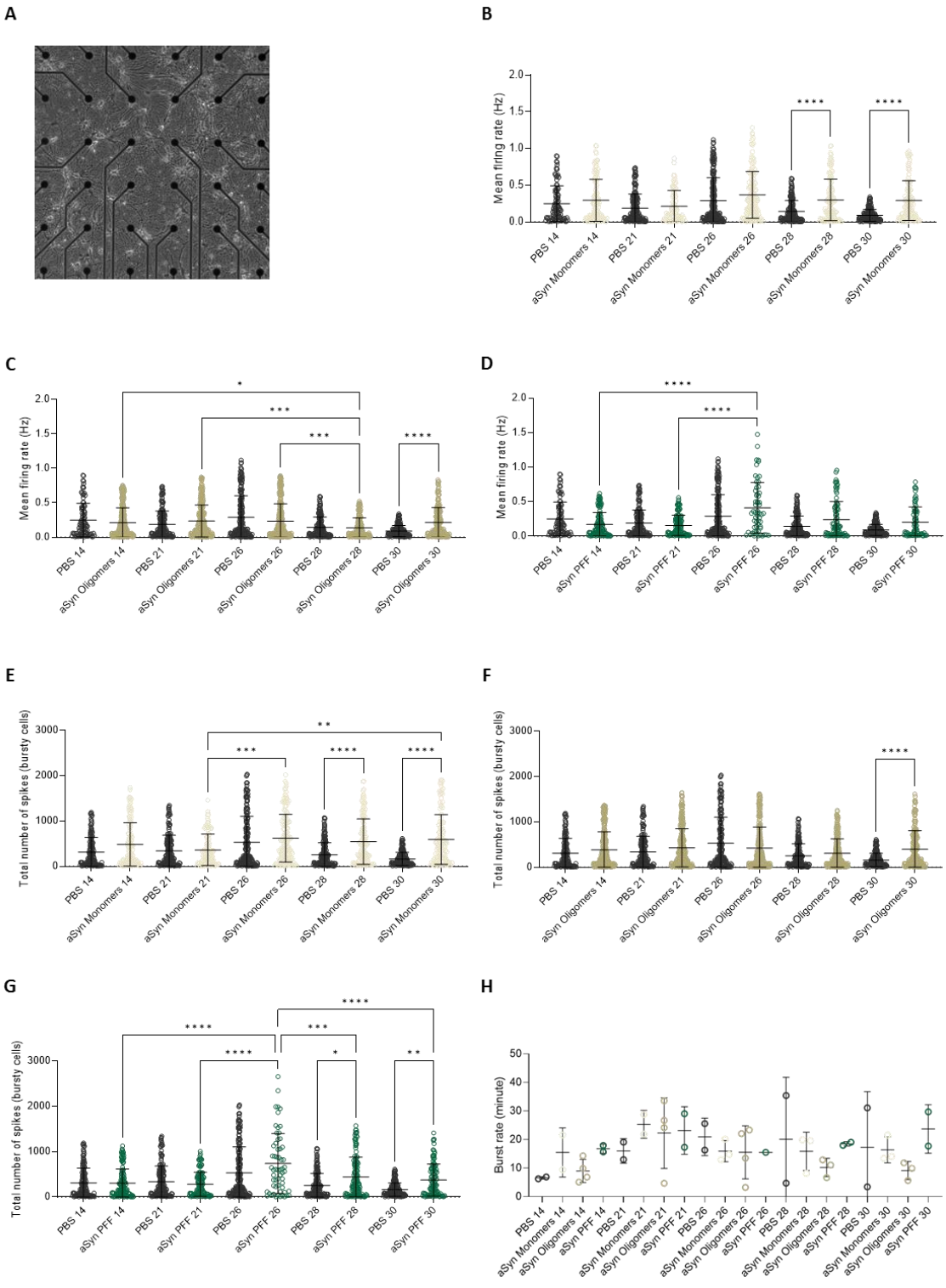
4.3. Alterations in neuronal function

4.3.1. Treatment with aSyn does not modify spontaneous neuronal activity in hippocampal neurons

We observed aSyn treated neuronal cells presented similar levels of aSyn, but this increase did not cause cytotoxicity and did not alter the cellular morphology or induce significant differences in synaptic protein levels. Afterwards, we assessed the functional relevance of increased aSyn levels. Since aSyn is a presynaptic protein and synaptic dysfunction might be considered a prodromal stage of PD, we hypothesized that neuronal function might be affected by increased aSyn levels. Therefore, we used microelectrode arrays (MEAs) to evaluate the effect of aSyn increased levels on spontaneous activity in primary hippocampal neuronal cultures. Cells were cultured in MEA chambers until DIV14 to allow the establishment of mature neuronal networks, and spontaneous firing activity was recorded 10, 17, 22, 24 or 26 days after incubation with 100nM of recombinant monomeric, oligomeric or PFF aSyn species or PBS as a negative control.

We observed an increase in the mean firing rate in bursty cells (Figure 16B and C) and an increase of total number of spikes (Figure 16E-G) for the neurons treated with aSynMono at DIV 28 and DIV 30 and aSynOligo at DIV30.

However, there were no alterations in the number of bursts during the recording (Figure 16H), in the burst duration (Figure 16I) and in the number of spikes within a burst (Figure 16J-L). These results indicate an increase in the number of spikes outside the burst, suggesting that a desynchronization could be happening. Interestingly, an increase in the inter-burst intervals (Figure 16M-O) corroborate these results. Altogether, these results suggest that recombinant monomeric, oligomeric or PFF aSyn species can modulate some parameters of neuronal spontaneous activity and make the cultured neurons fire in a less synchronized and more irregular way, highlighting the potential of aSyn in modifying the neuronal activity.


A
B
Mean firing rate (Hz)
PBS 14
aSyn Monomers 14
PBS 21
aSyn Monomers 21
PBS 26
aSyn Monomers 26
PBS 28
aSyn Monomers 28
PBS 30
aSyn Monomers 30
C
Mean firing rate (Hz)
aSyn Oligomers 14
aSyn Oligomers 21
aSyn Oligomers 26
aSyn Oligomers 28
aSyn Oligomers 30
D
Mean firing rate (Hz)
aSyn PFF 14
aSyn PFF 21
aSyn PFF 26
aSyn PFF 28
aSyn PFF 30
E
Total number of spikes (bursty cells)
F
Total number of spikes (bursty cells)
aSyn Oligomers 14
aSyn Oligomers 21
aSyn Oligomers 26
aSyn Oligomers 28
aSyn Oligomers 30
G
Total number of spikes (bursty cells)
aSyn PFF 14
aSyn PFF 21
aSyn PFF 26
aSyn PFF 28
aSyn PFF 30
H
Burst rate (minute)
aSyn Monomers 14
aSyn Oligomers 14
aSyn PFF 14
aSyn Monomers 21
aSyn Oligomers 21
aSyn PFF 21
aSyn Monomers 26
aSyn Oligomers 26
aSyn PFF 26
aSyn Monomers 28
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aSyn Monomers 30
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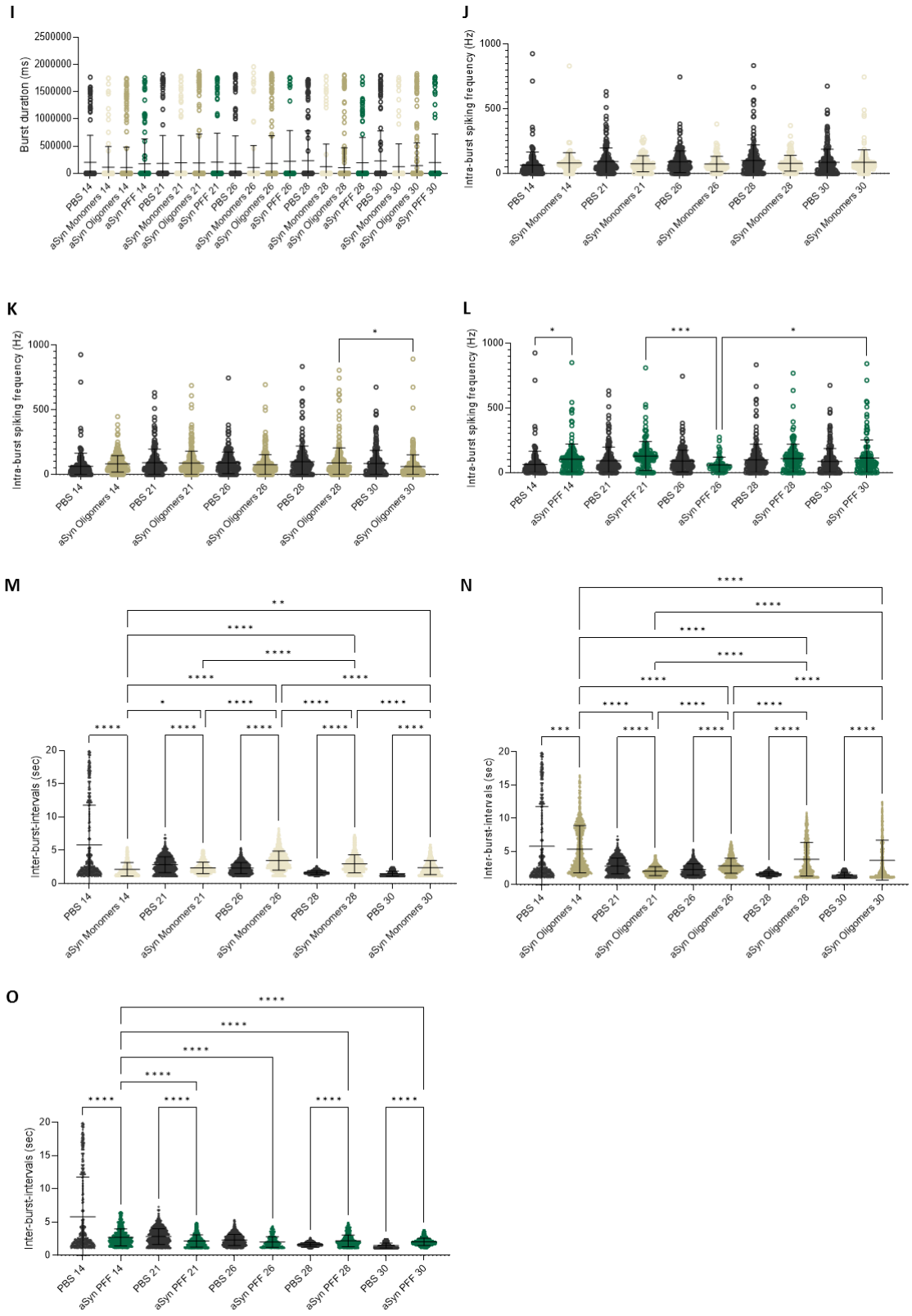


Figure 16 | Recombinant aSyn species can modulate some parameters of neuronal spontaneous activity. (A) Representative image of primary hippocampal neurons cultured on an MEA chamber. Quantification of the mean firing rate **(B-D)**, total number of spikes **(E-G)**, burst rate (per minute) **(H)**, burst duration (ms) **(I)**, intra-burst spiking frequency (Hz) **(J-L)**, inter-burst intervals **(M-O)** of primary hippocampal neurons incubated with PBS, as a negative control, or with 100nM of aSyn species (monomers, oligomers and PFFs) for 10, 17, 22, 24 or 26 days, recorded using 60-electrode MEAs. Data from at two to three

independent experiments for condition. All data are expressed as mean ± SD; One-way ANOVA, post-hoc Bonferroni's test; * p-value ≤ 0.05, ** p-value ≤ 0.01, *** p-value ≤ 0.001, **** p-value ≤ 0.0001.

4.4. Identification of interacting partners of aSyn in isolated synaptic vesicles

4.4.1. Immunoisolation of crude synaptic vesicles

Although aSyn role is still unknown (Burré, 2015), its presence at the presynaptic termini indicates that it may be involved in synaptic plasticity and learning (George et al., 1995; Murphy et al., 2000). A lot of evidence suggests a role of aSyn in the regulation of the homeostasis of SVs during neurotransmitter release (Auluck et al., 2010; Burré et al., 2010, 2014; Diao et al., 2013; Soper et al., 2008). Taking this in mind, we aimed to investigate if there were differences in SVs from wild-type (WT) mice or transgenic mice and whether the increased levels of aSyn would interfere with its interacting partners in these structures. Four and one independent SVs isolations from WT mice and Thy1-WTaSyn, respectively, were evaluated to allow the undoubted assignment of proteins. For this, together with a colleague from the same research group, SVs were purified from wild-type (WT) and transgenic overexpressing the human wild-type aSyn (Thy1-WT aSyn; WTaSyn) mice brain. Briefly, the protocol used starts with initial mild homogenisation of the brain (BH) to reduce fragmentation and vesiculation of large membranes (P1) and culminates in the release of SVs from damaged cells (S1) (Figure 9). Next, differential centrifugation steps are performed to separate large cell fragments and nuclei from free vesicles and synaptosomes. Synaptosomes (P2) are then pelleted by centrifugation. The resulting supernatant (S2), which is contaminated with cell fragments is discarded. SVs enclosed in synaptosomes are released with a hypo-osmotic shock and centrifugation, leaving small particles and SVs in the supernatant (LS1), while large components containing mainly active zone-enriched presynaptic membranes with docked vesicles attached to a portion of PSD are pelleted (LP1). The supernatant fraction with SVs (LS1) is then centrifugated at a high speed to obtain an enriched SV fraction (LP2).

