Characterization of the molecular interaction between α-synuclein and prion protein

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AFFIRMATION

I hereby declare that the doctoral thesis entitled "Characterization of the molecular interaction between α-synuclein and prion protein" was written independently and with no other sources and aids than quoted.

Göttingen, March 2023 *Renato Garcia Domingues*

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this, but always gave inspiration, especially when times get hard, I know you got my back somehow and somewhere, and a lot of this is for you.

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LIST OF ABBREVIATIONS

AD - Alzheimer’s disease

AEC - Anion exchange high-performance liquid-chromatography

ALP - Autophagy-lysosomal pathway

ALS - Amyotrophic lateral sclerosis

aSyn - α-synuclein

ATP - Adenosine triphosphate

aβ - Amyloid-β

BiFC - Biomolecular fluorescence complementation

BSA - Bovine serum albumin fraction V

CC - Charged clusters

CJD - Creutzfeldt-Jakob disease

CNS - Central nervous system

DAPI - 4′,6-diamidino-2-phenylindole

DLB - Dementia with Lewy bodies

DMEM - Dulbecco's modified eagle medium

EDC - 1, ethyl-3-(3-dimethylaminopropyl) carbodiimide

EDTA - Ethylenediaminetetraacetic acid

ER - Endoplasmic reticulum

FBS - Fetal bovine serum

FTLB - Frontotemporal lobar degeneration

GCI - Glial cytoplasmic inclusions

GFP - Green fluorescent protein

GPI - Glycosylphosphatidylinositol

GuHCl - Guanidine hydrochloride

HD - Hydrophobic domain

HEK293 - Human embryonic kidney cells

HS - Heparan sulfate

HSP73 - Heat-shock protein 73

HSQC - Heteronuclear single quantum coherence

ICC - Immunocytochemistry

IPTG - Isopropyl-1-thio-β-D-galactopyranoside

K_a - Association constant

K_d - Dissociation constant

K_D - Equilibrium constant

KDN - Knock-down

KO - Knock-out

LAG3 - Lymphocyte-activation gene 3

LAMP1/2 - Lysosomal-associated membrane protein 1/2

LBs - Lewy bodies

LB - Lysogeny broth

LN - Lewy Neurites

mGluR5 - Metabotropic glutamate receptor 5

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

MSA - Multiple system atrophy

MWΔCS - Mean weighted chemical shifts displacements

NAC - Non-amyloid-β

NKA - Na^+ /K^+ -ATPase

NMR - Nuclear magnetic resonance

O/E - Overexpression

OD - Optical density

OR - Octarepeat region

PAGE - Polyacrylamide gel electrophoresis

PBS - Phosphate-buffered saline

Pen/Strep - Penicillin-streptomycin

PFA - Paraformaldehyde

PFFs - Pre-formed fibrils

PMSF - Phenylmethylsulfonyl fluoride

PNS - Peripheral nervous system

ppm - parts per million

PrP^{C} - Cellular prion protein

PrP^{Sc} - Scrapie prion protein

PTEN - Phosphatase and tensin homolog

PTM - Post-translational modification

REM - Rapid-eye movement

PD - Parkinson's disease

ROS - Reactive oxygen species

rpm - rotation per minute

RT - Room temperature

RT-QuIC - Real-time quaking-induced conversion

SDS - Sodium-Dodecyl-Sulphate

SMA - Spinal Muscular Atrophy

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

SOD1 - Superoxide dismutase 1

TBS - Tris-buffered saline

ThT - Thioflavin T

UPS - Ubiquitin–proteasome system

VAMP2 - Vesicle associated membrane protein 2

WT - Wild-type

ABSTRACT

The interaction between α-synuclein (aSyn) and prion protein (PrP) has been implicated in the pathogenesis of synucleinopathies. However, the nature of this interaction remains poorly understood. Here, we employed a combination of biophysical and cellular assays to characterize the interaction between aSyn and PrP. Our results demonstrate that the C-terminal region of aSyn interacts with PrP in an orientation-specific manner through electrostatic interactions, and suggest that tryptophan residues on PrP may also bind to the tyrosine residues of aSyn through aromatic interactions. We further demonstrate that PrP potentiates seeded aSyn aggregation kinetics, as evidenced by an increase in monomer incorporation rate in the real-time quaking-induced conversion assay. Additionally, we observed colocalization between PrP and phosphorylated aSyn, suggesting a possible role of PrP in aSyn phosphorylation. Our findings provide valuable insights into the nature of the aSyn-PrP interaction and highlight potential therapeutic targets for synucleinopathies. In particular, targeting the C-terminal of aSyn or the N-terminal of PrP could be a promising strategy for preventing or reversing the effects of aSyn aggregation and spreading. Future studies could investigate the role of post-translational modifications of aSyn and the conformational states of aSyn in the interaction with PrP, as well as the effect of aSyn aggregates formed via PrP-aSyn interaction on cellular toxicity and spreading.

CHAPTER 1

Introduction

1.1. POPULATION AGING AND NEURODEGENERATIVE DISEASES

1.1.1. Synucleinopathies

With the increasing life expectancy and aging of the global population, a growing risk of age-related diseases follows, most notably cancer, inflammatory diseases, and neurodegenerative diseases (Jaul and Barron, 2017).

Aging is the leading risk factor for the development of neurodegenerative disorders. Furthermore, it is projected that in the next 30 years, the current 9.6 population over 65 will almost double, reaching 17%, forecasting a rise in neurodegenerative disease cases (Zheng and Chen, 2022). These worrying predictions may cause not only difficulties for millions of families, but also cause a great economic burden for countries experiencing an aging population, such as the United States of America, where Parkinson's disease (PD), Alzheimer's disease (AD), amyotrophic lateral sclerosis (ALS), and spinal muscular atrophy (SMA) had a combined expense of 655 billion dollars in 2020 ('2021 Alzheimer's disease facts and figures', 2021).

Synucleinopathies are a variety of neurodegenerative disorders characterized by the progressive aggregation and intracytoplasmic accumulation of α-synuclein (aSyn) in multiple cells from the nervous tissue, such as neurons and glial cells. Within this classification, we can find diseases such as PD, multiple system atrophy (MSA), and dementia with Lewy bodies (DLB) (Brás *et al.*, 2020). Within these pathologies, PD is the most prevalent one, being the second most common neurodegenerative disease and affecting 4% of the population aged 80 or above (Lee and Gilbert, 2016; Ou *et al.*, 2021).

Different synucleinopathies may share common features, but they differ in the symptoms progressions, main brain region affected, and disease progression after onset (Kahle, 2008). DLB displays cognitive decline, hallucinations, rapid-eye movement (REM) sleep disorders, and dementia before motor symptoms, having the neocortex as the central affected region (Mayo and Bordelon, 2014). In contrast, in MSA, the accumulations are primarily found in glial cytoplasmic inclusions (GCI), leading to glial degeneration mainly in the striatonigral and olivopontocerebellar regions, showing symptoms such as motor weakness, cognitive decline, and autonomic failure (Yoshida, 2011). It is still unclear what causes the differences between synucleinopathies; however, it is hypothesized that different strains of aSyn may cause the differences found in these diseases.

1.1.2. Parkinson's disease

James Parkinson first described PD in 1817 in "An essay on the shaking palsy" based on his observations of the motor clinical manifestations of the disease (Parkinson, 2002).

The main brain region affected in PD is the *substantia nigra pars compacta*, located in the midbrain (Davie, 2008). This region is highly enriched with dopaminergic neurons (Hajós and Greenfield, 1994), playing a role in the modulation of motor movement and reward function (Schultz, 1992; Pioli *et al.*, 2008). The progressive loss of dopaminergic neurons causes a reduction of striatal dopamine (Scherman *et al.*, 1989), resulting in motor symptoms such as tremors, bradykinesia, and rigidity; and non-motor symptoms such as depression, anxiety, personality changes, and sleep behavior disorders (Schrag *et al.*, 2015; Kozlovski *et al.*, 2019; Postuma *et al.*, 2019; Oliveira *et al.*, 2021).

In similarity with the other synucleinopathies, PD is characterized by the presence of intracellular inclusions containing aSyn, commonly known as Lewy bodies (LBs) and Lewy neurites (LN) (Spillantini *et al.*, 1997, 1998; Baba *et al.*, 1998) however this is not a determinant for the development of the disorder (Gaig *et al.*, 2007).

PD can appear sporadically (80% of the cases), where the cause of the disease is unknown and associated with aging (Savica *et al.*, 2013; Reeve, Simcox and Turnbull, 2014) and environmental factors (William Langston *et al.*, 1983); or familial/inherited, in which case the condition is linked to specific genetic mutations. Genes that are involved in the pathology are recognized as *PARK* genes (Benson and Huntley, 2019). Within these, the *SNCA* gene, the gene encoding aSyn, was the first one to be identified, where its duplication and triplication can hasten the onset of the pathology (Singleton *et al.*, 2003) and multiple point mutations, most notably in its N-terminal (L8I, A18T, A29S, A30P, A30G, E46K, H50Q, G51D, A53E, A53T, A53V, E57D, K58N, E83Q and P117S) (Polymeropoulos *et al.*, 1997a; Krüger *et al.*, 1998; Zarranz *et al.*, 2004a; Appel-Cresswell *et al.*, 2013; Proukakis *et al.*, 2013; Hoffman-Zacharska *et al.*, 2013; Kiely *et al.*, 2013; Lesage *et al.*, 2013; Pasanen *et al.*, 2014; Fujioka *et al.*, 2014; Ghosh *et al.*, 2014; Yoshino *et al.*, 2017; Chen *et al.*, 2020; Kapasi *et al.*, 2020; Liu *et al.*, 2021; Guo *et al.*, 2021)have been linked to not only earlier development of the disease, but also increased severity (Polymeropoulos *et al.*, 1997b; Polymeropoulos, 1998; Guan *et al.*, 2020). Other genes associated are the *PARK7* gene, encoding DJ-1 (Goldberg *et al.*, 2005), *PRKN* (Truban *et al.*, 2017), and *LRRK2* (Gaig *et al.*, 2014), which have functions ranging from chaperon activity to phosphorylation.

PD diagnosis focuses on the patient's medical history and present symptoms, with imaging techniques such as dopamine transporter single-photon emission computer tomography scans (Bajaj, Hauser and Grachev, 2013) and magnetic resonance imaging (Burciu *et al.*, 2017) as additional tools to further verify or clarify the diagnosis.

The causes of neuronal cell death and the nature of the pathology still need further characterization, and there is no known way of halting the progression of the disease. Most available therapeutic options aim to help relieve the symptoms with drugs such as dopamine replacements (namely levodopa), dopamine agonists, and monoamine oxidase-B inhibitors, and they depend on the set of symptoms and progression shown (Gray *et al.*, 2014). In addition to these drugs, both rehabilitative therapies and deep brain stimulation can also be used to aid with the management of the symptoms (Bratsos, Karponis and Saleh, 2018).

1.2. α-Synuclein

1.2.1. α-Synuclein: Function and Structure

aSyn is an intracellular protein encoded in the *SNCA* gene, located at the position 21 of the chromosome 4, and is a main player in synucleinopathies (Polymeropoulos *et al.*, 1997b). Synuclein gained its name due to its cellular location – "syn" from the synapse and "nuclein" from the nucleus, and in addition to aSyn, there are currently two isoforms known: β and γ synuclein (Maroteaux, Campanelli and Scheller, 1988; Clayton and George, 1998; Xing *et al.*, 1998).

aSyn comprises 140 amino acids, and is abundantly expressed in neurons (Murphy *et al.*, 2000). Although its function is yet to be further described, its cellular localization and knock-out (KO)/knock-down (KDN) or overexpression(O/E) experiments gave insights into its multiple roles. Most notably, aSyn is known to be a regulatory protein in neurotransmitter release, synaptic function, plasticity, and regulation of synaptic vesicles in the pre-synaptic terminals (Burré *et al.*, 2010; Marques and Outeiro, 2012; Burré, 2015; Brás *et al.*, 2020; Domingues *et al.*, 2022), while in the nucleus aSyn is assumed to have an impact on transcriptional regulation and regulation of histone acetylation (Goers *et al.*, 2003; Kontopoulos, Parvin and Feany, 2006). Furthermore, one-third of aSyn is found in a membrane-bound state (Visanji *et al.*, 2011) in a folded form that connects its N-terminal with the central region, maintaining the C-terminal unstructured (Jao *et al.*, 2008; Runfola *et al.*, 2020).

aSyn-KO mice have demonstrated synaptic vesicle modifications, decreased striatal dopamine and neurotransmission acceleration, and inhibition of the soluble N-ethylmaleimide-sensitive factor attachment protein receptors (SNARE) complex assembly, a modulator of exocytosis (Burré *et al.*, 2010; Butler, Sambo and Khoshbouei, 2017). Double and triple KO of the other members of the synuclein family showed decreased dopamine levels, alterations in pre-synaptic proteins, and higher lethality. In addition, it was revealed that neurons depleted of aSyn have impaired docked and reserve pool of synaptic vesicles, and defective replenishment of the synaptic vesicles (Cabin *et al.*, 2002). These results were corroborated using antisense oligonucleotides, which showed a reduction in the synaptic vesicle pool reserves in neurons (Murphy *et al.*, 2000).