Next, we assessed the purity of the SVs isolation protocol. Subfractions were collected from the purification procedure and immunoblotted to analyse SV integral membrane proteins. Synaptic vesicle integral membrane proteins like synaptophysin are enriched in the LP2 fraction in both WT (Figure 17A and C) and WTaSyn mice (Figure 17B and C).

We then used electron microscopy (EM) analysis to analyse the size and shape of SVs. In our study, purified WT and WTaSyn SV fractions exhibited a round sphere morphology, typical for negative stained vesicular structures by EM, and presented the expected diameter size (WT (34.8 ± 11.9), and WTaSyn (35.8 ± 9.4)) (Figure 17D and E). These results confirme the integrity of the vesicles and the uniform morphology suggest that SVs store a similar amount of neurotransmitters (Poudel and Bai, 2014).

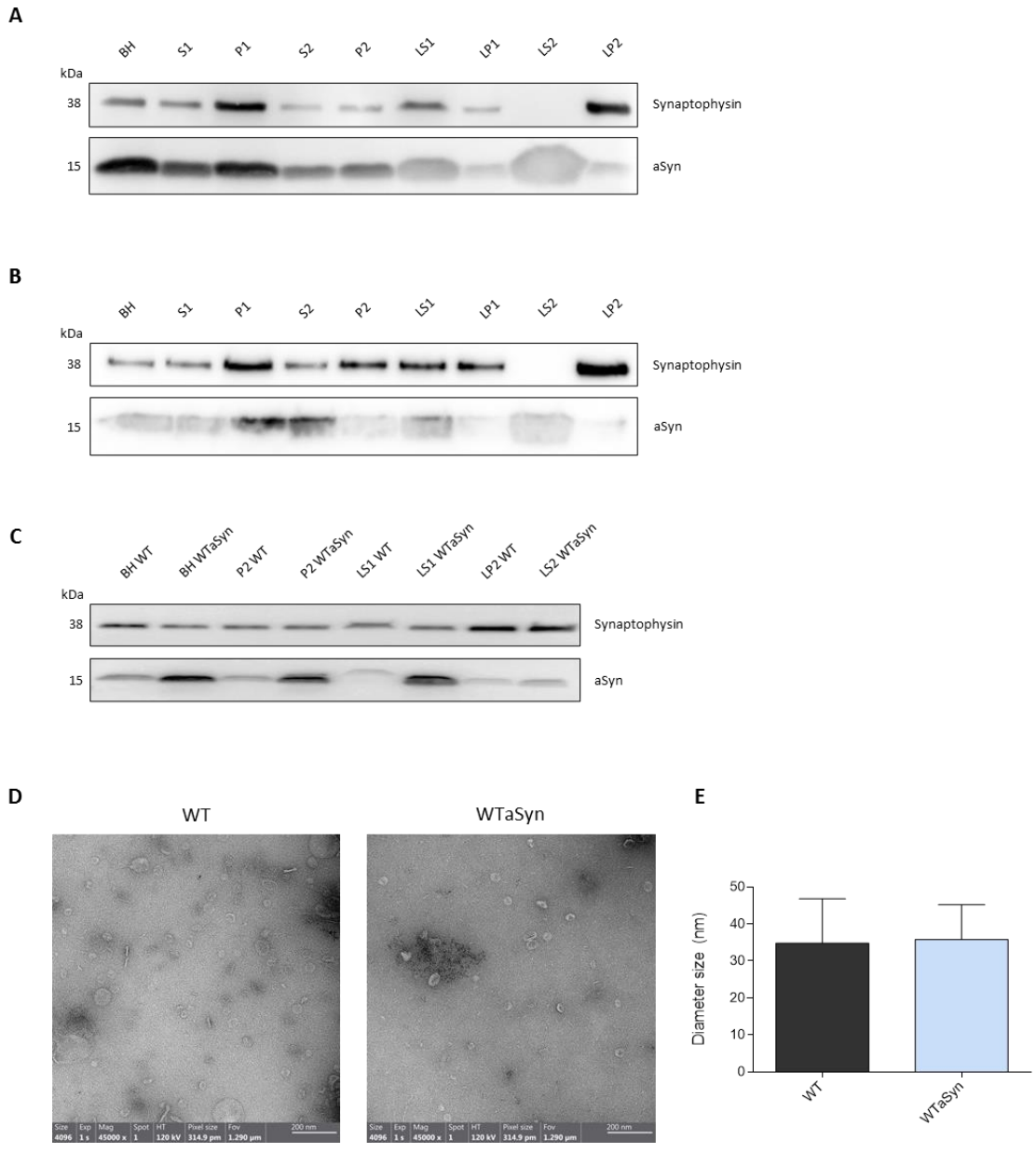


Figure 17 | Purified synaptic vesicles (SVs) isolated from WT and WTaSyn mice. Representative immunoblot of synaptophysin and aSyn in SVs isolated from WT **(A)** and WTaSyn **(B)** 3 m.o. mice, and comparison between both **(C)** (n=1-4). **(D)** Representative electron microscopy of SVs. Sample imaging was carried out by Dr Dietmar Riedel (Facility for Electron Microscopy, Max Planck Institute for Multidisciplinary Sciences). **(E)** Quantification of the SV diameters (nm). All data are expressed as mean ± SD; Student's t-test.

4.4.2. Co-Immunoprecipitation (Co-IP) of purified SVs

After assessing the purity of SVs, isolated by the protocol described, we wanted to isolate aSyn interacting partners on SVs. For this reason, together with a colleague from the same research group, a co-immunoprecipitation (Co-IP) against aSyn was performed on the LP2 fractions from WT and WTaSyn 3 m.o. mice. Briefly, Co-IP is the immunoprecipitation of intact protein complexes. This works by selecting an antibody that targets a known protein that is bound to other proteins that are part of a larger complex of proteins. This complex of proteins is then precipitated on a beaded support to which an antibody-binding protein is immobilized, and any proteins not precipitated on the beads are washed away. Then, it is able to pull the entire protein complex out and thereby identify unknown members of the complex.

The results show that the purified mouse anti-aSyn antibody was successfully coupled to the beads in the WT and WTaSyn mice isolated SVs, as observed by aSyn immunodetection in the aSyn-coupled beads elute fraction (Figure 18A and B). In contrast, there was no aSyn immunodetection in IgG-coupled beads elute fraction, used as a negative control (Figure 18A and B). Input, supernatants and 3rd washes were used as controls to confirm that the Co-IP works under the chosen conditions (Figure 18A and B). Input was used as a positive control to confirm that aSyn, our protein of interest (target), was present in the sample. Supernatants and washes were used to make sure that the co-IP was specific for aSyn and that our target was not lost in any other fractions during the protocol. Under both washing conditions, the antigen was not immunodetected, indicating that our target is not lost (Figure 18A and B). Immunodetection against VAMP2 was also used as a control. We observed a clear band in the input fraction, showing an enrichment of SVs in this fraction, and no presence of VAMP2 in the elute fraction, meaning that the elution fraction was successfully precipitated against aSyn-coupled beads and IgG-coupled beads (Figure 18C and D). Altogether, these results show an efficient affinity precipitation against aSyn on the LP2 fractions from WT and WTaSyn mice.

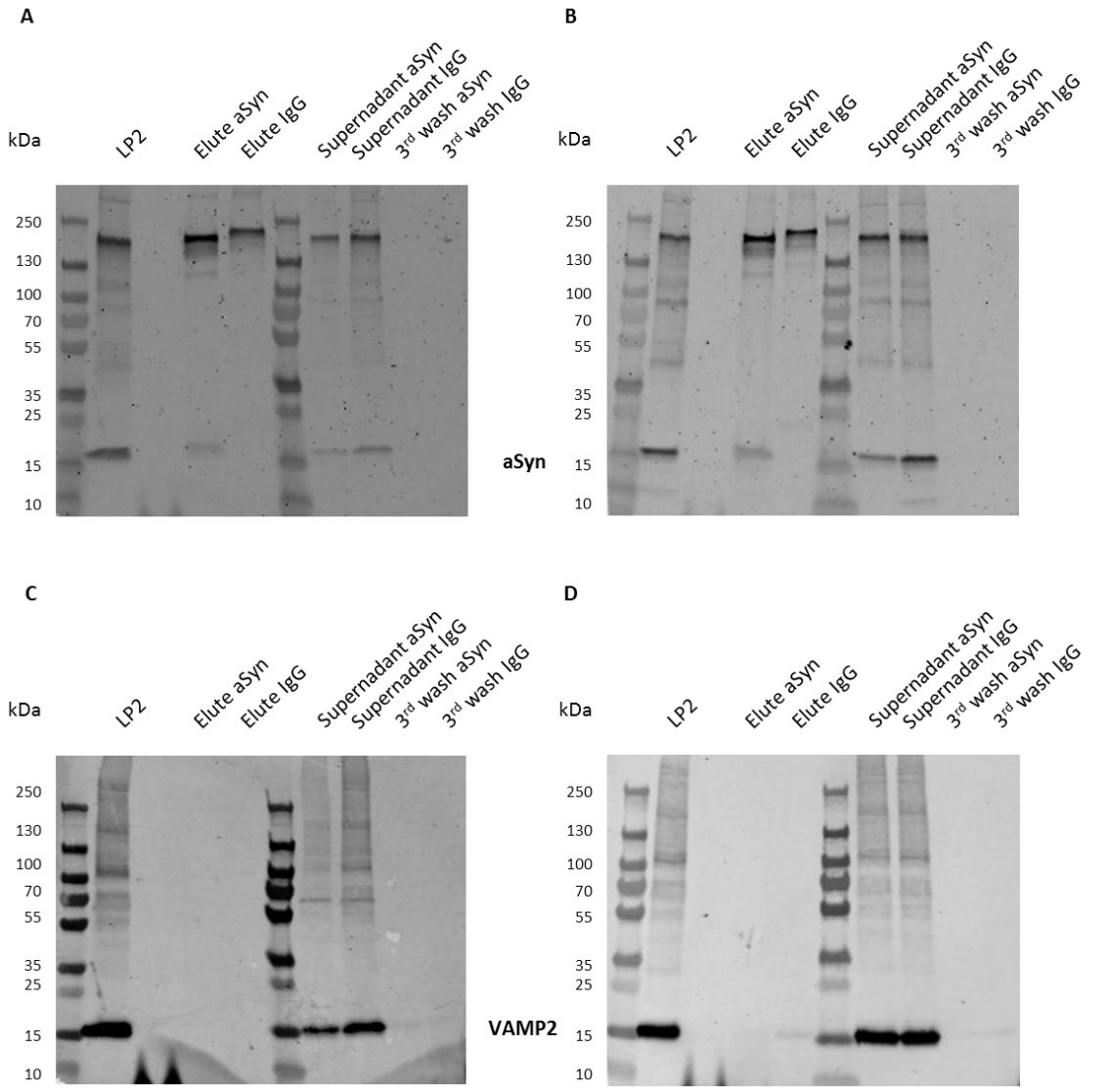


Figure 18 | Co-immunoprecipitation (co-IP) from LP2 fraction (SVs) isolated from WT and WTaSyn mice. Representative immunoblots of co-IP against aSyn **(A and B)** and VAMP2 **(C and D)** from LP2 fractions isolated from WT **(A and C)** and WTaSyn **(B and D)** mice. Magnetic Dynabeads were pre-incubated with aSyn, VAMP2 or mouse IgG (negative control) antibody before the LP2 incubation.

4.4.3. Mass spectrometry

4.4.3.1. Study of the proteins co-immunoprecipitated with aSyn by label-free quantitative mass spectrometry

After the successful immunoprecipitation of aSyn interacting partners on the LP2 fractions from WT and WTaSyn mice, mass spectrometry (MS) was performed on these samples to determine the protein composition. MS analysis was performed at the Proteomics Core Facility at the University Medical Center Göttingen. Immunoprecipitates containing aSyn and aSyn-interacting proteins were analysed in a label-free quantification approach combined with tandem mass spectrometry (nanoLC-MS/MS) (Zhu et al., 2010). The first step in sample preparation for mass spectrometry (MS) analysis is the reconstitution of the samples in the LDS Sample Buffer. This step is followed by protein separation by SDS-PAGE gel, in-gel tryptic digestion, extraction of digestive peptides from the gel pieces, and lastly their MS analysis. In our study, data obtained from three biological replicates in three technical repeats for WT mice and data obtained from one biological replicate in three technical repeats for WTaSyn mice were used. Immunoprecipitations in combination with MS data analysis must be performed at least in triplicate since the data validation is based on the Student's t-test statistics. Unfortunately, for the WTaSyn mice samples this was not possible, leading us to not being able to conclude results, but only to speculate about them.