On the other hand, the O/E of aSyn in mice and primary neurons induces a decrease in vesicle release, their recycling after endocytosis and causes mislocalization of SNARE proteins, leading to the sequestration of aSyn aggregates on SNAREs, such as synaptobrevin-2 causing a blockage on vesicle docking (Garcia-Reitböck *et al.*, 2010; Nemani *et al.*, 2010; Choi *et al.*, 2013). The excess of aSyn also reduces the dopamine reuptake via dopamine active transporter and production via tyrosine hydroxylase, causing impairment in neurotransmitter transport (Sisk *et al.*, 2000; Hansen *et al.*, 2013). Moreover, in cell models, O/E of aSyn reduces the pool of vesicles and inhibits its priming, ultimately inhibiting neurotransmitter release (Murphy *et al.*, 2000). In animal models, the O/E of aSyn redistributed the synaptic vesicles to locations further from the active zone, and in hippocampal neurons, it resulted in the loss of synaptic proteins and enlarged vacant vesicles, leading to abnormalities in vesicle priming, fusion, and docking (Janezic *et al.*, 2013).

aSyn has been known to bind to different proteins, such as calmodulin, regulating the secretory processes at the synapses (Martinez *et al.*, 2003).

aSyn can be divided into three major regions (Figure 1):

The amphipathic lysine-rich N-terminal domain (1-60) has an α-helix conformation and is responsible for the lipid-binding properties of aSyn, preferentially in the acidic headgroups and membranes with strong curvatures. This region also promotes the formation of the SNARE complex. In addition, it contains a multi-repeated imperfect consensus hexameric sequence (KTKEGV) and most point mutations associated with the familiar forms of PD are found in this region (Davidson *et al.*, 1998; Jo *et al.*, 2000; Burré *et al.*, 2010).

A hydrophobic central domain (61-95), commonly known as the non-amyloid-β component (shortened to NAC domain), is essential for aSyn aggregation and inclused additional KTKEGV repeats. It is the second major component of the amyloid plaques found in AD (Giasson *et al.*, 2001; Tuttle *et al.*, 2016; Li *et al.*, 2018).

The C-terminus (96-140) of aSyn interacts mainly with small molecules and proteins like vesicle associated membrane protein 2 (VAMP2) and Synaptobrevin-2, two regulators of synaptic exocytosis and endocytosis, respectively (Burré *et al.*, 2010; Sun *et al.*, 2019). This region is enriched in acidic residues and prolines, conferring a strong negative charge (Izawa *et al.*, 2012). In addition to its negative charge charge, this terminal also contains three tyrosine residues. The C-terminal is highly prone to post-translational modifications (PTMs).

In the first stage in oligomerization, aSyn exists as a soluble monomer that has the ability to form dimers and oligomers. After further oligomerization, aSyn is then able to assemble into fibrils, which are the major component of Lewy bodies.

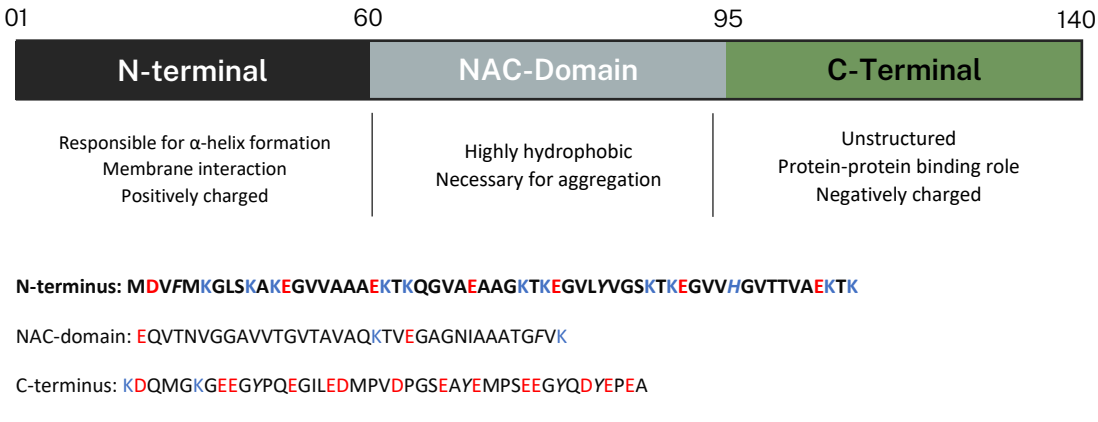


Figure 1. Structure and sequence of aSyn. It consists of 3 domains and 140 amino acids. The amphipathic positively charged N-terminal (black), the NAC region and the acidic C-terminal. Basic (blue), acidic (red) and aromatic (italic) residues can be seen.

Even though aSyn has two α-helix-structures in the N-terminal and NAC domain, it is considered an intrinsically disordered protein. In solution, aSyn takes the form of stable unfolded monomers that have been extensively characterized by different biophysical methods (Eliezer *et al.*, 2001b; Sang *et al.*, 2002; Hoyer *et al.*, 2004; Bertoncini *et al.*, 2005; Dedmon *et al.*, 2005; Cho *et al.*, 2007; Lee *et al.*, 2007; Uversky, 2007; Allison *et al.*, 2009). The structural characteristics of aSyn allow it to have high conformational flexibility that

may explain its different functions when interacting with diverse molecules, proteins, or cell compartments.

1.2.2. Familial mutations of Parkinson's disease

Several point mutations have been identified in the N-terminal domain of aSyn that are linked with familial forms of synucleinopathies, including L8I (Chen *et al.*, 2020), A30G (Liu *et al.*, 2021), A30P (Polymeropoulos *et al.*, 1997b), E46K (Zarranz *et al.*, 2004b), H50Q (Proukakis *et al*., 2013), G51D (Kiely *et al.*, 2013), A53E (Ghosh *et al*., 2014), A53T (Polymeropoulos *et al.*, 1997b), A53V (Yoshino *et al.*, 2017), E57D (Chen *et al.*, 2020), K58N, E83Q (Kapasi *et al.*, 2020) and P117S (Guo *et al.*, 2021), and rare variants such as A18T and pA29S that tend to be associated with late-onset PD (Hoffman-Zacharska *et al.*, 2013). Of these, A30P is the only mutation that appears to reduce the protein's propensity to adopt α-helical content (Balesh, Ramjan and Floriano, 2011). The N-terminal domain of aSyn is responsible for the protein's interaction with membranes, and mutations in this region have been shown to affect the protein's flexibility, intramolecular interactions, and ultimately alter its aggregation propensity, oligomerization, and morphological changes between the aggregates (Snead and Eliezer, 2014). Mutations H50Q, E46K, and A53T promote secondary structure changes leading to fibril formation (Ono *et al*., 2011; Rutherford *et al*., 2014), while A30P promotes rapid oligomerization with a delay in fibril formation (Narhi *et al*., 1999; Conway *et al*., 2000). The G51D mutation is associated with slower aggregation compared to wild-type (WT) aSyn. (Rutherford *et al.*, 2014).

In addition to the mutations mentioned previously, there are other mutations that have been identified in the aSyn gene that are associated with synucleinopathies. For example, the A18T and pA29S variants have been found to be associated with late-onset PD (Hoffman-Zacharska *et al.*, 2013). The A53V mutation has been identified in families with both PD and DLB (Yoshino *et al.*, 2017), while the P117S mutation has been associated with a rare form of familial PD (Guo *et al.*, 2021).

The N-terminal domain of aSyn is responsible for mediating its interaction with membranes, and mutations in this region can affect the protein's flexibility and lipid-binding properties. For example, the A30P mutation has been shown to reduce the protein's affinity for membranes and alter its ability to form stable oligomers (Jo *et al.*, 2002; Shu *et al.*, 2014). The E46K mutation also promotes membrane binding and may promote the formation of toxic oligomers and fibrils (Rovere *et al.*, 2019). These findings suggest that

mutations in aSyn can alter its interaction with cell membranes and ultimately contribute to its pathogenic effects in synucleinopathies.

Furthermore, there is evidence to suggest that genetic factors may interact with environmental factors to contribute to the development of synucleinopathies. For example, the H50Q mutation has been shown to have significant alteration when in the presence of copper when compared to WT aSyn. Similarly, the A53T mutation has been associated with an increased risk of PD in individuals exposed to pesticides (Norris *et al.*, 2007). These findings suggest that gene-environment interactions may play a role in the development of synucleinopathies.

Finally, it is worth noting that not all individuals with mutations in the aSyn gene develop synucleinopathies, and not all individuals with synucleinopathies have identifiable mutations in the aSyn gene. This suggests that other factors, such as epigenetic modifications or protein-protein interactions, may also play a role in the development of these disorders (Labbé, Lorenzo-Betancor and Ross, 2016). Overall, further research is needed to fully understand the complex interplay between genetic and environmental factors in the development of synucleinopathies.

1.2.3. Post-translational modifications of aSyn

aSyn undergoes several PTMs that are associated with physiological or pathological functions. A PTM is a chemical modification of specific amino acids that can alter the protein structure or regulate its activity, location, degradation, and binding affinity (Ramazi and Zahiri, 2021). Some of the PTMs of aSyn include glycation, nitration, acetylation, SUMOylation, truncation, ubiquitination, glutathionylation, glycosylation, acylation, oxidation, and phosphorylation (Figure 2).

The most commonly studied PTM of aSyn is phosphorylation at serine 129, due to its close association with pathology, as it is found deposited in the brains of synucleinopathy patients. Phosphorylation is a reversible PTM that plays a role in multiple cellular processes, such as cytoskeletal arrangement, cellular metabolism, and gene expression (Oueslati, Fournier and Lashuel, 2010; Ardito *et al*., 2017). Although the level of aSyn phosphorylation is low in physiological conditions, it is detectable in pathologically aggregated aSyn, affecting aSyn aggregation, oxidative stress, and inclusion formation. It is estimated that 90% of aggregated aSyn in PD patients is phosphorylated, compared to only 4% in healthy individuals (Fujiwara *et al.*, 2002). While its role in aSyn fibrillation remains debatable

(Chen *et al*., 2009; da Silveira *et al.*, 2009) phosphorylated aSyn at serine 129 has also been associated with interactions with vesicular trafficking, enzymes, and cytoskeletal proteins, suggesting an ability to interfere with their functions and utilize them as co-aggregates (Awa *et al*., 2022).

Phosphorylation of aSyn in serine 87 has been shown to inhibit aSyn aggregation and influence its interaction with membranes, but its functional role is yet to be fully elucidated (Paleologou *et al*., 2010; Oueslati *et al*., 2012). Enzymes such as kinases and phosphatases, including polo-like kinase 2, casein kinases, and G protein-coupled receptor kinases, balance aSyn phosphorylation, with the latter colocalizing with aSyn in Lewy bodies found in the brains of PD patients (Pronin *et al*., 2000; Waxman and Giasson, 2008; Inglis *et al*., 2009). Phosphorylation of aSyn has also been associated with increased reactive oxygen species (ROS) levels, which are caused by mitochondrial stress and exogenous toxins (Perfeito *et al*., 2014).