In total, 1234 proteins were identified in our study. The clustering of samples was evaluated by principal component analysis (PCA) and heatmap which revealed a clear separation between WT and WTaSyn mice (Figure 19A and B). Despite some overlap in identified proteins, hierarchical clustering revealed two distinct fractions with distinct proteomic profiles. These results suggest individual proteomic profiles of SVs isolated from WT and WTaSyn mice, supporting potential alterations in the behaviour of these two SVs.

To analyse the relationship or trend between these two groups, scatter plots were used. The protein content was defined by a strict log2FC cut-off (±1.00), and the statistical significance of the data was established by $P \leq 0.05$. The scatter plot between IgG WT over Syn1 WT showed 394 enriched proteins. 128 of these proteins were enriched in Syn1 WT, while the remaining 266 proteins were significantly and high fold-change enriched in the negative control (IgG WT) (Figure 20A). From the 394 identified proteins, 11 synaptic proteins were identified where the majority is present in the Syn1 WT sample. The top 10 proteins enriched in the Syn1 WT mice are listed in Table 2. Then, we

assessed the proteins enriched in IgG WTaSyn over Syn1 WTaSyn and we observed a total of 247 proteins enriched. From these proteins, 106 were significantly and high fold-change enriched in Syn1 WTaSyn, while the remaining 141 proteins were enriched in the negative control (IgG WT) (Figure 20B). From the total proteins identified, 4 were synaptic proteins and the majority of them were present in the IgG WTaSyn. The top 10 proteins enriched in the Syn1 WTaSyn mice are listed in Table 3. Next, we also evaluated the proteins enriched in Syn1 WTaSyn over Syn1 WT. We found a total of 631 enriched proteins. 377 of these proteins were enriched in Syn1 WTaSyn, while the other 254 proteins were enriched in the Syn1 WT (Figure 20C). 17 synaptic proteins were identified among the total enriched proteins where they were similarly distributed between WT and WTaSyn mice. The top 10 proteins enriched in the Syn1 WTaSyn mice are listed in Table 4, while the top 10 proteins enriched in the Syn1 WT mice are listed in Table 5. Moreover, we also analyse the biological background between WT and WTaSyn. We observed that the sample distribution was not homogeneous, suggesting that the biological background between WT and WTaSyn was different (Figure 20D).

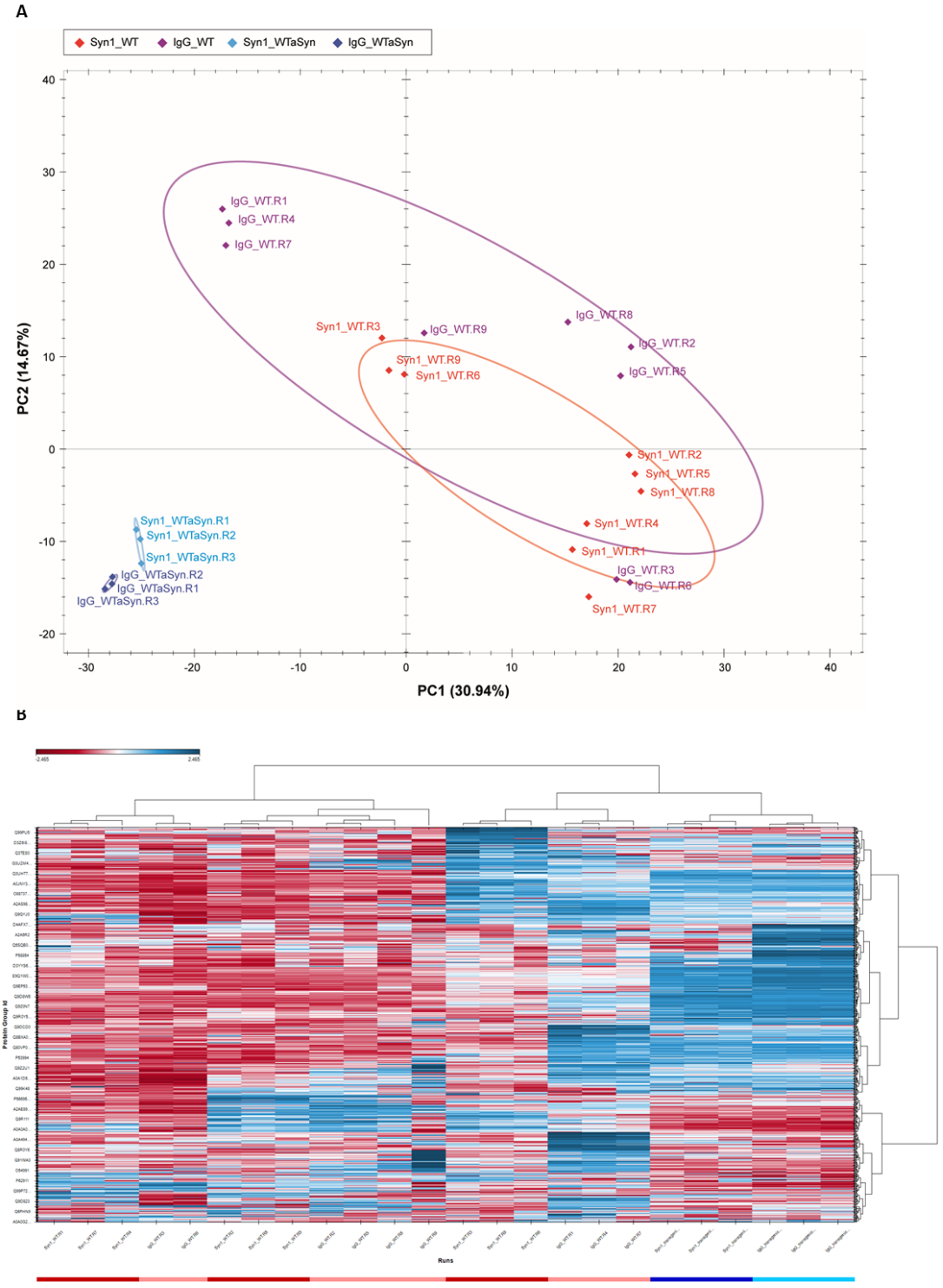
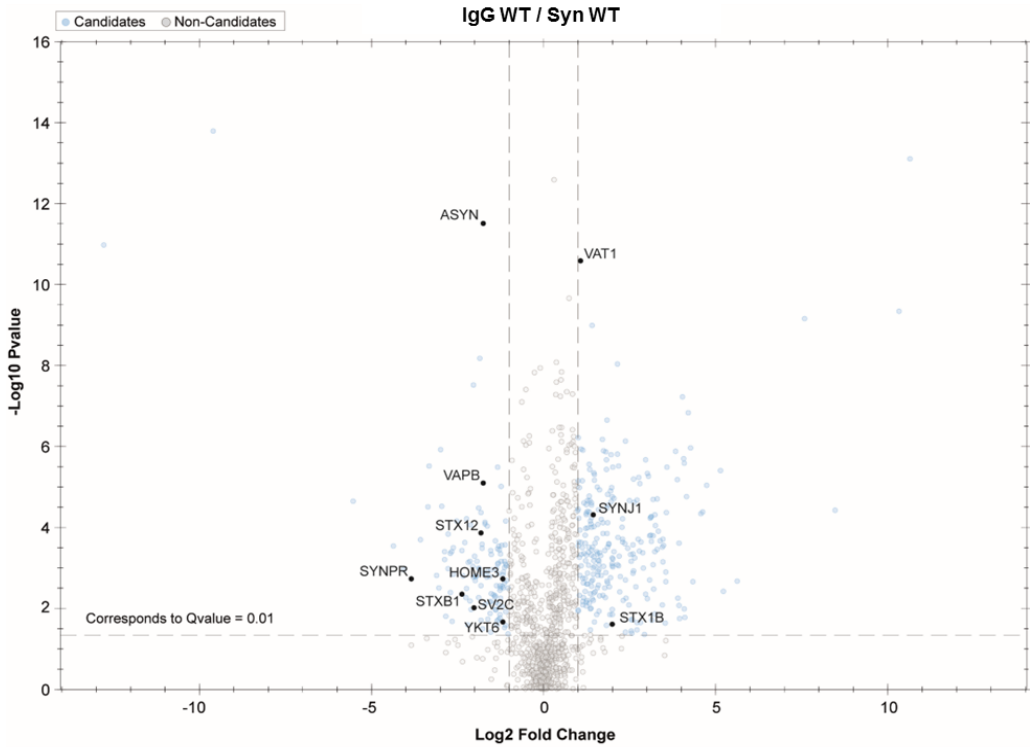
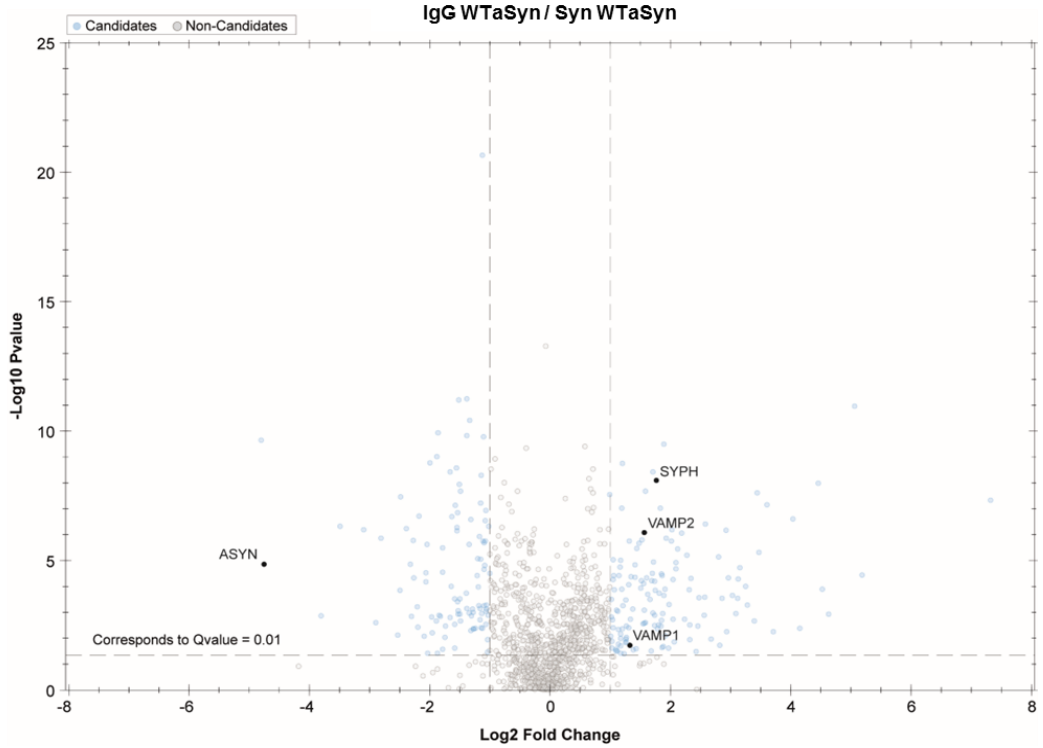


Figure 19 | Proteomic analyses of SVs using label-free quantitative mass spectrometry. (A) PCA of the quantitative differences in spectral counts between SVs isolated from WT (purple and red) and WTaSyn (light and dark blue) mice in three biological replicates. **(B)** Heatmap of the significantly different identified proteins from proteomic profiling of SVs isolated from WT and WTaSyn mice. High and low expression are shown in blue and red, respectively (protein intensity z-score -2.465 for dark red, protein intensity z-score for 2.465 dark blue). Data represented from four independent samples for WT mice and one independent sample for WTaSyn mice. Data processing was performed in Spectronaut software (v16.0.220606.53000 (Hawking)).