Ubiquitination is an important PTM for the degradation of excessive or aberrant aSyn. This process involves the attachment of ubiquitin, a small protein that is ubiquitously expressed in the central nervous system (CNS), to specific residues of a target protein (Hallengren, Chen and Wilson, 2013). Ubiquitin most commonly binds to lysine residues, but can also bind to cysteine, serine, and threonine (McClellan, Laugesen and Ellgaard, 2019). If a single molecule of ubiquitin is bound, the process is designated mono-ubiquitination, while if multiple ubiquitin molecules are bound, leading to the formation of a poly-ubiquitin chain, the process is referred to as poly-ubiquitination. Poly-ubiquitination leads to protein degradation primarily by the ubiquitin-proteasome system (UPS) *(Xu et al., 2009; Braten et al*., 2016). High amounts of ubiquitinated aSyn have been found in LBs. Monoubiquitination promotes oligomerization of aSyn, while polyubiquitination and SUMOylation promote its degradation, suggesting that the balance between these processes is important in regulating aSyn levels in the CNS (Beyer and Ariza, 2013).

aSyn can undergo glycation, a non-enzymatic reaction that involves the attachment of sugar molecules to proteins. This modification has been shown to promote the formation of aSyn oligomers, which are thought to be particularly toxic to neurons (Vicente Miranda *et al*., 2017). Interestingly, diabetes has been suggested as a risk factor for PD, and both diseases show altered sugar metabolism. This suggests a potential link between the two diseases that may have important implications for understanding PD pathology (Athauda *et al*., 2017; Brauer *et al*., 2020; Chohan *et al*., 2021; Chung *et al*., 2021). Furthermore,

studies in cell models of PD demonstrated that glycate aSyn has reduced mono-ubiquitination and degradation. Methylglyoxal-treatment showed impaired aSyn release and membrane binding (Vicente Miranda *et al.*, 2017; Konig, Miranda and Outeiro, 2018).

aSyn nitration is a PTM that involves the addition of a nitro group to the side chain of tyrosine residues, and is a hallmark of oxidative stress. Nitration of aSyn has been reported in the brains of PD patients, as well as in animal models of PD and *in vitro* studies using oxidative stress inducers. This modification has been shown to increase aSyn aggregation and toxicity, as well as impair its chaperone-mediated autophagy and lysosomal degradation, leading to the accumulation of aSyn in neurons (Hodara *et al.*, 2004)vvv. Additionally, nitration of aSyn has been associated with increased levels of ROS, indicating a potential positive feedback loop in oxidative stress-mediated aSyn pathology (Prigione *et al.*, 2010). Studies have also suggested a link between aSyn nitration and neuroinflammation, as nitrated aSyn has been shown to activate microglia and promote cytokine release (Reynolds *et al.*, 2008). Therefore, targeting aSyn nitration may be a potential therapeutic strategy for PD and other synucleinopathies.

Like many other proteins, aSyn is constitutively N-terminal acetylated. This PTM is common, affecting 85% of the proteins in eukaryotic cells. This modification involves the addition of an acetyl group to the N-terminus of aSyn, reducing its positive charges and affecting its biophysical properties (Dikiy and Eliezer, 2014; Runfola *et al.*, 2020)

aSyn truncation has also been shown to modulate aSyn cytotoxicity and aggregation. These truncated forms of aSyn are found in the brains of PD patients and reduce cell

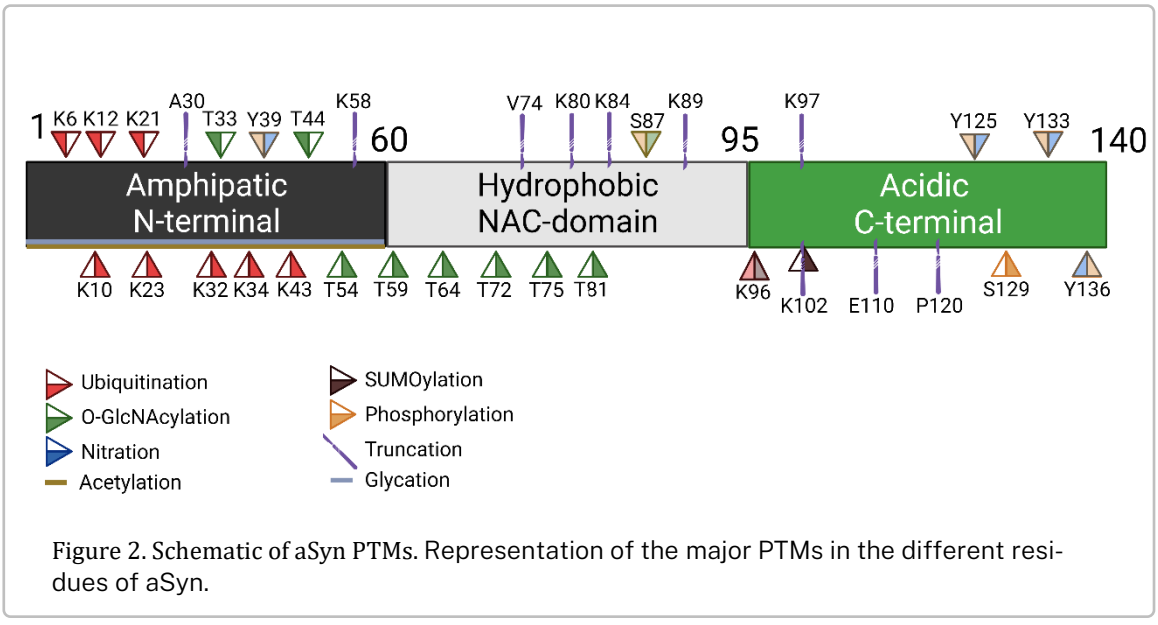


Figure 2. Schematic of aSyn PTMs. Representation of the major PTMs in the different residues of aSyn.

viability. These forms of aSyn are more toxic to the mitochondria than the WT counterpart (Zhang *et al.*, 2022).

1.2.4. Pathological aggregation and spreading of α-synuclein

The aggregation of aSyn is a complex process that involves multiple steps and factors, and has been the subject of numerous studies in recent decades. Different synucleinopathies, such as PD and MSA, present distinct aggregates that result from the ability of aSyn to generate divergent forms of aggregated structures, which have been coined as "strains" similarly to the protein accumulations found in prion diseases (Melki, 2015; Peelaerts *et al*., 2015; Peelaerts and Baekelandt, 2016; Lau *et al*., 2020). These conformational strains of aSyn can differ in several aspects, such as morphology, size, toxicity, seeding potential, and binding affinity with proteins and lipids (Peelaerts and Baekelandt, 2016; Peelaerts *et al.*, 2018). For example, oligomers and ribbons demonstrate greater spreading properties than fibrils, but only ribbons can cause the accumulation of phosphorylated aSyn, which is a hallmark of several synucleinopathies (Gribaudo *et al*., 2019; Rey *et al*., 2019). Therefore, understanding the pathways and triggers of aSyn aggregation is crucial for fully comprehending the pathology and diagnosing different synucleinopathies.

Current evidence suggests that aSyn oligomers and protofibrils, rather than the larger insoluble aggregates, are the most cytotoxic species (Tanaka *et al*., 2004; Winner *et al*., 2011; Cascella *et al*., 2021). Studies with aSyn variants that tend to form stable oligomers are associated with high cytotoxicity (Ingelsson, 2016; Fusco *et al*., 2017).

As stated previously, aSyn is an intrinsically disordered protein in solution, adopting multiple conformations, making it unclear how native unfolded aSyn acquires an aggregation and nucleation-prone structure. The NAC domain of aSyn, a region located between amino acid residues 61-95, plays a crucial role in aSyn aggregation (Periquet *et al*., 2007; Waxman, Mazzulli and Giasson, 2009). This stretch can potentiate monomeric accumulation and is also present in the core of the aSyn filaments (Tuttle *et al*., 2016; Li *et al*., 2018). The C-terminal of aSyn can shield the NAC domain and prevent aggregation, and truncating the C-terminal has been shown to increase the aggregation rate (Kanda *et al*., 2000; Ulusoy *et al*., 2010; Volpicelli-Daley *et al*., 2011; Bassil *et al*., 2016; Ma *et al*., 2018). The fibril core mainly contains the NAC and N-terminal domains, while the unstructured part outside the core is composed of the C-terminal (Farzadfard *et al*., 2022).

During the early stages of fibrillization, aSyn undergoes a partial folding into a pre-molten globule-like conformation (Uversky *et al*., 2001). This conformational change can result from various factors, including protein concentration imbalances, mutations, PTMs, and environmental factors such as changes in pH, salt concentration, inflammation, and polyamines (Antony *et al*., 2003; Sandal *et al*., 2008; Wu *et al*., 2009; Fujiwara *et al*., 2019; Guzzo *et al*., 2021; Manzanza, Sedlackova and Kalaria, 2021). The oligomerization process begins with the formation of dimers, which are followed by the formation of soluble and non-fibrillar oligomeric species that can have different morphologies, such as annular, chain-like, or spherical structures. Upon reaching a critical concentration, these oligomers convert into protofilaments, protofibrils, and high molecular weight amyloid fibrils that can be classified as amorphous or amyloid-like aggregates (Figure 3) (Hijaz and Volpicelli-Daley, 2020).

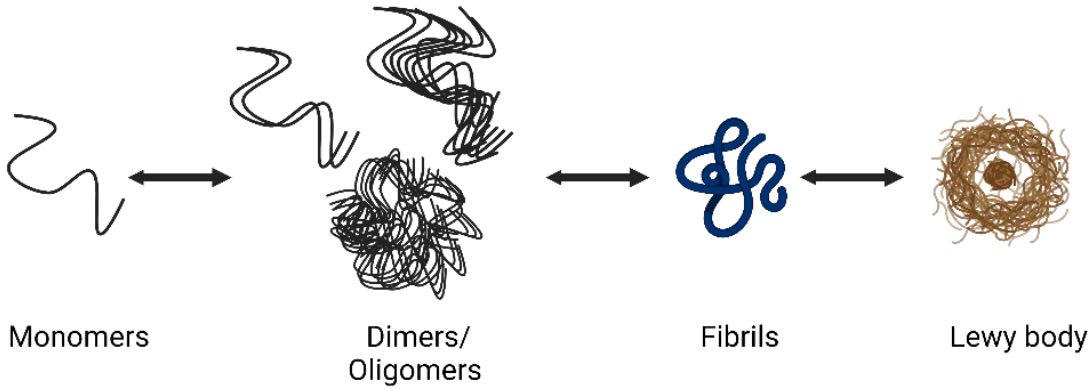


Figure 3. Schematic representation of the aggregation process of aSyn. In the first stage aSyn exists as a soluble monomer that has the ability to form dimers and oligomers. Due to further oligomerization, aSyn is able to then assemble into fibrils, that are the major component of LBs.

Under certain conditions, the spherical oligomers can be converted into ring-like structures, which have been shown to permeabilize membranes by forming pore-like structures that affect membrane potential and ion distribution (Lashuel *et al*., 2002; Kim *et al*., 2009).

Like many proteins, the levels of aSyn in the CNS are regulated by a balance between its synthesis, clearance, and aggregation (Kragh *et al*., 2012). Abnormal levels of aSyn can result from the failure of any of these mechanisms, leading to the formation of oligomers and fibrils. In healthy conditions, neurons have the ability to remove accumulated or dysfunctional proteins. However, if this mechanism fails, it can trigger or promote the pathogenic process (McNaught, Björklund, *et al.*, 2002; McNaught, Mytilin, *et al*., 2002).

The analogy between prion diseases and synucleinopathies is not exclusive to the concept of aggregate strains but also to the spread of the pathology from cell-to-cell, region-to-region, and even organ-to-organ (Holmqvist *et al.*, 2014).

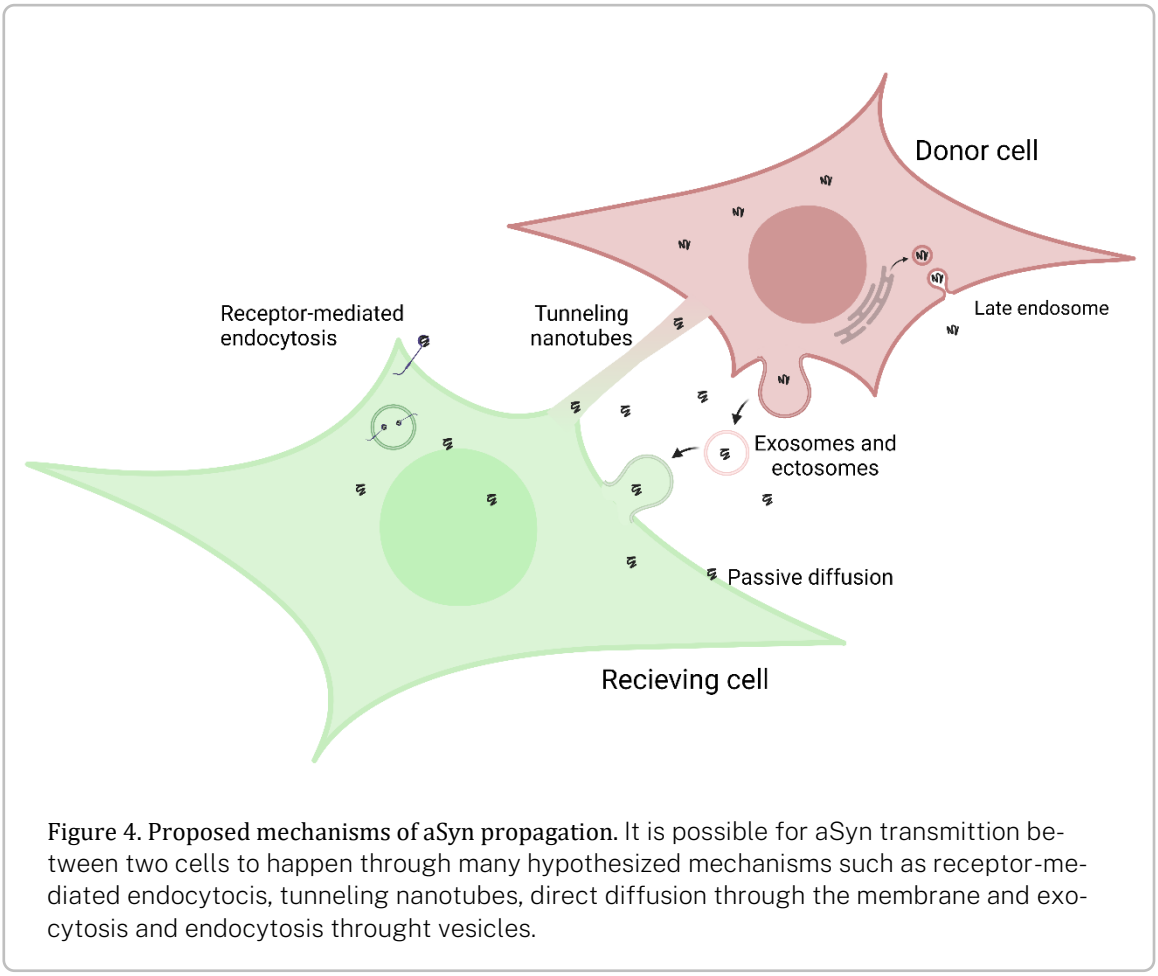


Figure 4. Proposed mechanisms of aSyn propagation. It is possible for aSyn transmittion between two cells to happen through many hypothesized mechanisms such as receptor-mediated endocytocis, tunneling nanotubes, direct diffusion through the membrane and exocytosis and endocytosis throught vesicles.

It was hypothesized by Braak and colleagues that the topography of synucleinopathies is related to the severity of the symptoms and that this progression could be separated into distinct stages (Braak *et al.*, 2003). In PD, the first stage is characterized by olfactory deficits, constipation, and sleep disturbances, which reflect the involvement of the olfactory bulb, enteric system, and caudal brainstem, respectively. In the subsequent stages, motor symptoms may correspond to the spread of pathology to the substantia nigra. In the final phase, the pathology reaches the neocortex, resulting in psychiatric symptoms and cognitive decline (Braak *et al.*, 2003; Fullard, Morley and Duda, 2017).

Several pathways have been identified as potentially involved in the transfer of aSyn between cells, including direct diffusion of aSyn through the cellular membrane, endoplasmic reticulum (ER)-Golgi dependent exocytosis, aSyn secretion in extracellular vesicles, tunneling nanotubes, or receptor-mediated endocytosis (Figure 4) (Emmanouilidou *et al.*,

2010; Danzer *et al*., 2012). Importantly, these pathways have been associated with specific assembly states of the protein.

1.2.5. Cytotoxicity in Parkinson's disease

The symptoms of PD reflect the neurodegenerative processes occurring in patients. To potentially halt the progressive nature of the disease, it is crucial to understand these phenomena. Several mechanisms have been proposed to explain the cytotoxicity seen in synucleinopathies, which can be grouped into three main categories: toxic gain of function of aSyn, loss of physiological function and disruption of normal cellular processes (as shown in Figure 5). These processes may not be mutually exclusive but can occur simultaneously. For example, the loss of function can result from trapping monomers in non-functional oligomers or fibrils, while these aggregation mechanisms can also trigger abnormal and toxic interactions.

aSyn can interact with multiple cytoskeletal proteins, including tau, which is associated with several neurodegenerative diseases. This interaction can regulate tau phosphorylation, which in turn influences tubulin stabilization and binding *(Chen et al., 2007; Sousa et al*., 2009; Dasari *et al*., 2019). The association of aSyn with α and β-tubulin, and their presence in the LBs, suggests a physiological role of this interaction and a possible link between aSyn aggregation and microtubule polymerization (Kovács *et al*., 2004; Carnwath, Mohammed and Tsiang, 2018). aSyn also interacts with actin, affecting its polymerization and, ultimately, the stability of the cytoskeletal network (Zhou *et al*., 2004; Esposito *et al*., 2007; Sousa *et al*., 2009). By inactivating cofilin, aSyn impairs axon elongation and growth cone turning, inhibiting proper neuronal function (Tilve, Difato and Chieregatti, 2015).

ER stress is a characteristic feature observed in dopaminergic neurons in PD brains (Mou *et al*., 2020). In animal models, the accumulation of oligomeric aSyn in the ER occurs before the onset of the disease, suggesting that ER dysfunction may be an early event in the pathology (Miraglia *et al*., 2020). Diffused ubiquitinated aSyn is found to colocalize with ER stress markers, and mouse PD models revealed that neurons affected by aSyn display ER dysfunction, morphological abnormalities, and an up-regulation of ER chaperones (Conn *et al*., 2004; Smith *et al*., 2005; Görlach, Klappa and Kietzmann, 2006; Bellucci *et al*., 2011; Credle *et al*., 2015). Furthermore, inhibiting ER stress has been shown to reduce the accumulation of aSyn oligomers and delay the symptomatic disease stage (Boyce *et al*., 2005; Colla *et al.*, 2012).

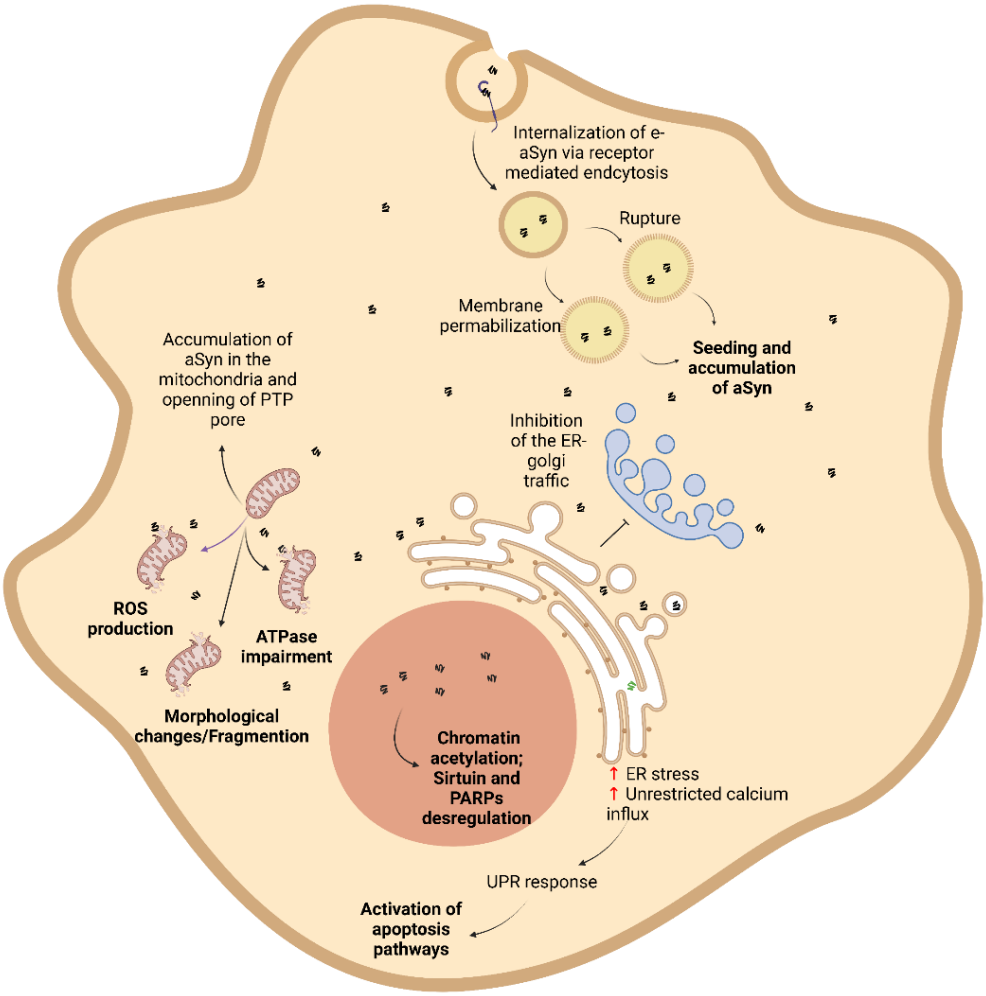


Figure 5. Proposed mechanisms of aSyn cytotoxicity. Multiple cell processes are affected such as mitochondrial function, ER-Golgi trafficking and molecular changes. Adapted from Domingues *et al*. 2022.

ER dysfunction also affects the ER-Golgi network, leading to fragmentation of the ER (Gitler *et al*., 2008; Paiva *et al*., 2018). O/E of aSyn has been shown to delay the ER-Golgi

transport in a concentration-dependent manner and the dispersion of the Golgi (Thayanidhi *et al.*, 2010).

One of the major complications in PD is the deterioration of the protein clearance mechanisms, such as the autophagy-lysosomal pathway (ALP) and the UPS, which play a role in the protein quality control and cleanse of unnecessary, damaged, or misfolded proteins. PD brains show lysosomal depletion, evident by a reduction in multiple lysosomal markers such as lysosomal-associated membrane protein 1 (LAMP1) and heat-shock protein 73 (HSP73), especially in neurons containing aSyn inclusions (Andringa *et al*., 2006; Dehay *et al*., 2010). This impairment is considered one of the possible causes that trigger aSyn aggregation and pathology (Pan *et al*., 2008). Furthermore, dysfunction of the ALP upon aSyn-mediated inhibition of Rab1 and sequestration of ALP components in aSyn aggregates ultimately impacts macroautophagy (Gitler *et al*., 2008; Winslow *et al*., 2010). aSyn also inhibits chaperone-mediated autophagy by binding to LAMP2, a lysosomal receptor, which further contributes to aSyn accumulation and loss of cellular homeostasis (Issa *et al*., 2018). O/E of aSyn inhibits proteasomal activity, which leads to the accumulation of ubiquitin-positive inclusions and cell death (Zondler *et al*., 2017). This process is thought to be due to aSyn interaction with multiple proteasome particles and the colocalization of proteasomal proteins with oligomers and fibrils (Lindersson *et al.*, 2004).

PD is characterized by a deficiency in mitochondrial activity, which is a primary hallmark of the disease. Toxins such as 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine (MPTP) and rotenone are used in many PD models to induce mitochondrial dysfunction (Maegawa and Niwa, 2021). Mutations in genes involved in cellular protection against mitochondrial stress, including *Parkin* and PTEN-induced putative kinase 1, are linked to familial forms of PD (Beilina *et al*., 2005; Dawson and Dawson, 2010; Billingsley *et al.*, 2019). aSyn has also been found to accumulate in the mitochondria of neuronal cells in PD patients, which is associated with impaired activity of the complex 1 and genetic dysregulation of genes involved in the mitochondria electron transport chain. The accumulation of aSyn in the mitochondria has been shown to impact the mitochondria's morphology and dynamics by permeabilizing the membrane and increasing ROS levels. The N-terminal of the protein, which has lipid-binding properties, regulates these effects (Nakamura *et al*., 2011; Perfeito *et al*., 2014).

1.2.6. Interactions with membranes and membrane-associated proteins

As mentioned previously, aSyn can spread cell-to-cell, disseminating the neurodegeneration to different neuroanatomical regions and affecting multiple tissues.

When aSyn spreads, the first cellular barrier it interacts with is the extracellular matrix. The N-terminal of aSyn has the ability to interact with phospholipids and fatty acids in the membrane (Eliezer *et al*., 2001a). The negative charge of the membranes becomes a primary target to the positively charged lysine residues, affecting membrane properties such as thickness and curvature (Braun *et al.*, 2012; Zarbiv *et al*., 2014). Furthermore, this interaction can affect the structure of aSyn itself, causing an increase in α-helix content and promoting oligomerization (Davidson *et al*., 1998) (Figure 6).