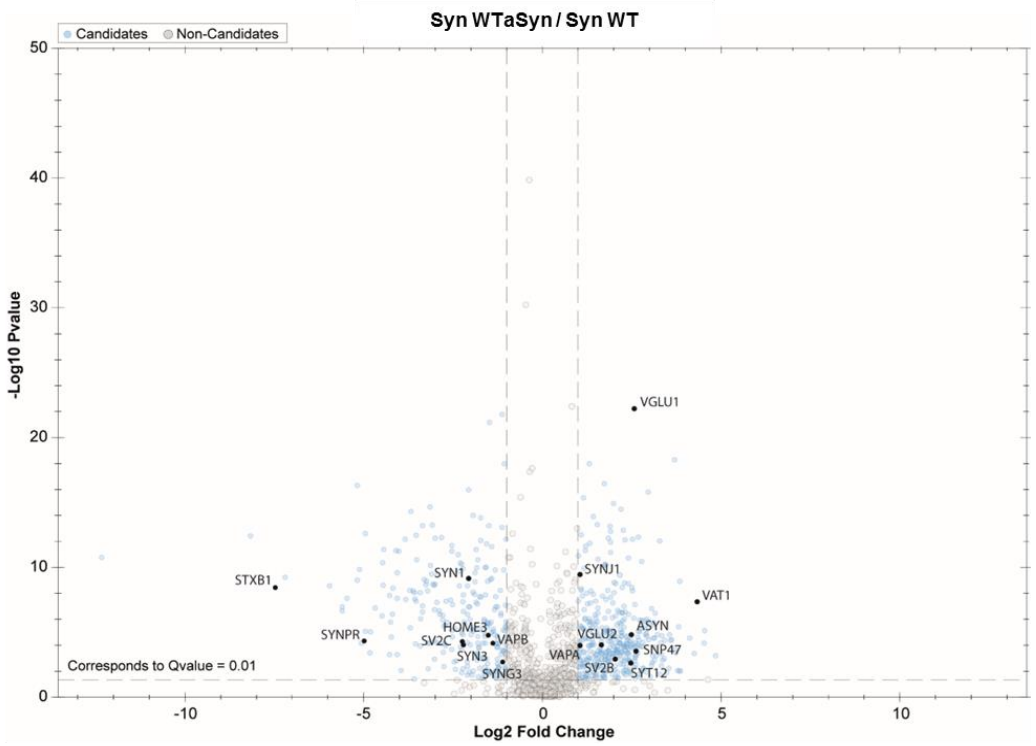
A


IgG WT / Syn WT
Candidates
Non-Candidates
-Log10 Pvalue
Log2 Fold Change
ASYN
VAT1
VAPB
SYNJ1
STX12
SYNPR
HOME3
STXB1
SV2C
YKT6
STX1B
Corresponds to Qvalue = 0.01

B


IgG WTaSyn / Syn WTaSyn
Candidates
Non-Candidates
-Log10 Pvalue
Log2 Fold Change
SYPH
VAMP2
ASYN
VAMP1
Corresponds to Qvalue = 0.01

C



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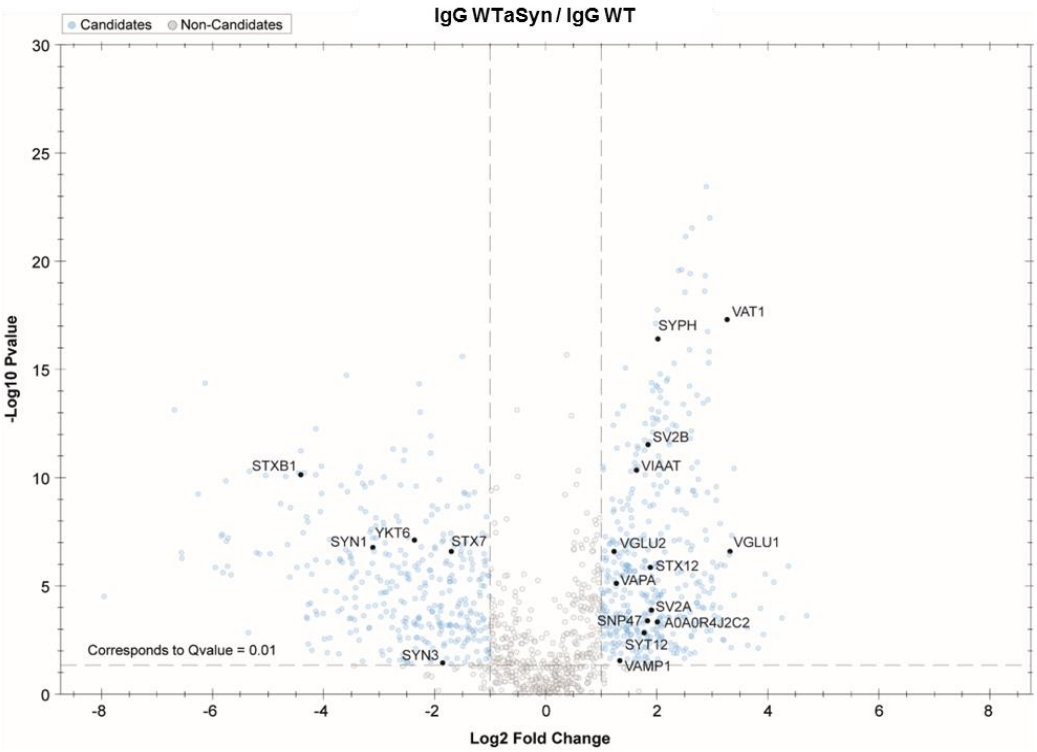

Figure 20 | Scatter plots of quantitative differences in proteins in SVs isolated from WT and WTaSyn mice. Logarithmic ratio of protein intensities difference was plotted against negative p-values. **A)** Scatter plot of quantitative differences in proteins in SVs isolated from WT mice. Significantly, enriched proteins in Syn1WT (left in the plot) and negative control (right in the plot) are represented as blue dots above the dashed lines. Grey dots represent the proteins that were not significantly enriched. **B)** Scatter plot of quantitative differences in proteins in SVs isolated from WTaSyn mice. Significantly, enriched proteins in Syn1WTaSyn (left in the plot) and negative control (right in the plot) are represented as blue dots above the dashed lines. Grey dots represent the proteins that were not significantly enriched. **C)** Scatter plot of quantitative differences in proteins in SVs isolated from WT and WTaSyn mice. Significantly, enriched

proteins in Syn1WT (left in the plot) and Syn1WTaSyn (right in the plot) are represented as blue dots above the dashed lines. Grey dots represent the proteins that were not significantly enriched. **D)** Scatter plot of quantitative differences in proteins in SVs isolated from WT and WTaSyn mice. Significantly, enriched proteins in IgGWT (left in the plot) and IgGWTaSyn (right in the plot) are represented as blue dots above the dashed lines. Grey dots represent the proteins that were not significantly enriched.

Table 2 | Top 10 proteins enriched in the Syn1WT (in relation to IgGWT) co-IP samples. Proteins are listed according to their P value in a decreasing order.

Protein ID	Gene name	Protein name	-log$_{10}$ (p-value)
A0A0B4J1H7	Igkv1-135	Immunoglobulin kappa variable 1-135 (Fragment)	10.98
A0A0A0MQC1	Ighv3-5	Immunoglobulin heavy variable 3-5	13.79
O35127	Grcc10	Protein C10	4.64
P51881	Slc25a5	ADP/ATP translocase 2	3.54
A0A140LI98	Uqcrc2	Cytochrome b-c1 complex subunit 2, mitochondrial (Fragment)	2.95
Q8BGN8-2	Synpr	Isoform 2 of Synaptoporin	2.73
D3Z2J6	Tmx2	Thioredoxin-related transmembrane protein 2	3.69
Q8VDQ8	Sirt2	NAD-dependent protein deacetylase sirtuin-2	4.51
Q8BLY2	Tarsl2	Threonine-tRNA ligase 2, cytoplasmic	5.51
E9Q455	Tpm1	Tropomyosin alpha-1 chain	2.81

Table 3 | Top 10 proteins enriched in the Syn1WTaSyn (in relation to IgGWTaSyn) co-IP samples. Proteins are listed according to their P value in a decreasing order.

Protein ID	Gene name	Protein name	-log$_{10}$ (p-value)
A0A0A0MQC1	Ighv3-5	Immunoglobulin heavy variable 3-5	9.65
O55042	Snca	Alpha-synuclein	4.85
Q08189	Tgm3	Protein-glutamine gamma-glutamyltransferase E	2.85
A2A513	Krt10	Keratin, type I cytoskeletal 10	6.30
Q8BLY2	Tarsl2	Threonine-tRNA ligase 2, cytoplasmic	6.18
Q7TQI3	Otub1	Ubiquitin thioesterase OTUB1	2.60
D3YZ06	Hspb1	Heat shock protein beta-1	5.86
Q9D8B3	Chmp4b	Charged multivesicular body protein 4b	2.10
Q61598	Gdi2	Rab GDP dissociation inhibitor beta	3.84
Q62266	Sprr1a	Cornifin-A	7.45

Table 4 | Top 10 proteins enriched in the Syn1WTaSyn (in relation to SynWT) co-IP samples. Proteins are listed according to their P value in a decreasing order.

Protein ID	Gene name	Protein name	-log$_{10}$ (p-value)
Q99P58	Rab27b	Ras-related protein Rab-27B	3.19
G3UZW8	Psmb8	Proteasome subunit beta (Fragment)	5.08
Q8BXA0	Lrfn5	Leucine-rich repeat and fibronectin type-III domain-containing protein 5	4.16
Q62465	Vat1	Synaptic vesicle membrane protein VAT-1 homolog	7.32
Q9EST1	Gsdma	Gasdermin-A	3.36
Q9D8W5	Psmd12	26S proteasome non-ATPase regulatory subunit 12	4.52
B1ASW6	Trappc1	Trafficking protein particle complex subunit	2.01
P62137	Ppp1ca	Serine/threonine-protein phosphatase PP1-alpha catalytic subunit	8.92
B7ZCU0	Abi1	Abl interactor 1	2.00
Q91Z83	Myh7	Myosin-7	5.41

Table 5 | Top 10 proteins enriched in the Syn1WT (in relation to SynWTaSyn) co-IP samples. Proteins are listed according to their P value in a decreasing order.

Protein ID	Gene name	Protein name	-log$_{10}$ (p-value)
A0A0B4J1H7	Igkv1-135	Immunoglobulin kappa variable 1-135 (Fragment)	10.74
Q9CPN9	2210010C04Rik	RIKEN cDNA 2210010C04 gene	12.41
O08599-2	Stxbp1	Isoform 2 of Syntaxin-binding protein 1	8.41
P63213	Gng2	Guanine nucleotide-binding protein G(I)/G(S)/G(O) subunit gamma-2	9.21
A0A338P6R8	Gm49601	Predicted gene	8.55
H3BIX4	Nptn	Neuroplastin (Fragment)	6.61
Q3UZP7	Palm	Paralemmin-1	6.96
A0A0U1RPY2	Fxyd7	FXYD domain-containing ion transport regulator	7.60
A0A075B5N7	Igkv6-13	Immunoglobulin kappa variable 6-13	16.29
P21279	Gnaq	Guanine nucleotide-binding protein G(q) subunit alpha	9.01

4.4.3.2. Gene ontology enrichment terms of the proteins enriched in SVs isolated from WT and WTaSyn mice

To further understand the distinct biological roles of proteins involved in SVs from WT and WTaSyn mice, we performed gene ontology (GO) enrichment analysis.

Translation as well as proteasomal protein catabolic biological processes were enriched in Syn1WT samples (Figure 21A). Furthermore, most of the proteins identified were associated with synapse, cytoplasm and cytosol cellular components, correlating with the molecular functions of these proteins (Figure 21B and C).

In Syn1WTaSyn samples, several of the enriched biological processes were related to the regulation of biological and cellular processes, heterochromatin assembly, intermediate filament organization, and nucleosome (Figure 21D and E). As expected, the molecular function hits included protein binding, structural constituent of ribosome, and ubiquitin protein ligase binding (Figure 21F). Overall, these data indicate that the increase of aSyn levels in the WTaSyn animals changes the levels and composition of proteins present in the SVs and it drives changes in the brain of these animals.