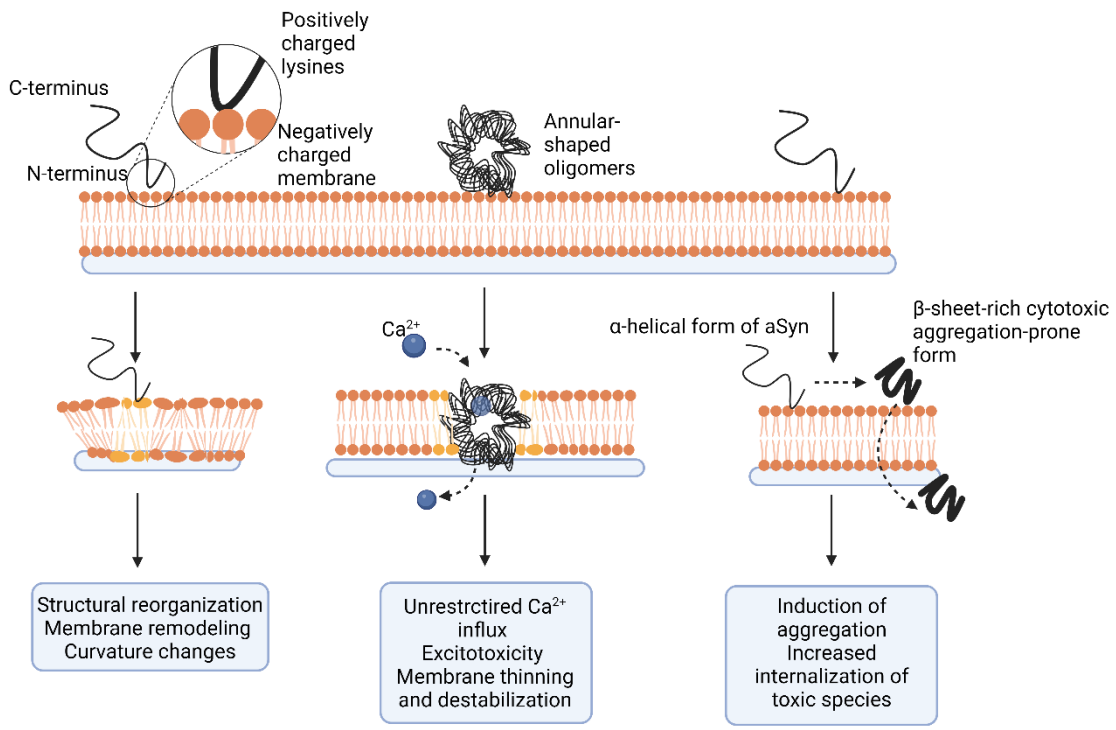


Figure 6. Diagram of the multiple effects caused by aSyn interaction with the membrane. aSyn can not only cause reorganization of the membrane, but also incorporate and oligomerize after interaction. Adapted from Domingues *et al*. 2022.

Intracellular acetylated aSyn can bind to membranes, and displays reduced aggregation propensity compared to its non-acetylated form (Bartels *et al*., 2014; Dikiy and Eliezer, 2014; O'Leary *et al*., 2018). This interaction is also modulated by other PTMs, mutations, and membrane composition (Lashuel *et al*., 2002; Volles and Lansbury, 2002; Zakharov

et al., 2007; Galvagnion *et al*., 2016). For example, phosphorylation of aSyn can inhibit its interaction with membranes, and mutations like A30P and E46K can alter its binding affinity to anionic lipids (Fiske *et al*., 2011; Kuwahara *et al*., 2012).

The binding of the N-terminus of aSyn with membranes still allows other partners to interact with the intrinsically disordered C-terminal, such as the SNARE complex, suggesting that this interaction may play a highly relevant role in the normal function of aSyn (Lou *et al*., 2017).

Proposed mechanisms for the membrane-mediated cytotoxicity are the disruption and permeabilization of membranes by integrating annular-shaped aSyn oligomers. These oligomers are incorporated into the lipid membrane, acting as channels and destabilizing membrane permeability, causing an unrestricted calcium influx and excitotoxicity (Lashuel *et al*., 2002; Zhu, Li and Fink, 2003; Quist *et al*., 2005; Zakharov *et al*., 2007). In addition, these pores can also directly affect the membrane by damaging and thinning it, which could affect diffusion transport (Chaudhary *et al*., 2016). The damage is, however, affected by both mutations and PTMs, supporting that distinctive organizational assemblies of oligomers may mediate different pathological pathways (Lashuel *et al*., 2002; Volles and Lansbury, 2002; Zakharov *et al*., 2007).

In addition to the membrane itself, aSyn can interact with multiple protein receptors and membrane proteins that play a role in neurodegeneration and the spreading of extracellular

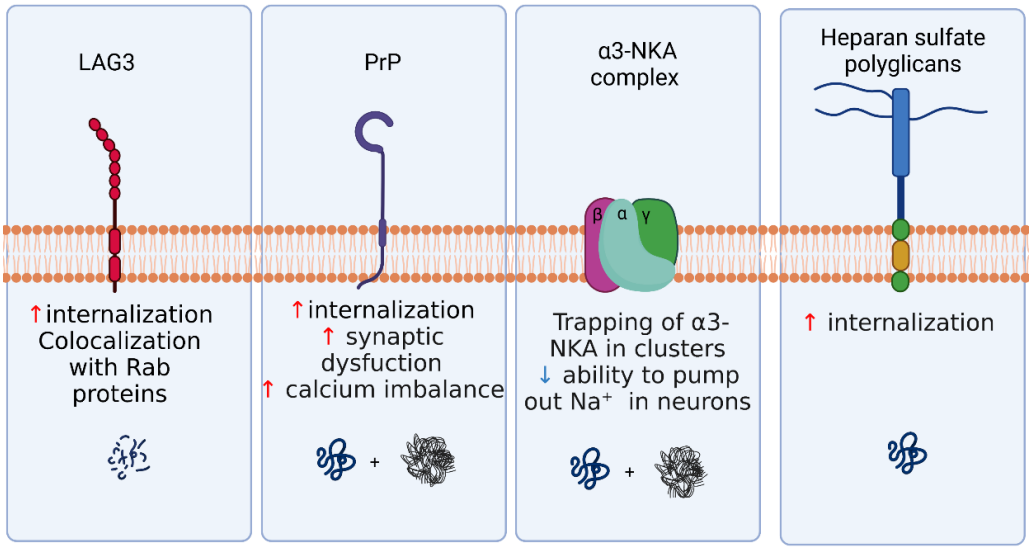


Figure 7. Multiple receptors that have shown to interact with aSyn. Receptors have multiple functions in synucleinopathies, ranging from modulation of the spreading to synaptic dysfunction and cytotoxicity. Adapted from Domingues *et al*. 2022.

aSyn (Figure 7). Experiments with unspecific proteases, which reduce the number of cell surface proteins, have demonstrated that reduced internalization of aSyn occurs in cells (Lee *et al*., 2008).

One of these proteins is lymphocyte-activation gene 3 (LAG3), an immunoglobulin family member expressed in neurons, microglia, and immune cells (Anderson, Joller and Kuchroo, 2016; Liu *et al*., 2018). LAG3 can bind with its D1 domain to murine mouse aSyn pre-formed fibrils (PFFs), colocalizing with multiple Rab proteins and endosomal GTPases implicated in the internalization of extracellular aSyn (Mao *et al*., 2017). Deletion or inhibition of LAG3 will cause a decrease in the endocytosis of aSyn and a reduction of dopaminergic loss *in vivo.* This protein can also be cleaved by metalloproteases and exist in a soluble form, shown to be increased in the serum of PD patients and is associated with increased severity of non-motor symptoms in patients (Li *et al*., 2007; Cui *et al*., 2019; Guo *et al*., 2019).

The Na^+/K^+-ATPase (NKA) is an enzyme located in the membrane of animal cells, responsible for importing two potassium ions and exporting three sodium ions using adenosine triphosphate (ATP), playing a role in various cell processes, including neuronal activity (Skou, 1998; Clausen, Hilbers and Poulsen, 2017). The NKA is composed of three subunits, and the α3 subunit is linked to several neurodegenerative diseases (Geering, 2008; Ohnishi *et al*., 2015; Shrivastava *et al*., 2015; Petrushanko *et al*., 2016; Ruegsegger *et al.*, 2016). This subunit interacts with amyloid-β (Aβ), superoxide dismutase 1 (SOD1), and aSyn, and is hypothesized to regulate the endocytosis of these proteins into neuronal cells (Ohnishi *et al*., 2015; Shrivastava *et al*., 2015; Ruegsegger *et al*., 2016). he interaction between aSyn and α3-NKA causes the α3-NKA to become trapped in aSyn clusters, which in turn reduces the ability of α3-NKA to export sodium effectively following an action potential. Ultimately, this can lead to dysregulation of the neuronal refractory period. α3-NKA was detected alongside neurexin 1α and 2α in a proteomic-based analysis, indicating its possible involvement in synapse function (Shrivastava *et al*., 2015).

The cellular prion protein (PrP^C) has been shown as a key regulator in the internalization of aSyn and the modulation of neuronal damage caused by aSyn oligomers (Ferreira *et al*., 2017).

Heparan sulfate (HS) in proteoglycans can function as a receptor, modulating the uptake of aSyn fibrils in cell lines. Soluble heparin inhibits the uptake of aSyn by competing and binding into aSyn fibrils (Holmes *et al*., 2013; Ihse *et al*., 2017; Karpowicz *et al*., 2017).

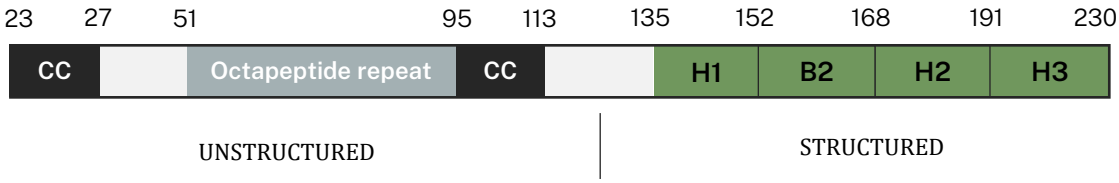
1.3. CELLULAR PRION PROTEIN

1.3.1. Cellular prion protein: Function and structure

PrP^C is a cell surface protein glycoprotein, strongly conserved across species and expressed in multiple organs and tissues with higher expression levels in the CNS and peripheral nervous system (PNS) (Bendheim *et al*., 1992; Wopfner *et al*., 1999).

The *PRNP* gene encodes PrP^C, and it is synthesized in the ER and processed for PTMs in the ER and Golgi (Chesebro *et al*., 1985; Oesch *et al*., 1985; Westaway *et al*., 1994; Chakrabarti, Ashok and Hegde, 2009). PrP^C has a disulfide bridge between cysteine 179 and cysteine 214 in the C-terminal globular domain and a glycosylphosphatidylinositol (GPI) anchor that binds PrP^C to the outer part of the membranes (Ning *et al*., 2014; Puig *et al*., 2019). In addition, oligosaccharides can bind to PrP^C, causing mono-glycosylation and di-glycosylation in the asparagine 181 and asparagine 197 residues (Haraguchi *et al*., 1989). Once in the extracellular membrane, PrP^C can go under constitutive endocytosis for recycling or degradation via clathrin-dependent and caveolin pathways (Shyng, Heuser and Harris, 1994; Peters *et al*., 2003; Sunyach *et al*., 2003; Morris, Parkyn and Jen, 2006; Fehlinger *et al*., 2017). PrP^C can also undergo proteolytic cleavage via ADAM metalloprotease (Liang and Kong, 2012).

The unprocessed PrP^C has 253 amino acids and is trimmed to 209 amino acids after the removal of the signaling peptides in the C- and N- terminus (Drisaldi *et al*., 2003). PrP^C can be divided into two structural areas: an N-terminal flexible tail and a C-terminal globular domain composed of three α-helices and two antiparallel β-sheets (Figure 8) (Cohen *et al*., 1993; Donne *et al*., 1997; Heske *et al*., 2004; Calzolai *et al*., 2005; Wille *et al*., 2009; Riek *et al*., 2021).



Unstructured: KKRPKPGGWNTGGSRYPGQGSPGGNRYPPQGGTWGQPHGGGWGQPHGGSWGQPHGGSWGQ-PHGGGWGQGGGTHNQWNKPSKPKTNLKHVAGAAAAGAV

Structured: VGGLGGYMLGSAMSRPMIHFGNDWEDRYYRENMYRYPNQVYYRPVDQYSNQNNFVHDCVNITIKQH-TVTTTTKGENFTETDVKMMERVVEQMCVTQYQKESQAYYDGRRS

Figure 8. Structure and sequence of PrP^{c}. It consists of 2 domains and 209 amino acids. The unstructured positively charged N-terminal and the structured C-terminal can be seen, along with their features such as the CC, octapeptide repeat (underlined in the sequence and in grey in the scheme), the α-helixes (H) and β-strands (B). Basic (blue), acidic (red) and aromatic (italic) residues are represented.