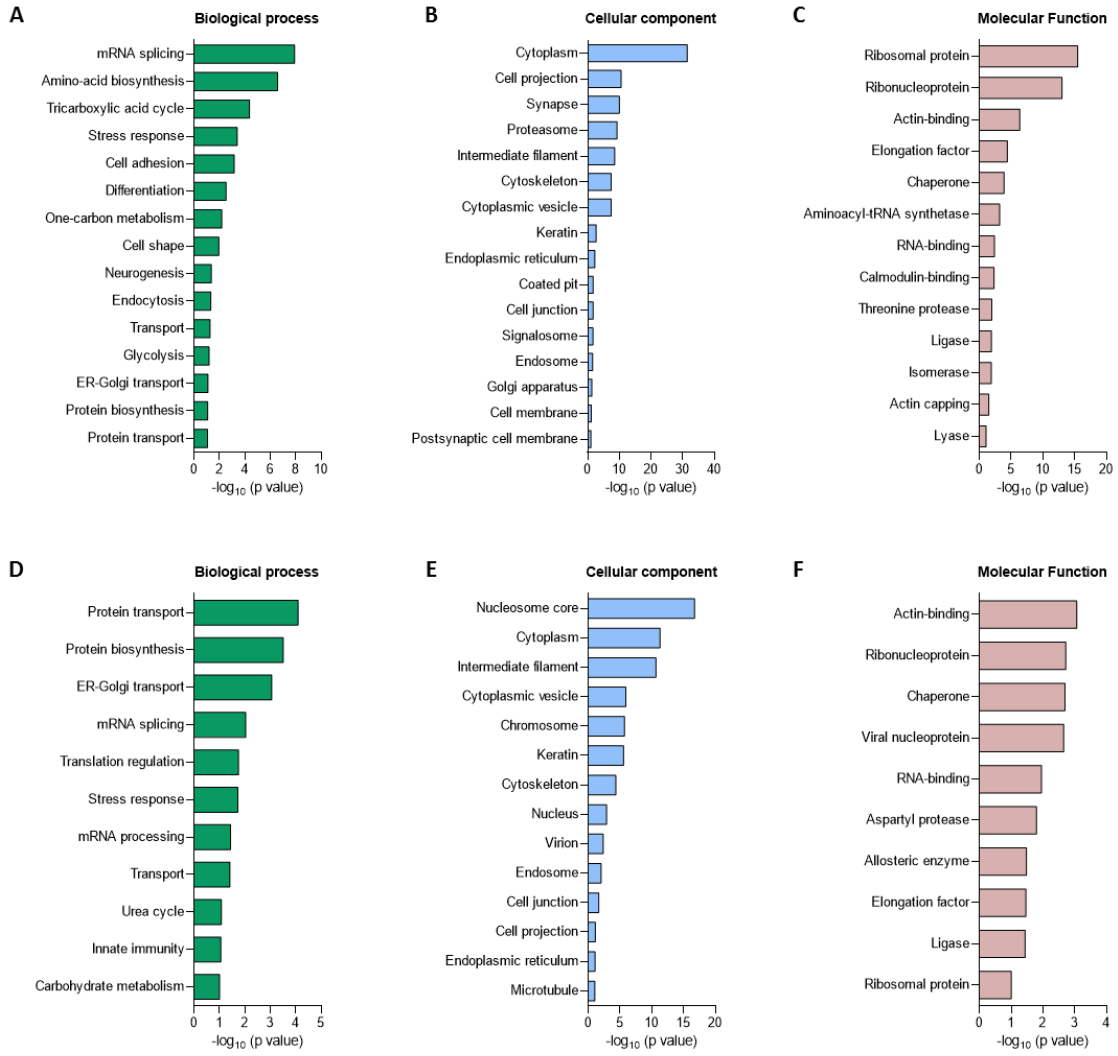

Figure 21 | Gene ontology (GO) enrichment terms for proteins enriched in WT (A, B and C) and WTaSyn mice (D, E and F). The following categories were evaluated: biological process **(A and D)**, cellular component **(B and E)**, molecular function **(C and F)**. Data from three independent biological replicates in three technical repeats for WT mice and data from one independent biological replicate in three technical repeats for WTaSyn mice was analysed using Spectronaut software (v16.0.220606.53000 (Hawking)).

4.5. Occurrence of synaptic proteins in PNS and CNS from WTaSyn and A30PaSyn mice

4.5.1. Quantification of aSyn in biofluids at 3,6 and 9 months of age in WTaSyn and A30PaSyn mice

CSF, the fluid bathing the CNS, provides a window to biochemical and neuropathological changes in the brain. By providing a metabolic-pathological profile of the CNS, CSF can help identify. patterns that differentiate between WT mice and those expressing either

WTaSyn or A30PaSyn. To investigate this, we assessed aSyn expression levels at different time points using ELISA. In particular, aSyn levels were assessed at 3, 6 and 9 m.o to associate the aSyn levels with a specific timeframe of the phenotype development.

Interestingly, no significant differences were observed in the CSF aSyn levels at 3 m.o, 6 m.o and 9 m.o WTaSyn mice (2461 ± 3481, 1053 ± 374.4 and 1768 ± 1054 pg/mL, respectively) when compared to age-matched controls (1105 ± 1131, 183.6 ± 281.1 and 718.1 ± 632.2 pg/mL, respectively) (Figure 22A). However, there was a tendency to increase the levels of CSF aSyn in WTaSyn mice in all the time points analysed when compared to the respective controls. Similarly, to WTaSyn mice, no significant differences were observed in the CSF aSyn levels at 3 m.o, 6 m.o and 9 m.o A30PaSyn mice (37986 ± 39933, 61246 ± 75608 and 10439 ± 5866 pg/mL, respectively) when compared to age-matched controls (1534 ± 2304, 1716 ± 2633 and 272.9 ± 283.4 pg/mL, respectively) (Figure 22F). However, there was a tendency to increase the levels of CSF aSyn in A30PaSyn mice at 3 m.o and 6 m.o and at 9 m.o it drops to almost basal levels.

Moreover, although CSF appears to be an ideal biofluid to check for biomarkers, the process of obtaining CSF involves an invasive and painful procedure, and there may be additional technical and ethical issues related to the collection procedure. For that reason, the look for alternatives and the investigation of PD biomarkers in other biofluids started to be more investigated. Taking this into consideration, we decided to investigate the levels of aSyn in blood and saliva. The easy accessibility of blood in clinical circumstances makes it a good alternative to the conventional use of CSF. Saliva is also a highly versatile biofluid that is easy to collect in a non-invasive manner. Similarly to CSF, blood was drawn from each mouse to be processed to obtain whole blood, plasma or serum and the aSyn levels were examined.

Serum aSyn levels showed only a slight increase in the 3 m.o, 6 m.o and 9 m.o Thy1-WT aSyn mice (2118 ± 391.0, 3712 ±1006 and 2670 ± 1162 pg/mL, respectively), when compared to age-matched controls (1032 ± 367.8, 2312 ± 1515 and 1200 ± 414.3 pg/mL, respectively) (Figure 22B). As WTaSyn mice, A30PaSyn mice showed an increase of serum aSyn levels at 3 m.o, 6 m.o and 9 m.o, however, in this mouse model these increases were significant (14614 ± 5588, 14276 ± 2387 and 21380 ± 3832 pg/mL, respectively) when compared to age-matched controls (2598 ± 263.5, 3674 ± 591.0 and 2430 ± 1392 pg/mL, respectively) (Figure 22G).

Moreover, plasma aSyn levels do not show any significant differences at 3 m.o, 6 m.o and 9 m.o WTaSyn mice (3627 ± 3711, 1976 ± 733.1 and 1067 ± 302.6 pg/mL, respectively), when compared to age-matched controls (1382 ± 926.2, 1479 ± 670.2 and 823.0 ± 216.5 pg/mL, respectively) (Figure 22C). There were also no differences between males and females. Contrarily, plasma levels of aSyn in A30PaSyn mice were found significantly increased at 3 m.o, 6 m.o and 9 m.o (8360 ± 2207, 12321 ± 2892 and 13214 ± 2418 pg/mL, respectively), when compared to WT mice (2324 ± 239.4, 3531 ± 257.7 and 2722 ± 485.9 pg/mL, respectively) (Figure 22H).

In addition, there were no significant changes observed in whole blood aSyn levels in WTaSyn mice (1984000 ± 1537000, 2254000 ± 902339 and 1096000 ± 172326 pg/mL, 3 m.o, 6 m.o and 9 m.o, respectively), when compared to age-matched controls (1652000 ± 1101000, 1792000 ± 764943 and 1105000 ± 184357 pg/mL, respectively) (Figure 22D). Similarly, aSyn levels in the whole blood of A30PaSyn mice in all the time points analysed show no differences (3 m.o: 3859000 ± 557584, 6 m.o: 3646000 ± 649594 and 9m.o: 3762000 ± 395342 pg/mL), when compared to WT mice (2635000 ± 347551, 4499000 ± 631350 and 3110000 ± 1812000 pg/mL, 3 m.o, 6 m.o and 9 m.o, respectively) (Figure 22I).

The last biofluid analysed was saliva, where a significant increase of the levels of aSyn was observed at 3 m.o-Thy-WTSyn mice (111.7 ± 60.28 pg/mL) when compared to age-matched wildtype mice (10.14 ± 9.104 pg/mL). The levels of aSyn got back to physiological levels at 6 m.o-Thy-WTSyn mice (20.92 ± 18.65 pg/mL) and kept similar levels until 9 m.o (14.67 ± 9.039 pg/mL) (Figure 22E). Furthermore, in A30PaSyn mice, the aSyn saliva levels were not significantly altered (3 m.o: 45.40 ± 38.53, 6 m.o: 139.10 ± 129.50 and 9m.o: 262.90 ± 245.20 pg/mL) when compared to WT mice (3 m.o: 24.98 ± 38.05, 6 m.o: 80.31 ± 63.68 and 9m.o: 26.54 ± 36.87 pg/mL) (Figure 22J). However, the levels of aSyn tend to increase with time in Tg mice. Although, with time the variability of aSyn levels between mice also increases.

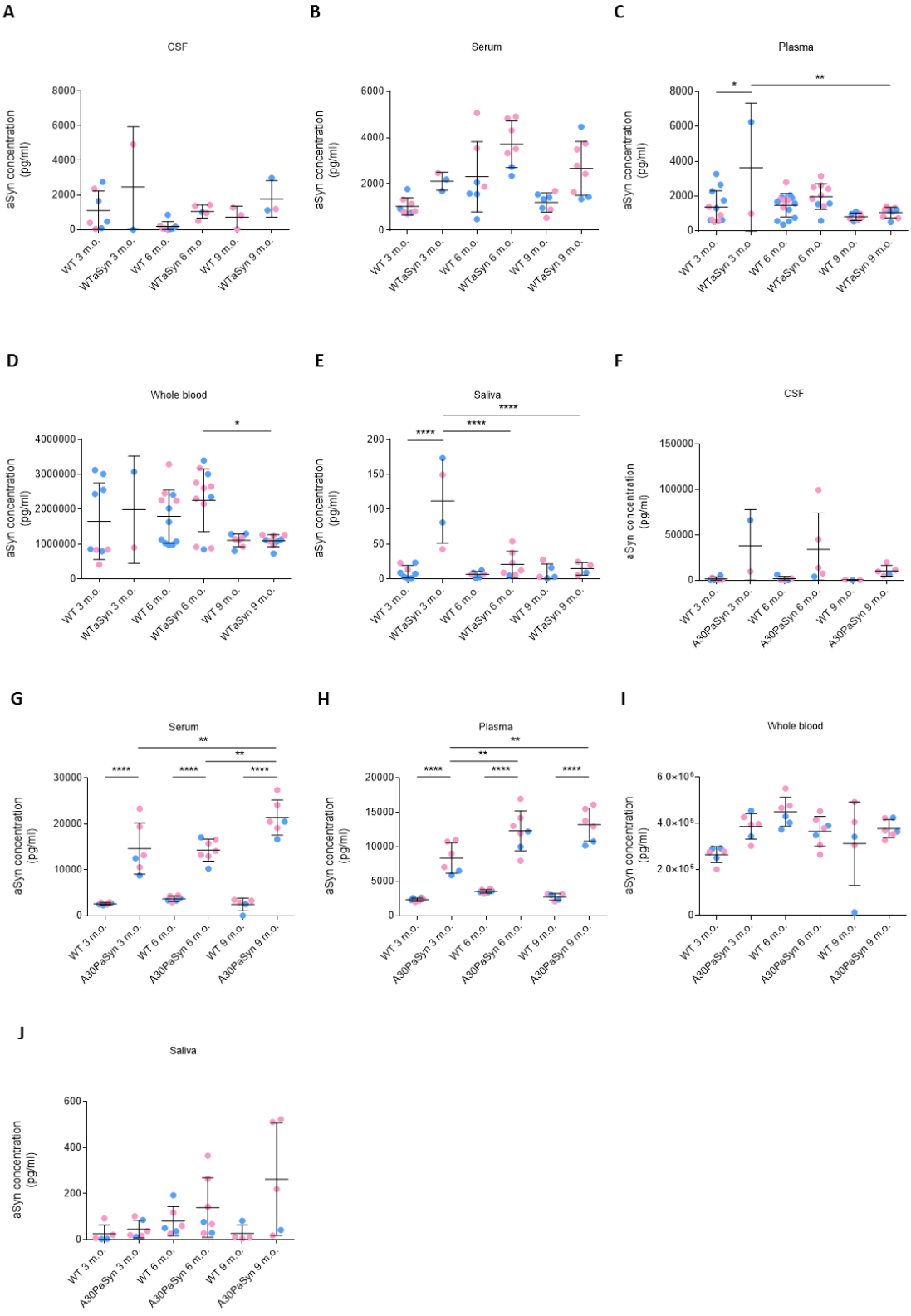

Figure 22 | aSyn concentration in biofluids at 3, 6 and 9 months of age in WTaSyn (A-E) and A30PaSyn mice (F-J). Quantification of aSyn concentration (pg/ml) in CSF **(A and F)**, serum **(B and G)**, plasma **(C and H)**, whole blood **(D and I)**, and saliva **(E and J)** (n=2-12). All data are expressed as mean ± SD; One-way ANOVA, followed by post-hoc Tukey's test.

4.5.2. Quantification of different proteins at 3,6 and 9 months of age in WTaSyn and A30P mice

After evaluating the levels of aSyn outside the CNS, we decided to investigate the levels of aSyn and other biomarkers in the brain. For these analyses, the protein levels were assessed by Western blot, and the immunoreactivity of each band was normalized by β-actin. In a similar way as before, the protein levels were assessed at 3, 6 and 9 m.o of WTaSyn and A30PaSyn mice.

Quantification of aSyn levels did not show any significant increase in WTaSyn mice (3 m.o: 2.880 ± 1.384, 6 m.o: 2.380 ± 1.464 and 9m.o: 1.952 ± 1.247245.20) when compared to WT animals (3 m.o: 1.343 ± 0.926, 6 m.o: 1.457 ± 0.770 and 9m.o: 1.681 ± 0.489). However, a tendency for an increase of the levels of aSyn is observed in these Tg animals when compared to the WT animals for three time points (Figure 23A). Consistent with these data, no significant changes were observed in aSyn levels of A30PaSyn mice when compared to age-matched controls (Figure 24A). Although no significant differences were observed in aSyn levels, interestingly, pS129 levels were significantly increased in both Tg mouse models at 3 m.o and 6 m.o, and at 9 m.o only for A30PaSyn mice (Figure 23B and 24B). Moreover, immunoblotting analysis did not show any changes of TH levels in Tg animals when compared to WT animals supporting no evidence of dopaminergic neuronal loss or neurotransmitter imbalance (Figure Figure 23C and 24C). Also, pre-synaptic proteins, markers of synaptic vesicles (VAMP2, synaptotagmin (SYT) and synapsin I (SYN)) and SNARE complex (SNAP25), were quantified. Quantification of these proteins showed no significant changes in WTaSyn and A30PaSyn mice compared to Wt animals (Figure 23D-I and 24D-H) indicating no loss in presynaptic structures. Furthermore, the post synaptic markers, PSD95 and Homer, do not show any significant changes in WTaSyn and A30PaSyn mice compared to WT mice, indicating no loss of post synaptic structures (Figure 23J AND K and 24I-J).

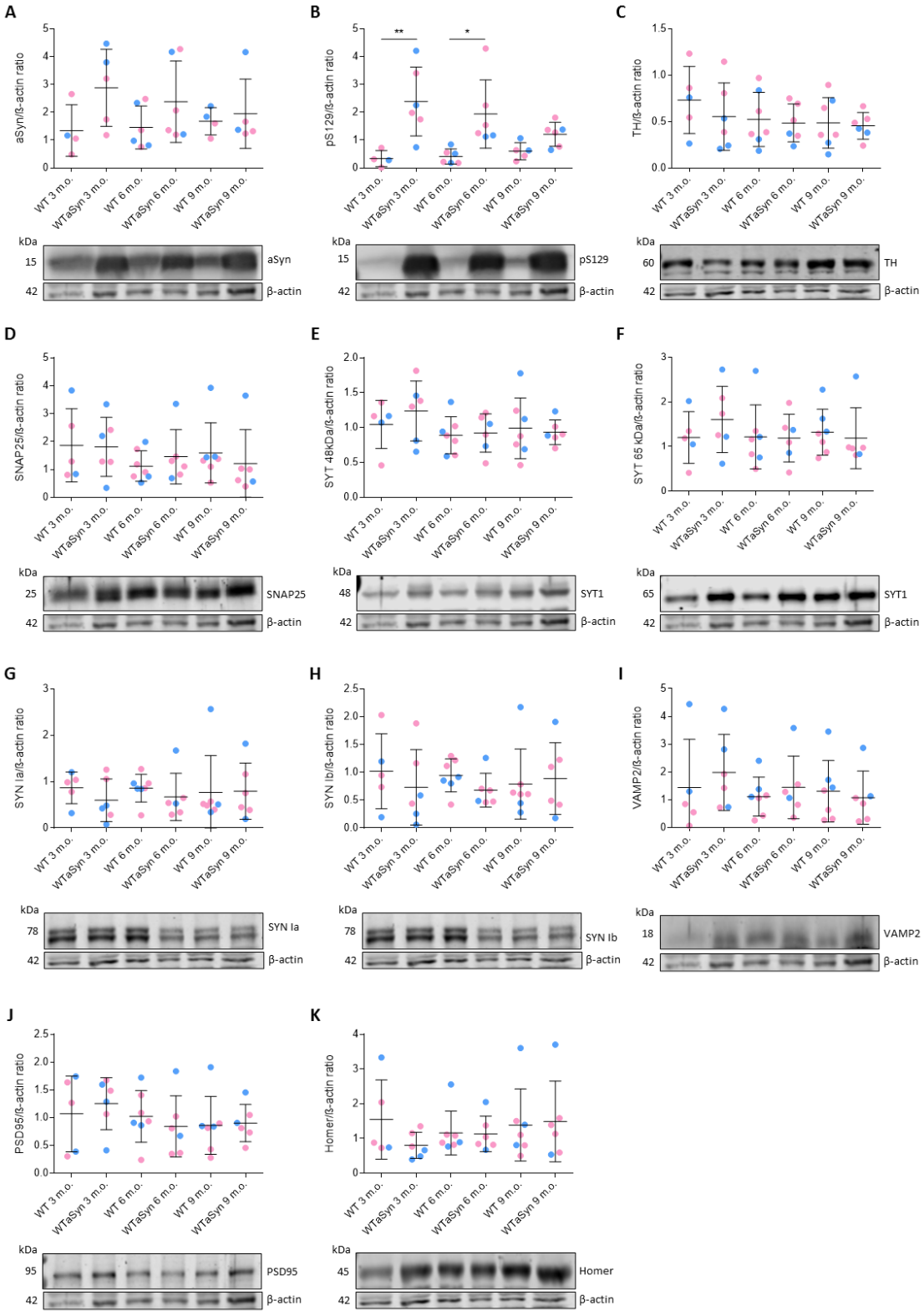

Figure 23 | Quantification of different proteins in the brain of 3, 6 and 9 months of age WTaSyn mice. Quantification of aSyn **(A)**, pS129 **(B)**, TH **(C)**, SNAP25 **(D)**, synaptotagmin **(SYT; E, F)**, synapsin I **(G and H)**, VAMP2 **(I)**, PSD95 **(J)**, and homer **(k)** levels (n=4-7). Pink dots represent the females and blue dots represent the males. All data are expressed as mean ± SD; One-way ANOVA, followed by post-hoc Tukey's test.

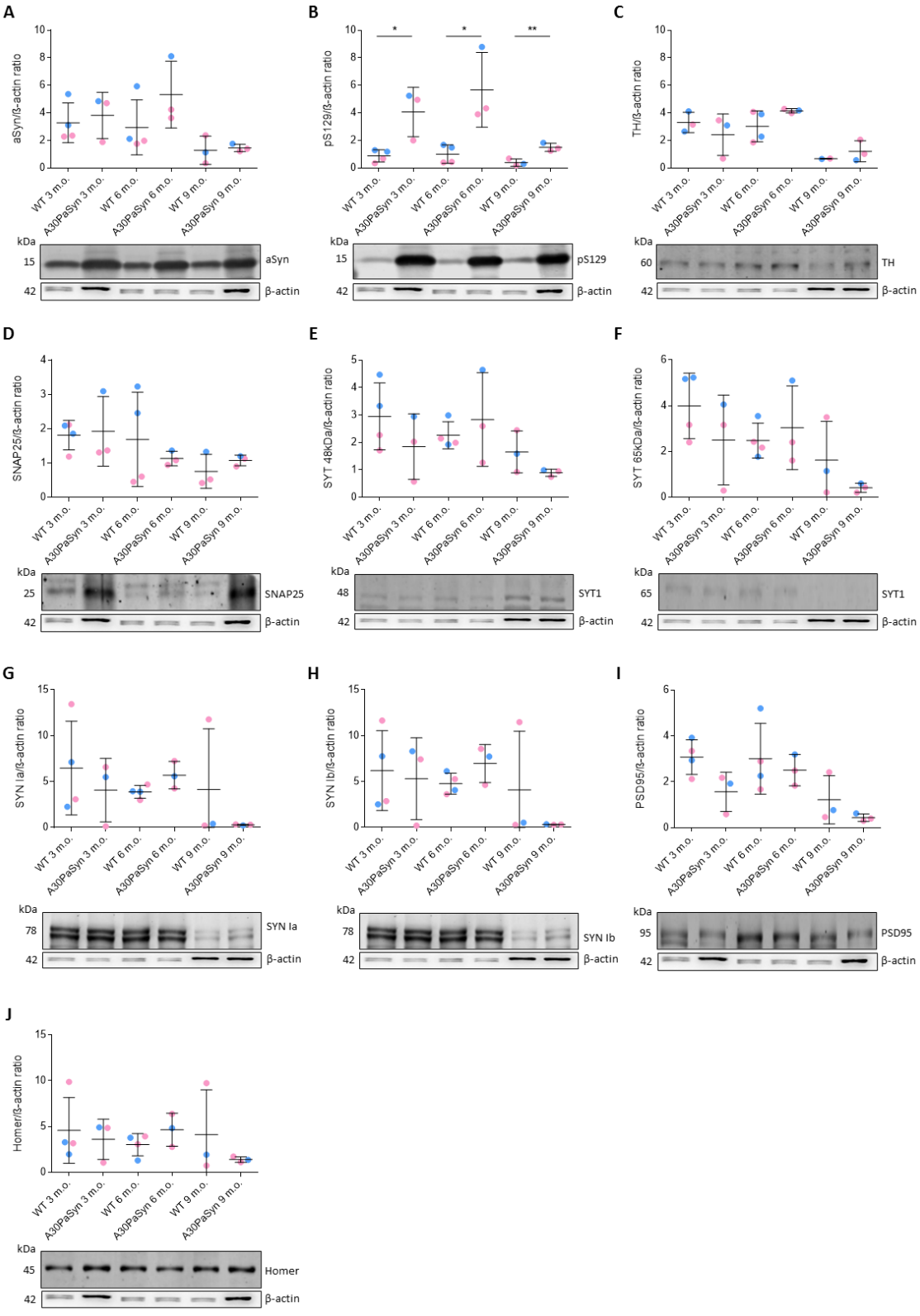

Figure 24 | Quantification of different proteins in the brain of 3, 6 and 9 months of age A30PaSyn mice. Quantification of aSyn **(A)**, pS129 **(B)**, TH **(C)**, SNAP25 **(D)**, synaptotagmin **(SYT; E, F)**, synapsin I **(G and H)**, PSD95 **(I)**, and homer **(J)** levels (n=4-7). Pink dots represent the females and blue dots represent the males. All data are expressed as mean ± SD; One-way ANOVA, followed by post-hoc Tukey's test.

CHAPTER 5

DISCUSSION AND CONCLUSIONS

After AD, PD is the second most common chronic neurodegenerative disease (de Lau and Breteler, 2006). It has been identified as a multifactorial disorder over the past few decades (Navarro-Sánchez et al., 2018; Rocha et al., 2018). Unfortunately, there is no cure for PD, although medicines, surgical treatments, and other therapies can often relieve some symptoms. A common treatment strategy involves medications that substitute dopamine or mimic its effect on dopamine receptors (Hayes, 2019; Lang and Espay, 2018; Milligan, 2019). However, current therapies are not capable of rescuing disease progression. The inefficiency of PD treatment is largely due to late diagnosis, which occurs after motor symptoms have appeared and brain degeneration is already advanced (Postuma et al., 2015). While current treatments can improve symptoms, they are not able to modify the course of the disease. More recently, the focus of PD research has shifted towards biochemical and molecular changes in early disease stages where interventions may have greatest impact (Ghiglieri et al., 2018; Schirinzi et al., 2016). Understanding the underlying mechanisms of the disease, particularly synaptic dysfunction is crucial for developing therapies that can halt or slow the progression of PD (Bae and Kim, 2017).