The N-terminal ranges from 23-124 amino acids. It contains two positively charged clusters (CC1 and CC2), an octarepeat region (OR), which has four to five repetitions of 8 amino acids (PHGGGWGQ), and a hydrophobic domain (HD) that is critical for the PrP biology and the most conserved region across species (Zahn, 2003; Coleman *et al.*, 2014; Martinez *et al.*, 2015; Evans *et al.*, 2016). In the octapeptide repeat sequence, the histidines have been shown to help coordinate the binding of metal ions, namely copper, zinc, nickel, and manganese (Hornshaw *et al.*, 1995; Yin, Zheng and Tien, 2003; Gaggelli *et al.*, 2005). Furthermore, the unstructured, flexible conformation of the N-terminus of PrP^{C} is not only implicated in the binding of different metals but also has been associated with protein-protein interactions (Freir *et al.*, 2011; Ferreira *et al.*, 2017).

The C-terminal of PrP^{C}, ranging from amino acids 125-230, is where most of the pathological mutations associated with increased aggregation propensity are found. The structured C-terminal was extensively characterized due to its role in PrP^{C} conversion to its scrapie form (PrP^{Sc}) (Wopfner *et al.*, 1999; Eghiaian *et al.*, 2004; Parkin *et al.*, 2007) and most PTMs occur in the C-terminal region.

The function of PrP^{C} has yet to be completely elucidated. In neurons, PrP^{C} has been shown to locate preferentially in the synaptic compartments of the nerve terminals (Peggion *et al.*, 2019). It is known that PrP^{C} plays a role in long-term potentiation (LTP) - the gained plasticity of the synapses to change their strength in response to previous stimuli (Maglio *et*

al., 2006; Sánchez-Alavez *et al*., 2007; Ferreira *et al*., 2017). PrP^C also has a role in sleep homeostasis and its continuity, based on the findings of PrP^C-ablation in pathology and mice models (Tobler, Deboer and Fischer, 1997). PrP^C-KO mice reported altered circadian rhythms and increased sleep fragmentation – a phenomenon rescued with the reintroduction of PrP^C expression (Cagampang *et al*., 1999; Collins, McLean and Masters, 2001). PrP-KO mice also showed reduced neuronal proliferation in development and adult neurogenesis (Steele *et al*., 2006; Haigh and Collins, 2014). PrP^C also appeared to play a neuroprotective role in models of ischemia, apoptosis, excitotoxicity caused by glutamate, and resistance to oxidative stress (Brown *et al*., 1997; Zeng *et al*., 2003; Kim *et al*., 2004; Weise *et al*., 2004; Dupiereux *et al*., 2008; Rangel *et al*., 2009; Nuvolone *et al*., 2013).

1.3.2. Prion protein in neurodegenerative disorders

The role of membrane receptors in neurodegeneration cannot be overstated. In this regard, PrP^C has shown to be one of the key receptors for the modulation of neuropathologies, both in neuronal toxicity and transmission of pathogenic aggregates to adjacent regions.

In the context of proteinopathies, PrP^C has been a well-characterized binding partner of aβ. This interaction was shown to be oligomeric-specific in the CC region of PrP^C (amino acids 23-27 and 94-110) (Freir *et al*., 2011). This interaction leads to phosphorylation of the Fyn kinase via the metabotropic glutamate receptor 5 (mGluR5) and subsequent phosphorylation of the NMDAR2B, leading to synaptic dysfunction in the hippocampus, LTP deficits, and destabilization of the dendritic spines. This phosphorylation cascade has also been associated with increased hyperphosphorylated Tau levels, affecting the progression of the disease (Um *et al*., 2012; Salazar and Strittmatter, 2017). This mechanism could explain why many early symptoms can appear years before the disease diagnosis and provides a potential therapeutic target for AD (Freir *et al*., 2011; Ostapchenko *et al*., 2013).

In addition, distinct isoforms and expression levels of PrP^C are found in AD brains (Zhang *et al*., 2019), with a change in the PrP^C profile with the progression and severity of the disease, giving a potential diagnosis and progression evaluation tool for AD. Evidence shows the presence of PrP^C in amyloid plaques in AD (Schwarze-Eicker *et al*., 2005; Takahashi *et al*., 2011).

There is still some debate regarding the function of PrP^C in AD. Studies show that a decreased expression of PrP^C in the hippocampus is associated with aging and increased

risk for AD (Whitehouse *et al*., 2010), suggesting a protective role in the pathology. This study was, however, complemented by different studies that focused on the PrP^C expression in different Braak stages, showing that PrP^C expression is increased in the first stages of the pathology, decreasing in later stages and subsequent neuronal death (Vergara *et al*., 2015). Other studies showed that there was no significant diversity in PrP^C expression between healthy groups and AD patients (Dohler *et al.*, 2014; Abu Rumeileh *et al.*, 2017), while others showed that there were no differences between total PrP^C levels, but differences regarding the glycosylated forms of PrP^C (Saijo, Scheff and Telling, 2011).

The binding of aβ to PrP^C is restricted to the N-terminus of PrP^C – blocking it with antibodies or deleting it can prevent synaptic plasticity deficits induced by oligomers of aβ (Laurén *et al*., 2009; Didonna *et al.*, 2015). Specifically, the antibodies 6D11, which blocks the residues 93-109, and an antibody specific for the region 23-111 were shown to rescue cognitive deficits in AD mouse models (Chung *et al*., 2010; Kudo *et al*., 2012), suggesting that the residues 23-27 and 92-110 are necessary for the binding and subsequent neuronal damages caused by aβ. PrP^C has also been shown to modulate the internalization of aβ oligomers dependent on low-density lipoprotein 1 and laminin receptor (Rushworth *et al*., 2013; Pinnock *et al*., 2015).

PrP^C has been shown to increase the uptake and modulate the toxicity of TDP-43 fibrils, a protein that affects neurons in ALS and frontotemporal lobar degeneration (FTLD) (Scialò *et al*., 2021).

In neurodegenerative disorders, PrP^C is mainly known to be the main culprit in prion diseases, such as scrapie in sheep, bovine spongiform encephalopathy in cattle, Creutzfeldt-Jakob disease (CJD) in humans, and chronic wasting disease in some cervids. Prion diseases are transmissible and fatal brain diseases. In human pathologies, we can catalog these diseases as spontaneous, acquired by another organism, or genetic. In these diseases, the cellular form of the protein converts into a protease-resistant pathogenic state known as PrP^{Sc}. Contrarily to its cellular isoform, PrP^{Sc} contains more β-sheets (>43%, compared to 3%) and a lower proportion of α-helices (30%, compared to 42%) (Caughey *et al*., 1991; Cohen *et al*., 1993).

Prion diseases have the particularity that their pathogenic and transmission agent is a protein capable of catalyzing the healthy isoform into a pathogenic and, subsequently, infectious one. PrP^{Sc} forms oligomers that can then be assembled into fibrils (Prusiner *et al*., 1983; Bieschke *et al*., 2004). These fibrils have small fragments that can detach and

spread the PrP^{Sc} seeds to the surrounding locations, tissues, and in some instances, even different organisms of distinct species (Saborio, Permanne and Soto, 2001; Baskakov *et al*., 2002). This mechanism inspired the term “prion-like spreading,” found in multiple other neurodegenerative diseases, to describe why some pathologies, such as PD and AD, can progress across neighboring cells, different brain regions, and tissues (Marciniuk, Taschuk and Napper, 2013). There is, however, some debate on whether prion pathologies are caused by a loss of function of the PrP^{C} or a toxic gain-of-function caused by PrP^{Sc}.

1.3.3. Prion protein in synucleinopathies

As referred to in the previous chapter, PrP^{C} is known to be an interacting partner to multiple types of amyloids; however, its function in synucleinopathies remains to be fully understood. Furthermore, previous literature has shown conflicting data regarding the interaction of aSyn with PrP^{C}, being therefore essential to characterize this interaction better to unlock a potential therapeutic prospect. In addition, it is unclear whether PrP^{C} has a receptor or a sensor role in the pathology.

Like previously discussed receptors, PrP^{C} was shown to act as a modulator of aSyn internalization in cell models. Although it is not essential for aSyn spreading, as seen in *PRNP*-KO models, it does increase the spreading when overexpressed. Stereotaxic injections of sonicated aSyn fibrils into the brains of mice either lacking PrP^{C} expression, WT, or O/E via Tg20 demonstrated that PrP^{C} expression is associated with increased spreading in an expression-dependent manner. The results in primary cultures of these neurons were insignificant, suggesting that PrP^{C} acts as a sensor rather than a direct culprit in aSyn internalization. Immortalized human embryonic kidney cells (HEK293) transfected with different PrP^{C} mutations demonstrated strong colocalization except when the CC was removed, suggesting an involvement of this region in the interaction (Urrea *et al.,* 2017, 2018). In addition, in mice, the presence of PrP^{C} displayed a higher amount of aggregated aSyn compared to *PRNP* KO.

This interaction affected not only the aSyn but also prion replication. aSyn fibrils were able to interfere with PrP^{C} and increase the formation of neuroprotective PrP^{C} fragments (Reyes *et al*., 2014; Aulić *et al.*, 2017).

This interaction can form micron-sized clusters with aSyn oligomers with relatively high affinity and slow dissociation, modulated by the residues 95-111 of PrP^{C} (Rösener *et al*., 2020).

Studies have demonstrated that the oligomeric form of aSyn can interact with PrP^C, resulting in the phosphorylation of mGluR5 through Fyn kinase. This activation of NMDAR2B ultimately leads to an elevation in intracellular calcium levels and the impairment of synaptic function. This dysfunction can cause abnormalities in long-term potentiation (Figure 9) (Ferreira *et al.*, 2017). Furthermore, this interaction has been proposed to encourage the formation of cofilin/actin rods, which can rearrange the cytoskeleton and impact actin dynamics, leading to axonal transport blockage (Brás, Lopes and Outeiro, 2018).

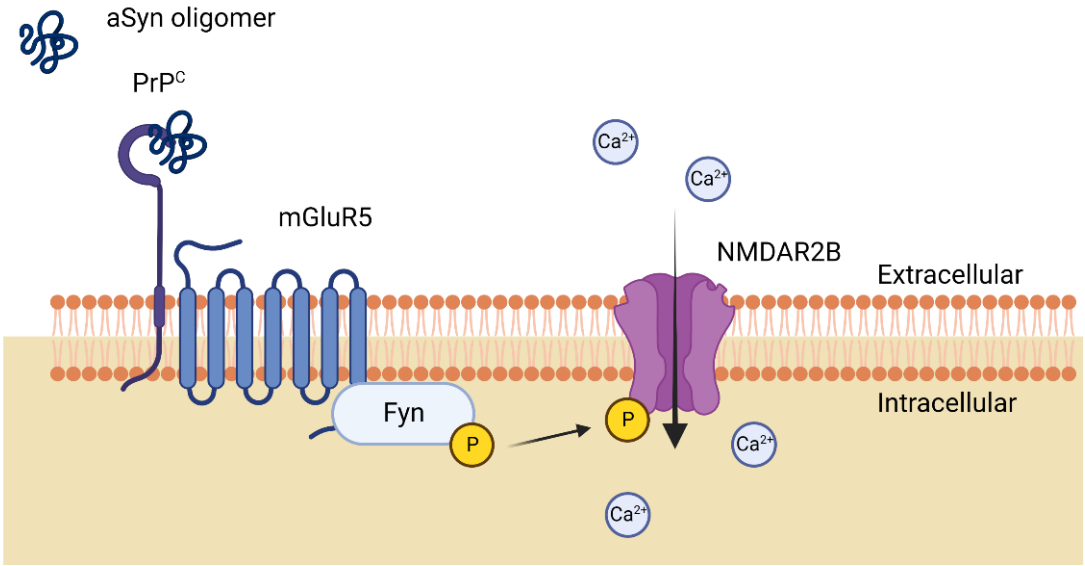


Figure 9. Diagram of the pathway triggered by the interaction of aSyn oligomers and PrP^C. Oligomeric aSyn triggers activation of the mGluR5 which leads to the phosphorylation and activation of the Fyn Kinase, and causes a phosphorylation of the NMDAR2B, leading to an increase in the amount of intracellular calcium.

In mice models of aSyn, the behavioral deficits and reduced lifespans found were ameliorated in mice where PrP^C expression was ablated. In addition, it was also found that PrP^C could facilitate aSyn internalization in SH-SY5Y cells O/E PrP^C compared to WT SH through clathrin-mediated endocytosis and changing the location of aSyn oligomers. The interaction could also be detected through surface plasmon resonance (SPR) (Thom *et al.*, 2021).

It was also shown that aSyn and PrP form by complex coacervation that is a highly dynamic, thermos-responsive, and reversible liquid droplet through the interaction of the N-terminal of PrP^C and C-terminal of aSyn (Agarwal *et al.*, 2022).

The potential of this interaction to modulate synucleinopathies makes it an enticing therapeutic target to try to slow the progression of the disease with therapies such as antisense oligonucleotides or immunotherapies.

1.4. CELLULAR MODELS OF NEURODEGENERATIVE DISORDERS

In order to study synucleinopathies, several cellular models have been developed to simulate specific characteristics of the disease. However, all models have particular advantages and disadvantages, so it is essential to use the correct models and a combining them to offset each other's limitations.

The use of patient tissue, although quite valuable since it is the model most closely related to the disease, contains the limitation of being heterogeneous and its ethical considerations (Falkenburger, Saridaki and Dinter, 2016).

Although also limited by ethical considerations, animal models offer the advantage of being able to be genetically modulated and allow us to follow the course of the disease from the start until a certain point. In addition, multiple tissues can be collected or cultured; however, it is limited by survivability (in primary culture), differences regarding species, and experimental time (Hartung, 2008).

Cell lines allow easily handled and reliable experiments while reproducing certain specific parts of the disease while ignoring others. These have, however, the limitation of overgroWTh, which limits the ability to monitor the more prolonged effects of synucleinopathies (Hartung, 2007).

Within the cell line models, the biomolecular fluorescence complementation assay (BiFC) is a valuable tool for studying protein-protein interactions. This method is based on the discovery of two non-fluorescent fragments of a fluorescent protein that reconstitute the protein complex, allowing visualization of a signal when in close proximity. This complex is formed when each fragment is fused to specific proteins that are interacting (Figure 10) (Outeiro *et al.*, 2008).

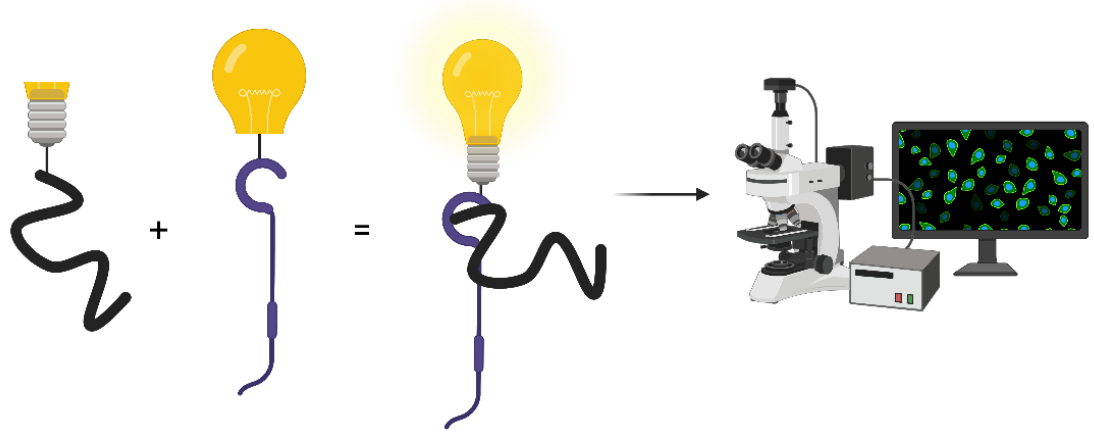


Figure 10. Schematic representation of BiFC. The non-fluorescent fragments of Venus fluorescent protein are attached to two different proteins. If the proteins interact, the complex is formed and the fluorophore is reconstituted.

CHAPTER 2

Aims of the Project

aSyn-associated pathologies cause cytotoxicity and spreading through a plethora of cellular events, ranging from calcium imbalance to mitochondrial dysfunction. The characterization of these events is therefore essential to the better understanding of the pathologies and consecutively, important for the identification of potential therapeutic targets.

Membrane proteins, most notably PrP^C,have recently been shown to be modulators of aSyn-pathology, acting as a sensor for misfolded forms of aSyn, and activating signaling cascades that result in the abnormal activation of the NMDA receptor and, consequently, dysregulation of the neural function. However, the precise molecular determinants of the interaction need to be further elucidated.

In this project, we aimed to characterize the molecular interaction between aSyn and PrP^C so that we can better understand the full potential role of this interaction. Our main goals were the:

- Characterization of the molecular interaction between α-synuclein and cellular prion protein *in vitro*.
- Investigation of the molecular interaction between α-synuclein and cellular prion protein in cell models.

For the first goal of the project, we aimed to characterize the biophysical and structural properties of the interaction, analyzing its effects using *in vitro* approaches, such as NMR or RT-QuICs assays.

For our second goal, we generated aSyn and PrP^C tagged with fragments of Venus fluorescent protein in order to characterize the interaction using BiFC assay and aggregation assays in human cell lines such as HEK293 and H4 cells.

CHAPTER 3

Materials and Methods

3.1. PURIFICATION OF RECOMBINANT PROTEIN

3.1.1. Murine α-synuclein purification and characterization

3.1.1.1. Purification of α-synuclein

Murine aSyn was obtained by transforming *E. coli* BL21 (DE3) ultracompetent cells with a plasmid containing its cDNA sequence (pET21a-aSyn WT). ^{15}N isotopically labeled and N-terminal acetylated aSyn were obtained by co-transforming *E. Coli* BL21 with both pET21a-aSyn WT and a plasmid encoding the components of yeast NatB acetylase complex.

Transformed cells were cultured in a narrow-necked baffled-designed flask in slow stirring at 37°C in lysogeny broth (LB) medium in the presence of ampicillin (200 µg/mL) until the optical density (OD) at 600 nm reached 0.6. Protein expression was then induced by adding 1 mM of isopropyl-1-thio-β-D-galactopyranoside (IPTG), and the culture was incubated for 2 hours. For ^{15}N isotopically labeled proteins, M9 medium was enriched with $N^{15}H4Cl$.

The cells were pelleted (6600x*g*, 15 minutes at 4°C) and resuspended in lysis buffer (750 mM NaCl, 10 mM TRIS, 1 mM EDTA, pH 7.6) with 1 mM of PMSF protease inhibitor (La Roche, Basel, Switzerland). The mixture was sonicated and incubated at 95°C for 15 minutes. After briefly cooling down the sample, we centrifuge the sample (16000x*g*, 20 minutes at 4°C) and collect the supernatant for dialysis. The supernatant was added to a dialysis tubing (3000 molecular weight cut-off), which was added to the dialysis buffer (10 mM TRIS, 1mM EDTA, 50 mM NaCl, pH 7.6).

Anion exchange high-performance liquid chromatography (AEC) was carried out on an Äkta-HPLC Purifier (GE Healthcare). The dialyzed mixture was injected into a HiTrap Q HP column (GE Healthcare). After injection of the sample, a gradient was run from buffer A (25 mM TRIS, pH 7.7) to buffer B (25 mM TRIS, 1 M NaCl, pH 7.7), and fractions were collected for further analysis.

Samples deemed pure by polyacrylamide gel electrophoresis (PAGE) were selected for size exclusion chromatography using a Superdex 200 10/300 (GE Healthcare Life Sciences). Protein concentration was estimated with spectrofluorometer (Jasco Inc, MD, USA) from the absorbance at 274 nm using a molar extinction coefficient of 5960 M^{-1} cm^{-1}. The protein was aliquoted and kept frozen at -80°C for later use.

3.1.1.2. Purification of aggregated forms of α-synuclein

To model *in vitro* amyloid variants of aSyn found in pathological conditions, monomeric aSyn needs to be subjected to certain standardized conditions and controls. To obtain fibrils of aSyn, we first need to centrifuge monomeric aSyn at high speeds (to prevent contamination with self-aggregated high molecular weight species) and dilute it into PBS to a final concentration of 5 mg/mL. The tube is then placed into a thermomixer at 37°C and shaken at 1000 rotations per minute (rpms) for 7 days (Eppendorf Thermomixer Comfort, Eppendorf, USA). After incubation, the mixture should appear turbid, composed of fibrillary forms of aSyn, which are then confirmed by Thioflavin T (ThT) assays.

ThT assay consists of incubating a total concentration of 0.05 mg/mL of purified fibrillar aSyn into 25 μM of ThT. The absorbance is then read using an Infinite M200 PRO (Tecan Ltd., Maennedorf, Switzerland) with an excitation of 450 nm and an emission of 500 nm.

Fibrils were sonicated according to previous descriptions (Luk *et al.*, 2012) to produce PFFs to study aSyn prion-like activity.

3.1.2. Murine prion protein purification and characterization

Murine mature 23-230 PrP^C was obtained by transforming *E. coli* BL21 (DE3) ultracompetent cells with a plasmid corresponding to its cDNA sequence (pET22b-mPrP23-230).

Transformed cells were cultured in a narrow-necked baffled-designed flask in slow stirring at 37°C in LB medium in the presence of ampicillin (100 μg/mL) until the OD at 600 nm reached 0.6. Protein expression was then induced by adding 1 mM of IPTG, and the culture was incubated for 5 hours.

The cells were pelleted (5000x*g*, 20 minutes at 4°C) and resuspended in 1x BugBuster Master Mix (Merck Millipore, Billerica, MA, USA). The culture was then sonicated and incubated for 20 minutes at room temperature (RT). The supernatant was discarded and the pellet was resuspended in 1X BugBuster Master Mix, sonicated under the same conditions, and incubated (15 minutes at RT). The mixture was centrifuged (8000x*g*, 15 minutes at 4°C), and after discarding the supernatant, the pellet was resuspended in 0.1x Bug Buster reagent. The mixture was centrifuged (16000x*g*, 15 minutes at 4°C). The supernatant was discarded. The pellet was resuspended in denaturing buffer (8 M GuHCl, 10 mM Tris, 100 mM $NaPO_4$, pH 8), sonicated, and incubated for 1 hour at RT. After incubation,

the pellet was centrifugated (13000x*g*, 5 minutes at 4°C), and the resulting supernatant was mixed with Ni-NTA SuperFlow resin (Qiagen) preequilibrated in denaturing buffer. The mixture was incubated for 1 hour at RT and then packed into an XK 16/20 glass chromatography column (GE Healthcare) and added to an Äkta-HPLC Purifier (GE Healthcare).

To discard other proteins, we ran a gradient from denaturing buffer to refolding buffer (10 mM Tris–HCl, 100 mM $NaPO_4$, pH 8.0), followed by the elution of our protein by adding the elution buffer (10 mM Tris–HCl, 100 mM NaPO4, 500 mM imidazole, pH 5.8). Fractions with OD_{280}>1000 were added to a dialysis tube (7000 MWCO) and dialyzed 1.5h against dialysis buffer (10 mM $NaPO_4$, pH 5.8), changing it every 30 minutes and dialyzing other 1.5 hours in ddH_2O under the same conditions. The protein stocks were filtered through a 0.22 µm filter unit and then frozen in aliquots at -80°C.

3.2. CELL CULTURE AND MOLECULAR BIOLOGY

3.2.1. Cell Lines

For *in vitro* studies, we used HEK293, and genetically modified HEK293 stably expressing green fluorescent protein (GFP)-tagged aSyn were kept at 37°C and 5° CO_2 environment in Dulbecco's Modified Eagle Medium (DMEM) (PAN, Germany), supplemented with 1% penicillin-streptomycin (Pen/Strep) (ThermoFisher) and 10% fetal bovine serum (FBS) (ThermoFisher). Cells were seeded 24 hours before transfection into well-plates at a density of 10^5 cells per ml. Cells were counted using a Neubauer counting chamber.

At a confluence of around 90%, the cells were resuspended and transferred into a new culture flask. Cells were washed briefly with PBS, followed by incubation with trypsin/EDTA. The mixture was transferred into new cell culture medium.

All handling was done under sterile conditions under a laminar flow hood.

3.2.2. Transient transfection method

For BiFC, aggregations, and internalizations experiments, cells were seeded in a 12-well-plate 24 hours before the transfection. All transfections were done using Metafectene (Biontex) according to the manufacturer’s instructions. Shortly, the medium was exchanged for a new culture medium 1 hour before the transfection. 1 µg total Plasmid DNA was diluted in serum and antibiotic-free DMEM media, and 2 µL Metafectene was diluted into

serum and antibiotic-free DMEM media on another tube. The DNA solution was then combined with the lipid solution, and the mixture was incubated for 20 minutes at RT. The final solution was then added dropwise to the medium, and cells were incubated for 24 hours for further tasks.