Some studies have linked synaptic dysfunction to the onset of nonmotor symptoms (Bae and Kim, 2017), while neurodegeneration and cell loss occur later in the disease (Milnerwood and Raymond, 2010; Picconi et al., 2012; Schulz-Schaeffer, 2010). Hence, synaptic dysfunction could be considered a precursor to axonal abnormalities, which eventually lead to neuronal soma degeneration (Bae and Kim, 2017; Calo et al., 2016; Fang et al., 2017; Grosch et al., 2016; Kouroupi et al., 2017; Lu et al., 2014; Morales et al., 2015; Roy, 2017; Schulz-Schaeffer, 2015; Scott et al., 2010; Tagliaferro and Burke, 2016). aSyn-rich LB inclusions are present in axonal processes and in neuronal cell bodies (Braak et al., 1999). Some studies described the presence of aSyn in axonal dystrophic neurites in PD (Duda et al., 2002) and DLB patients (Galvin et al., 1999). According to these studies, synucleinopathy appears to occur in presynaptic terminals and axons, which correlates with the primary localization of aSyn in these terminals (Lücking and Brice, 2000; Maroteaux et al., 1988; Yang et al., 2010). These early phenotypes, as synaptic impairments, can be attributed to the accumulation of toxic aSyn species within synapses, or the trapping of synaptic proteins by aSyn, ultimately leading to a process of dying back-like neurodegeneration (Fogarty, 2019; Li et al., 2003; Santos and Outeiro, 2020; Schirinzi et al., 2016; Spires-Jones and Hyman, 2014). Synaptic dyshomeostasis is seen as an early event in the pathogenesis of synucleinopathies in both human studies and animal models (Bellucci et al., 2016). In order to fully understand the aSyn impact on PD development, underlying synaptic changes in early stages need

to be studied. Therefore, in this study, we focused on aSyn-associated alterations at the synapse in cellular and mouse models of PD.

For this, we employed a primary neuronal culture model of synucleinopathy. *In vitro* models are useful to replicate important aspects of synucleionopathies, and primary neuronal cultures have been used for research on aSyn pathology progression (Lázaro et al., 2017).

These models use mostly recombinant aSyn species and investigate cell-to-cell transmission, revealing many pathways involved in synucleinopathies pathogenesis (Volpicelli-Daley et al., 2011). Recent studies showed a protocol using lower levels of recombinant aSyn species for long periods of incubation time reproducing some events of aSyn pathology, including disruption of cellular functions such as synaptic dysfunctions (Mahul-Mellier et al., 2020; Wu et al., 2019).

Based on this model, we chose to assess synaptic protein levels in primary neurons exposed to recombinant monomeric, oligomeric and PFFs aSyn and PBS as control. Similar to the previous model, we used the primary neurons but instead of exposing them to the recombinant protein, we transduced them with lentiviral vectors encoding for WT or mutant forms (A30P or A53T) of aSyn, for longer periods, for intracellular expression of aSyn.

As expected, an increase was observed in the aSyn levels after exposure to recombinant aSyn and after transduction (Figures 11 and 12). Then, to assess the impact of this increase in aSyn levels on the health of the cells over time, we used the ToxiLight assay. Interestingly, no cytotoxicity or cytolysis was observed in both neuronal cultures for all the conditions, as reflected by the no significant release of AK to the surrounding medium (Figure 13). These results indicate that changes in the levels of aSyn were not due to treatment-induced cell death. According to the literature, we were expecting to observe some neurotoxicity in a time-dependent manner (Mahul-Mellier et al., 2020; Volpicelli-Daley et al., 2011). The discrepancies in the results could be related to distinctions in aSyn experimental toxicity model (Wong and Krainc, 2017), the animal species used for the primary cultures, or slight variabilities in the production of recombinant aSyn species, that could generate strain discrepancies with differences in structure, seeding, propagation, and toxicity (Guo et al., 2013). It is important to note that different models have been used to investigate the mechanisms underlying synucleinopathies, and each of these models has its own advantages and disadvantages (Lázaro et al., 2017).

aSyn has been shown to play a crucial role in various aspects of synaptic function. For example, studies have demonstrated that aSyn is involved in the regulation of SVs trafficking, SNARE exocytosis, and neurotransmission release (Abeliovich et al., 2000; Nemani et al., 2010; Spillantini and Goedert, 2000). Moreover, aSyn has been found to be incorporated in exosomes and SVs as well as secreted into the extracellular compartment, further implicating in the regulation of synaptic function (El-Agnaf et al., 2003; Emmanouilidou et al., 2010). Due to the high affinity of aSyn to membranes of high curvature, some species of this protein, monomers and oligomers, are incorporated into vesicles, leading to disturbances in pathways related to vesicles (Credle et al., 2015; Lee et al., 2005; Ouberai et al., 2013). Indeed, it has been demonstrated *in vitro* that large aSyn oligomers can bind to the N-terminal of Vamp2 (Choi et al., 2013, 2018), and it has been proven the co-localization between aSyn and SNAP-25, VAMP2 and syntaxin-1a, some proteins of the SNARE complex, in cortical primary neurons (Almandoz-Gil et al., 2018). It is essential for synapses that the homeostatic mechanisms leading to neuronal plasticity that can interfere with neuronal networks are maintained. The susceptibility to synaptic damage can increase if minor synaptic imbalances occur. Given that aSyn is involved in neuronal plasticity, the functional consequences of its increased expression were explored at both presynaptic and postsynaptic levels.

We have found that the increase in the levels of aSyn does not alter the levels of SNAP25 and PSD95 evaluated in our conditions (Figure 14). However, the literature reports inconsistencies in the effect of aSyn on the expression and function of synaptic and axonal proteins (Chu et al., 2012; Dijkstra et al., 2015; Lim et al., 2011; Prots et al., 2018; Rockenstein et al., 2014; Scott and Roy, 2012; Volpicelli-Daley et al., 2011). These inconsistencies are mostly linked to different approaches (Cardinale et al., 2021). For example, Volpicelli-Daley and colleagues reported a loss of VAMP2 and SNAP25 in primary neuronal cultures exposed to preformed aSyn fibrils (Volpicelli-Daley et al., 2011). However, a systematic review of 18 studies found that SNAP-25 expression levels were not significantly affected in half of the studies and reduced in six studies (Murphy and McKernan, 2022). Also, in another study, the levels of SNAP-25 and syntaxin remained unchanged (Lim et al., 2010). Similarly, some studies reported reduced levels of PSD95 in the post-mortem tissue of PD patients (Kramer and Schulz-Schaeffer, 2007), and in Tg mice overexpressing the A53T mutation of aSyn (Lim et al., 2010). However, another group showed no change in the levels of this protein in Tg mice (Wihan et al., 2019). These inconsistencies in the effects of aSyn at both pre- and post-synaptic sites, make it more difficult to identify or predict a pattern of events that occur throughout the brain. Therefore, it is important to continue studying the effects of aSyn on synaptic

proteins in different experimental models and conditions to better understand its role in synapse function and dysfunction.

To investigate further, since no changes were observed in the synaptic markers, we evaluated the dendritic length of neurons exposed to increased levels of aSyn. Dendritic pathology can undergo significant changes in many neurodevelopmental, neurodegenerative, and psychiatric disorders (Cook and Wellman, 2004; Dierssen and Ramakers, 2006; Moolman et al., 2004; Radley et al., 2004; Sousa et al., 2000; Teskey et al., 2006; Villalba and Smith, 2010; Yamada et al., 1988). However, the exact cause of dendrite pathology in these diseases is still unclear. At early stages of the disease, the dendrite pathology differs phenotypically from the dendrite degeneration observed in dying neurons. As neurons die, they exhibit blebbing/beading dendrites, followed by extensive dendritic cleavages, accompanied by rapid disruption of dendritic cytoskeletons (Park et al., 1996). On the contrary, the dendrites affected at the early stage by neurodegenerative diseases tend to undergo slow and progressive modifications, or maintain these alterations for some time (Lee et al., 2011). Some studies have observed reduced dendritic length and complexity in neurons overexpressing WTaSyn and A53TaSyn (Czaniecki et al., 2019; Winner et al., 2012). A decrease in dendritic length was also reported in cortical neurons in DLB, medium spiny neurons of the striatum in PD and in dopaminergic neurons of the SN (Kramer and Schulz-Schaeffer, 2007; Patt et al., 1991; Zaja-Milatovic et al., 2005). In primary cultured hippocampal neurons (Dächsel et al., 2010) and newly generated DG neurons (Sepulveda et al., 2013; Winner et al., 2011b) in the LRRK2-mutant mice, the dendritic length and branching were also reduced. However, a previous study in MPTP-lesioned mice showed that the dendritic complexity in some hippocampal subregions was not altered (Weerasinghe-Mudiyanselage et al., 2021). Based on previous literature, we hypothesized that aSyn overexpression would cause a reduction of dendrite length. In contrast, we did not find any significant difference in both apical or basal dendritic length when compared to controls (Figure 15). Therefore, further studies are needed to confirm the possible effects of aSyn pathology on dendritic pathology in the hippocampus.

After the administration of recombinant aSyn to neural networks, we recorded spontaneous electrophysical activity. Individual neurons were still intact and functioning, confirmed by the large number of electrodes that remained active, whose action potential shapes and amplitudes were unaffected. This was also supported by cell death and metabolic assays. The burst rate, the bursts/minute, the burst duration, and the inter-burst frequency did not change, meaning that aSyn administration was not able to

change the burstiness (Figure 16). Neuronal bursting plays a significantly important role in neuronal communication. Bursting neurons are particularly important for the generation of motor patterns and synchronization (Hassink et al., 2018). However, there was an increase in the total number of spikes in bursty cells, as well, as an increase in mean firing rate (Figure 16), indicating that there is an increase of single spikes between bursts. This is also corroborated by the increase in time between the burst (inter-burst-interval). Altogether, our results suggest that, after exposure to aSyn species, the firing of cultured neurons is more irregular, highlighting the potential of aSyn in regulating significant aspects of spontaneous neuronal activity and coordination of neuronal communication.

As mentioned above, aSyn interacts with SVs and participates in vesicle aggregation, initiation, fusion and recycling (assembly and disassembly of SNARE complex) (George et al., 1995; Jensen et al., 1998; Lashuel et al., 2013; Maroteaux et al., 1988). aSyn binds to the plasma membrane through the N terminus and simultaneously interacts with VAMP2 through the C terminus, creating cross-bridges between the plasma membrane and the vesicles to facilitate vesicle docking (Burré et al., 2010; Lou et al., 2017). Recent studies report that VAMP2 and synapsin interact to promote SVs clustering and regulate SV recycling (Atias et al., 2019; Sun et al., 2019). Nonetheless, other research showed that aSyn's overexpression reduces the release of neurotransmitters by breaking the SVs docking in exocytosis (Larsen et al., 2006). Moreover, some studies do not support the opinion that aSyn facilitates the assembly of the SNARE complex. Darios and colleagues showed that there is no direct interaction between aSyn and SNARE proteins (Darios et al., 2010). Further investigation is needed to understand the mechanisms behind aSyn's divergent roles in the assembly of SNARE complex and exocytosis and the interacting partners.

To better understand this, we isolated crude SVs from WTaSyn mice and non-transgenic mice by a linear gradient centrifugation protocol and we observed the successful isolation of SVs with a uniform round sphere morphology (Figure 17). Then, the SVs were co-immunoprecipitated against aSyn to study the interacting partners of aSyn in these SVs. These interacting partners were identified by mass spectrometry (Figures 18,19 and 20). Overall, these findings suggest that proteins that interact with aSyn in SVs of WT mice are proteins that may be associated with translation and proteasomal protein catabolic processes, and may be located in cytoplasm and synapse. On the other hand, proteins that interact with aSyn in Tg mice’s SVs may be associated with intermediate filament organization and heterochromatin assembly and may be located in cytoplasm and

nucleosome. Collectively, these data indicate that aSyn overexpression-driven changes in the organization of SVs in the brain of Tg animals.

To induce robust cell loss in PD mouse models, most use toxins such as 6-hydroxydopamine (6-OHDA), MPTP, rotenone, and paraquat (Betarbet et al. (2000); Meredith and Rademacher 2011; Manning-Bog et al. 2002; Ungerstedt 1968) or introduce aSyn species in different brain regions (Luk et al. 2012; Mason et al. 2016; Paumier et al. 2015). Although these models replicate many of the phenotypic characteristics of PD, their major disadvantage is that they do not mimic the chronic progressive course of the disease. On the other hand, transgenic mouse models overexpressing high levels of human WT aSyn or mutant aSyn forms develop gradually neuropathology and phenotype with middle-age onset (Masliah et al. 2000; Kahle et al. 2000; Giasson et al. 2002), which more closely resemble the chronic progression of PD.

The Tg mouse model overexpressing human WT aSyn under the Thy1 promoter is one of the most largely characterized animal models of PD and reproduces several features of the sporadic disease (Chesselet et al., 2012; Rockenstein et al., 2002). Particularly, these Tg mice develop progressive changes in the striatum and release of dopamine, molecular and biochemical pathways, aSyn pathology, and motor and nonmotor impairments. However, they lack the loss of *substantia nigra* dopaminergic neurons, a hallmark of PD, nevertheless, they have a progressive loss of these cells' terminals. At 1 month of age, small punctate aggregates are abundant in the olfactory bulb and dorsal nucleus of the vagus, whereas bigger aggregates are found in the dorsal lateral *geniculate nucleus, locus coeruleus, thalamus*, and *cerebellum* (Chesselet et al., 2012). Fine motor skills are impaired at 3 months (Rabl et al., 2017). Later, at 4 to 6 months of age, mild cognitive dysfunction can be observed in WTaSyn mice, and its presence may expect later development of more severe cognitive deficits (Janvin et al., 2005). At 5 months of age, aggregation of proteinase K-resistant aggregates of variable sizes are found in the *substantia nigra* (Chesselet et al., 2012). In contrast, these aggregates are not present in the cerebral cortex. In older mice, aggregates increase in size and number, a pattern that is consistent across ages. Before 14 months of age, the general health of WTaSyn mice is similar to those of WT mice (Chesselet et al., 2012). Robust motor deficits appear early, but parkinsonian-like motor impairment only develops around 14 months, with a robust loss of dopamine in the striatum.

Human mutant A30P aSyn-expressing mice have been widely characterized phenotypically and neuropathologically. This model expresses the mutant form of aSyn, A30P, under the Thy-1 promoter and presents a two-fold increase in the aSyn expression

relative to the endogenous levels (Kahle et al. 2000). Moreover, accumulation of the protein is observed in both soma and neurites of neurons in SN, neocortex, cerebellum, and brainstem (Kahle et al. 2000). At 2 months of age, mice show fine motor impairments and hyperactivity that progressively deteriorate with age (Ekmark-Lewén et al. 2018; Freichel et al. 2007). Later, at 8 m.o., misfolded phosphorylated aSyn inclusions start appearing, together with locomotor impairments and reduced TH levels (Ekmark-Lewén et al., 2018; Neumann et al., 2002). By 12 months, mice display severe locomotor deficits and cognitive impairment (Freichel et al. 2007). In sum, this model approximates to age-dependent neuropathology of synucleinopathies and cognitive declines reproducing DLB.

The WTaSyn and A30P mouse is broadly studied, however, the studies about the biological processes linked to the synapses are still minimum in these models. Here we analysed the hippocampal protein content by immunoblotting at 3, 6 and 9 m.o Tg mice and littermate controls. Until the age of overt dopamine depletion, at 14 m.o., WTaSyn mice represent a model of preclinical PD, notably useful to study early neurodegenerative processes and therapeutic interventions. Based on the characterisation of the A30PaSyn mouse model, at 3 and 6 m.o, aSyn aggregates are still not formed and at 9 m.o these aggregates have already started to appear and neuronal function is altered while neurons and synapses are still intact. By assessing neurons known to degenerate from aSyn increase before they die, it is possible to identify the processes that cause these losses to occur.

We assessed the levels of aSyn and unexpectedly, no significant differences were observed between the Tg animals and the non-Tg animals (Figures 23A and 24A). However, a slight trend towards increase was observed in the Tg mice when compared to the age-matched WT controls. According to previous studies, we expected a moderate 1.5-2-fold increase in the WTaSyn mice when compared to WT mice (Chesselet et al., 2012; Rockenstein et al., 2002). These differences in the results could be related to the approach used to estimate the aSyn levels that were different to the one used in the mentioned studies. There are several approaches that can be used, however, none is completely reliable, because the protein can take on various conformations that can react differently with antibodies. Moreover, changes in the conformation can also affect the migration of aSyn in the gel, leading to differences in the protein readout. Therefore, it is impossible to be sure that all transgenic protein was detected. On the other hand, high levels of pS129 were significantly observed in Tg animals already since 3 m.o (Figures 23A and 24A). These results were consistent with the data from Chesselet and her

colleagues (Chesselet et al., 2012). Next, the TH levels were assessed and Tg mice did not show any reduction in the number of this enzyme (Figures 23C and 24C). This enzyme is a rate-limiting enzyme for dopamine synthesis, in the *substantia nigra pars compacta*, and a decrease in its levels indicate a compromised dopaminergic function. The results were consistent with other studies where they report no changes in TH levels up to 14 m.o WTaSyn mice and 11 m.o A30PaSyn mice (Behere et al., 2021; Chesselet et al., 2012; Neumann et al., 2002). Finally, several presynaptic markers as markers of SVs (VAMP2, SYT and SYN I) and SNARE complex (SNAP25), and postsynaptic markers as PSD95 and Homer were assessed in both mouse models. There were no differences, neither in the levels of presynaptic markers nor in the levels of postsynaptic markers, between Tg mice and control mice (Figures 23D-K and 24D-J), indicating no loss in synaptic structures. To our best knowledge, this study is the first attempt to assess synaptic markers in the A30PaSyn mouse model hippocampus, contrary to the WTaSyn mouse model. There is one study where the authors assessed the levels of synaptophysin, PSD95 and SNAP25 in the WTaSyn mouse model, where a decrease of synaptophysin and PSD95 levels were observed, contrary to no changes in SNAP25 levels (Coulombe et al., 2018). The contradicting result could be due to the different brain regions used.

After evaluating the levels of aSyn and other biomarkers in the brain, we decided to investigate the levels of aSyn in the PNS. aSyn has been examined extensively as a biomarker in PD because of its involvement in PD pathogenesis and its presence in different body fluids. The distribution of aSyn species in biofluids may provide unique opportunities to develop non-invasive diagnostic and prognostic tools based on measuring individual or multiple aSyn species levels that reflect the pathology or progression of the disease (Fayyad et al., 2019; Ganguly et al., 2021; Parnetti et al., 2019). Moreover, aSyn is promptly secreted into extracellular spaces and can be found in different forms in body fluids such as CSF (Eusebi et al., 2017; Foulds et al., 2012; Hong et al., 2010; Mollenhauer et al., 2011, 2013; Shi et al., 2010; Tokuda et al., 2006, 2010; Wang et al., 2012b), blood components (Abd-Elhadi et al., 2015; Barbour et al., 2008; Bryk and Wiśniewski, 2017; Cariulo et al., 2019; Foulds et al., 2011, 2013; Gorostidi et al., 2012; Ishii et al., 2015; Klatt et al., 2020; Koehler et al., 2015; Shi et al., 2014), saliva (Devic et al., 2011; Kang et al., 2016; Shaheen et al., 2020; Vivacqua et al., 2019) and tears (Hamm-Alvarez et al., 2018; Maass et al., 2020) as well as in peripheral tissues (e.g., skin, esophagus, colon) (Chung et al., 2016; Fenyi et al., 2019; Tanei et al., 2021; Wang et al., 2021), and it is transported bi-directionally between PNS and CNS (Sui et al., 2014). This makes it even more challenging to interpret and correlate

the alterations of aSyn levels in body fluids with brain PD pathology. Moreover, since this protein can also exist in different forms (i.e., total, oligomeric/ aggregated) and can be translationally modified, the complexity of the measurement of this protein is increased and there is not yet a consensus on which form or forms constitute reliable biomarker(s) for early diagnosis or following disease progression.

To address the challenges in studying aSyn, we examined its levels in various bodily fluids including CSF, serum, plasma, whole blood and saliva from WTaSyn and A30PaSyn mice and control mice. Our findings showed no significant differences in the CSF aSyn levels at 3 m.o, 6 m.o and 9 m.o Tg mice when compared to age-matched controls (Figure 22A and F). However, previous studies on aSyn levels in CSF have produced inconsistent results, with some reporting a lower level of total aSyn in CSF of PD patients while other studies did not find any significant difference between PD and control groups (van Dijk et al., 2013; Hall et al., 2012; Hansson et al., 2014; Hong et al., 2010; Mollenhauer et al., 2008, 2011, 2013; Öhrfelt et al., 2009; Park et al., 2011; Parnetti et al., 2011; Shi et al., 2011; Tokuda et al., 2006; Toledo et al., 2013; Wennström et al., 2013). Moreover, the specificity of the CSF aSyn measurement was low in most studies (Aerts et al., 2012; Chahine et al., 2020; L. et al., 2015; Mollenhauer et al., 2011, 2013; Zhou et al., 2015). Also, there was any correlation between total aSyn levels and disease progression/severity (Aerts et al., 2012; Eusebi et al., 2017; Foulds et al., 2012; Hong et al., 2010; L. et al., 2015; Mollenhauer et al., 2011, 2013; Shi et al., 2010; Tokuda et al., 2006, 2010; Wang et al., 2012b; Xiong et al., 2015).

We also investigated the levels of aSyn in blood and saliva, since there are a lot of technical and ethical issues related to CSF collection. The easy accessibility and the collection in a non-invasive manner of blood and saliva make them a good alternative to the conventional use of CSF (Fayyad et al., 2019).

Serum aSyn levels showed only a slight trend to increase in the 3, 6 and 9 m.o WTaSyn mice, when compared to age-matched controls (Figure B). Moreover, plasma aSyn levels do not show any significant differences in WTaSyn mice (Figure 22C). Further, A30PaSyn mice show a significant increase in serum and plasma aSyn levels at the three-time points analysed compared to WT mice (Figure 22G and H). This is consistent with the study by Chang and colleagues that reported increased levels of aSyn in PD plasma and serum, and the serum levels were correlated with disease severity (Chang et al., 2020). The increase in plasma levels of aSyn in PD patients was also observed in other studies, although the correlation between this parameter and motor disability varied (Ding et al., 2017; Lee et al., 2006; Lin et al., 2017; Wang et al., 2020). Nevertheless,

several other studies observed a decrease in the levels of aSyn in PD patients' plasma or failed to observe any significant difference in this parameter (Li et al., 2007; Shi et al., 2010). These inconsistent results for the changes in the aSyn level in the blood of PD patients could be due to technical reasons such as contamination from erythrocyte hemolysis, different approaches of sample collection, use of different assay techniques, and the differential ability of antibodies to bind to different forms of aSyn (Lee et al., 2006; Li et al., 2007; Lin et al., 2017). Overall, the potential of aSyn as a blood biomarker for PD remains unclear and it is still unclear if it can be used in clinical practice. Although the studies conducted so far have shown promising results, further research is still necessary.

The final biofluid analysed was saliva, where a significant increase in the levels of aSyn was observed at 3 m.o WTaSyn mice when compared to control mice (Figure 22E). The levels of aSyn got back to physiological levels at 6 m.o. and kept similar levels until 9 m.o. Furthermore, in A30PaSyn mice, the saliva aSyn levels were not significantly altered (Figure 22J). Some studies reported a decrease in the total levels of aSyn in the saliva of PD patients (Al-Nimer et al., 2014; Devic et al., 2011; Vivacqua et al., 2016). Contrary to these studies, Kang and colleagues did not observe any significant difference between both groups (Kang et al., 2016). In conclusion, it is currently unclear the implications of studies on aSyn as a saliva biomarker for PD are, as the research is still in the early stages. However, due to the non-invasive nature of the saliva collection and the potential of aSyn as a biomarker make it a valuable area for further research. Despite this, replicating and validating many of these studies remains challenging. There are various reasons for this, including inconsistencies among different studies, small sample sizes, patients with different degrees of disease severity and onset, the efficacy of the antibody used to detect a range of aSyn species or specific modified or aggregated forms of the protein, variations in biomarkers measurement techniques and protocols, and variations in sample storage methods. Overcoming these limitations and using a combination of biomarkers can improve diagnostic accuracy and contribute to predicting motor progression or cognitive impairment of PD. To obtain the most accurate results, novel biomarkers should be combined with existing clinical predictors rather than simply replacing clinical assessment with a biomarker alone.

Overall, this study provides insights into the early events in the pathogenesis of PD and despite the increase of aSyn levels in the used PD models, no major significant changes were observed in the cytotoxicity, neuronal morphology, biochemical analyses and functional analyses. Moreover, the analyses of aSyn levels in PNS or other proteins in

the brain do not show robust results. Altogether, these results challenge the dogma that aSyn toxicity is the primary driver of PD and suggest that increased levels of aSyn in PD models do not necessarily lead to an increase in toxicity. This hints that aSyn pathology alone is not sufficient to cause neurodegeneration and the development of PD symptoms, reinforcing the fact that PD is a multifactorial disorder.

In line with this evidence, several studies have shown that overexpression of aSyn in rodents does not necessarily lead to the degeneration of dopaminergic neurons or the development of PD-like symptoms (Decressac et al., 2012; Watson et al., 2012). Moreover, some studies have reported that knocking out or reducing aSyn expression in mice does not have a significant impact on dopaminergic neuron survival (Abeliovich et al., 2000; Cabin et al., 2002). In addition to animal studies, there are also several human studies that support the fact that aSyn toxicity is not the sole driver of PD pathology. A recent study found that patients with incidental Lewy body disease had similar levels of aSyn pathology as PD patients, but significantly less neurodegeneration (Frigerio et al., 2011). Another study found that approximately 10% of individuals over 80 years old had LBs in their brains, but only some of these individuals developed clinical PD. This suggests that maybe LBs are not always associated with the development of PD symptoms.

In conclusion, while aSyn pathology is undoubtedly an important feature of PD, there is still a debate, about whether aSyn loses function or gains cytotoxic function, and what are the consequences. These findings have important implications for the development of therapies targeting aSyn and suggest that a more comprehensive understanding of PD pathology is necessary to develop effective treatments for this devastating disease.

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