The medium was removed or exchanged after 18 hours.

3.2.3. Treatment of cells with α-synuclein

Twenty-four hours after transfection with different plasmids, different forms of recombinant aSyn were added directly to the cell medium until a total concentration of 500 nM was reached. The culture incubated for 24 hours. Cells were treated 24 hours after transfection. Cells were washed 3 times with 1x PBS and 1 time with diluted trypsin to remove residual protein from the plate.

3.2.4. Protein extraction and quantification

Cells were solubilized 24h post-treatment (either transfection or treatment with recombinant protein). Cells were lysed using radioimmunoprecipitation assay (RIPA) buffer ((50 mM Tris pH 8.0, 150 mM NaCl 0.1% Sodium-Dodecyl-Sulphate (SDS), 1% Nonidet P40, 0.5% sodium deoxycholate, α-complete protease inhibitor cocktail (La Roche, Basel, Switzerland). The cell lysates were centrifuged (10000x*g*, 15 minutes at 4°C), and the supernatant was transferred to a new recipient. Protein concentration was measured through Bradford's assay. 1 µLof cell lysate was diluted with 49 µL of H_2O and 150 µL of Bio-Rad Protein Assay Dye Reagent Concentrate (Bio-Rad Laboratories, Inc., Hercules, CA, USA). Absorbance was measured using a TECAN Infinite M200 PRO plate reader. All samples were measured in triplicates

3.2.5. Western blot analysis

The detection of proteins and quantifications of relative amounts were verified by immunoblotting. 20 µg of samples were added to 5x laemmli buffer (250 mM Tris pH 6.8, 10% SDS, 1.25% Bromophenol Blue, 5% β-Mercaptoethanol, 50% Glycerol), and loaded for SDS-PAGE with 12% resolving gel and 7% stacking gel (Acrylamide 37.5 (Carl Roth GmbH, Karlsruhe, Germany), 0.01% SDS in ddH_2O, 0.1% tetramethylethylenediamine (TEMED), and 1% ammonium persulfate (APS), with 5µL PageRuler Plus Protein Ladder (Perbio Science Deutschland, Bonn, Germany).

Electrophoresis was performed with an SDS-Running buffer (125mM Tris, 960 mM Glycine) for 20 minutes at 70 Volts followed by 1 hour with 110 Volts in a Bio-Rad Mini-Protean 3 Mini Vertical Electrophoresis System (Bio-Rad Laboratories, Inc., Hercules, CA, USA).

Following the electrophoresis run, the protein was transferred into a nitrocellulose membrane by dry transfer using an iBlot 2 Gel Transfer Device (Thermofisher Scientific, MA, USA), according to the manufacturer's manual.

The membrane was fixed with 0.4% PFA, and protein was detected with Ponceau S.

The membrane with the protein was then blocked using 5% bovine serum albumin fraction V (BSA), and diluted in TBS-Tween for 1h at RT. The primary antibodies were dissolved in TBS to immunolabel specific proteins and incubated under mild shaking overnight at 4°C. Membranes were washed three times for 15 minutes with TBS-Tween and incubated with secondary antibodies for 2 hours at RT. The fluorescence signal was visualized using Odyssey® DLx Imaging System (Li-cor).

3.2.6. Cloning of prion plasmids

For the production of plasmids expressing the venus-fragment tagged VN-PrP, the vector VN-pcDNA3.1. was selected. We digested for 1 h at 37°C 5µgof PrP-GFP to obtain an insert encoding for the mature human PrP RNA, and the vector in 2µL of FastDigest Green Buffer (Thermo Fisher Scientific, MA, USA), 1µL of restriction enzymes NheI (Thermo Fisher Scientific, MA, USA) and AflII (Thermo Fisher Scientific, MA, USA) at a final volume of 20µL. The final mixture was loaded into a 1.5% agarose gel in TAE buffer and 40mM of Tris, supplemented with 0.5ug/µL of ethidium bromide. Electrophoresis was done using a PowerPac Universal Power supply (BioRad) at 120V, and the DNA bands were observed using a FUSION Xpress chamber with a UV transilluminator (PEQLAB Biotechnologie, Germany). DNA Insert band was removed, and DNA was extracted from the agarose gel using a QIAquick gel extraction kit (Qiagen) following the manufacturer's protocol.

For the venus-fragment tagged PrP-VC, we perfumed the digestion under the same conditions changing only the restriction enzymes to XhoI (Thermo Fisher Scientific, MA, USA) and ApaI (Thermo Fisher Scientific, MA, USA).

For the WT-PrP plasmid with a translation initiator enhancer (Kozak Consensus sequence), we performed the digestion under the previously described conditions but with

BamHI (Thermo Fisher Scientific, MA, USA) and XbaI (Thermo Fisher Scientific, MA, USA) as restriction enzymes.

3.3. IMMUNOFLUORESCENCE MICROSCOPY

3.3.1. Immunocytochemistry

For immunocytochemistry (ICC), the cells were seeded in plates with previously coated glass coverslips.

Cells were seeded at 8.5*10^4 on the coated plate. Density was determined using a Neubauer chamber. After treatments (transfection and transfection with aSyn treatment), the media was removed, and the slides were washed three times with 1x PBS. The cells were then fixed with 4% PFA diluted in water. Next, permeabilization was done using 0.1% Triton X-100 (Sigma Aldrich, St. Louis, MO, USA), diluted in 1x PBS, and incubated for 10 minutes at RT, followed by the blocking of the non-specific binding sites with 3% BSA (PAA, Cölbe, Germany) in 1x PBS and incubation for 1 hour. The primary antibody is then added and incubated overnight. After washing the plates 3 times for 10 minutes with 1x PBS to remove any residual unbound antibodies, we incubated the secondary antibodies for 2 hours at RT. Cells were then rewashed under the same conditions and stained with DAPI (Life Technologies- Invitrogen, Carlsbad, CA,USA). Finally, after 5 minutes of incubation, the slides were washed and mounted on a glass slide with Mowiol.

Primary antibodies are listed in Table 1.

PRIMARY ANTIBODIES			
Antibody	Species	Dilution	Company
Prion protein (SAF32)	Mouse IgG2	1:1000	Bertin Pharma
A-synuclein (MJFR1)	Rabbit monoclonal IgG	1:1000	Abcam
B-Actin (A5441)	Mouse IgG1	1:4000	Sigma-Aldrich
Phosphorylated synuclein (EP1536Y)	Rabbit monoclonal IgG	1:1000	Abcam

Table 1. List of antibodies used in this study.

3.3.2. Microscopy and image analysis

Microscopy images were obtained using a Leica inverted microscope DMI6000 (Leica, Wetzlar, Germany), using 40x (HCX Pl Fluotar) or 63x objectives (HCX Pl Fluotar).

Images were processed in ImageJ and colocalization analysis for graphs X were obtained using JaCoP colocalization plugin for ImageJ.

3.4. STRUCTURAL BIOLOGY

3.4.1. RT-QuIC Assay

For the real-time quaking-induced conversion (Figure 11) (RT-QuIC), 0.01 mg/mL of monomeric aSyn and/or 0.001 mg/mL of PFFs was diluted in RT-QuIC buffer (150 mM NaCl, 0.0002% SDS, 1 mM EDTA, 10 mM of ThT, all diluted in 1x PBS). Samples were measured in a COSTAR 96 flat black plate covered with transparent plastic.

Samples were measured in an Infinite M200 PRO (Tecan Ltd., Maennedorf, Switzerland) plate reader, with temperatures between 41 and 42°C. Excitation (440 nm) and emission (480 nm) were measured for 250 cycles. Each cycle had 2 minutes of incubation and 60 seconds of orbitally shaking at 432 RPMs. To obtain monomer incorporation rate we used the previously described function (Dominguez-Meijide *et al.*, 2020) $y = y_0 + A/(1 + \exp(-k(t - t_{0.5})))$ where y_0 is the pre-transition baseline, A is the amplitude of transition, k is a groWTh rate previously calculated, and $t_{0.5}$ is a midpoint.

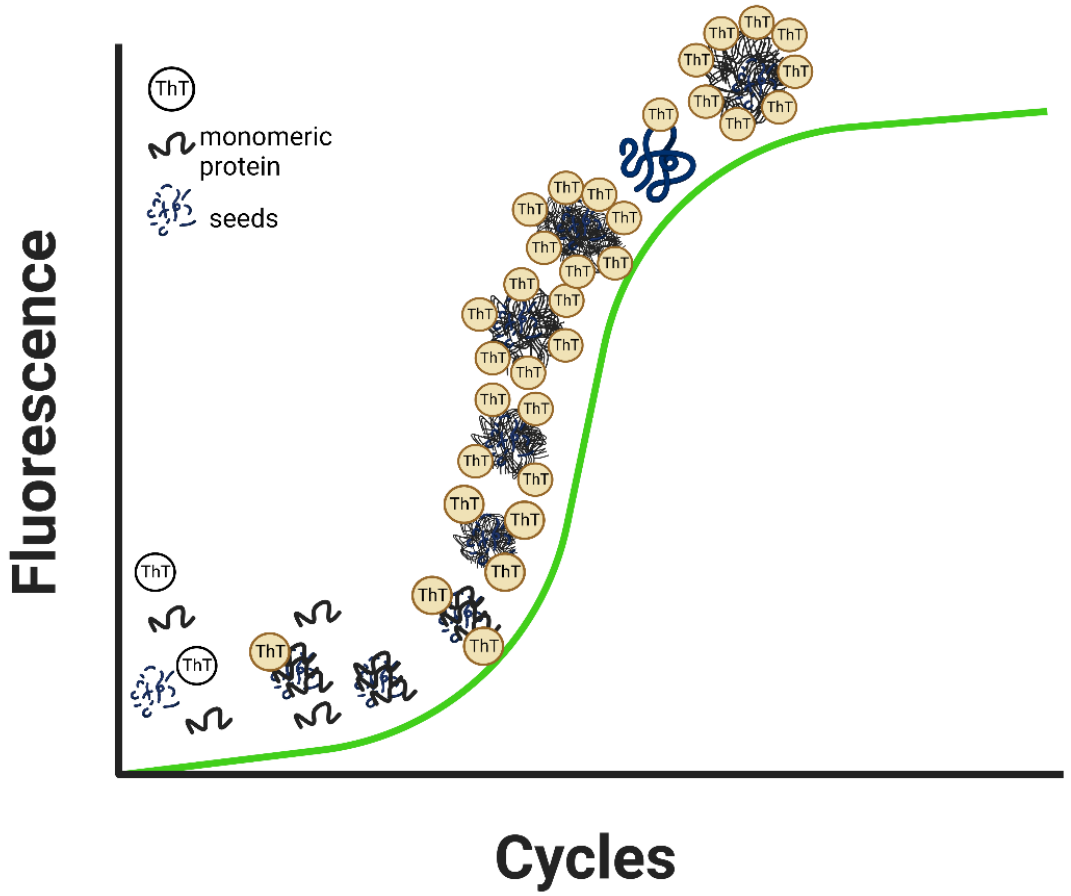


Figure 11. Schematic representation of RT-QuIC. PFFs of aSyn act as seeds to monomeric aSyn, recruiting them into β-sheet-rich structures that can be detected by ThT.

3.4.2. Surface Plasmon Resonance Spectroscopy

This method is used to detect the quantitative binding of two or more unmarked molecules.

The concept is based on polarized light going through as prisms on a reflective surface while a detector reads the reflection (Figure 12). The surface has an immobilized ligand and a constant flow of sample buffer. After the injection of the analyte into the immobilized ligand layer, any changes in the surface of the gold chip would cause a correspondent change in the angle of the reflection of the light that could be measured. These measurements were performed using a ProteOn XPR36 (Bio-Rad).

When adding the analyte, it is possible to measure the association constant (K_a), while the dissociation constant (K_d) can be measured when depleting the ligand of the analyte and measuring the release of bound analyte from the ligand. Using these measurements, it is

possible to calculate the equilibrium constant (K_D) which represents the point at which association and dissociation are equal and represents $K_D=\frac{K_d}{K_a}$.

For protein-protein interaction studies, the GLC sensor chip was used. The initialization step was performed with 50% glycerol. The surface was then activated with 400 mM of 1ethyl-3-(3-dimethyl aminopropyl) carbodiimide (EDC) and 100 nM of N-hydroxysulfosuccinimide. Protein ligand was added and immobilized at a concentration of 20 µg/mL, and the analyte was flowed at five different concentrations to obtain the K_D.

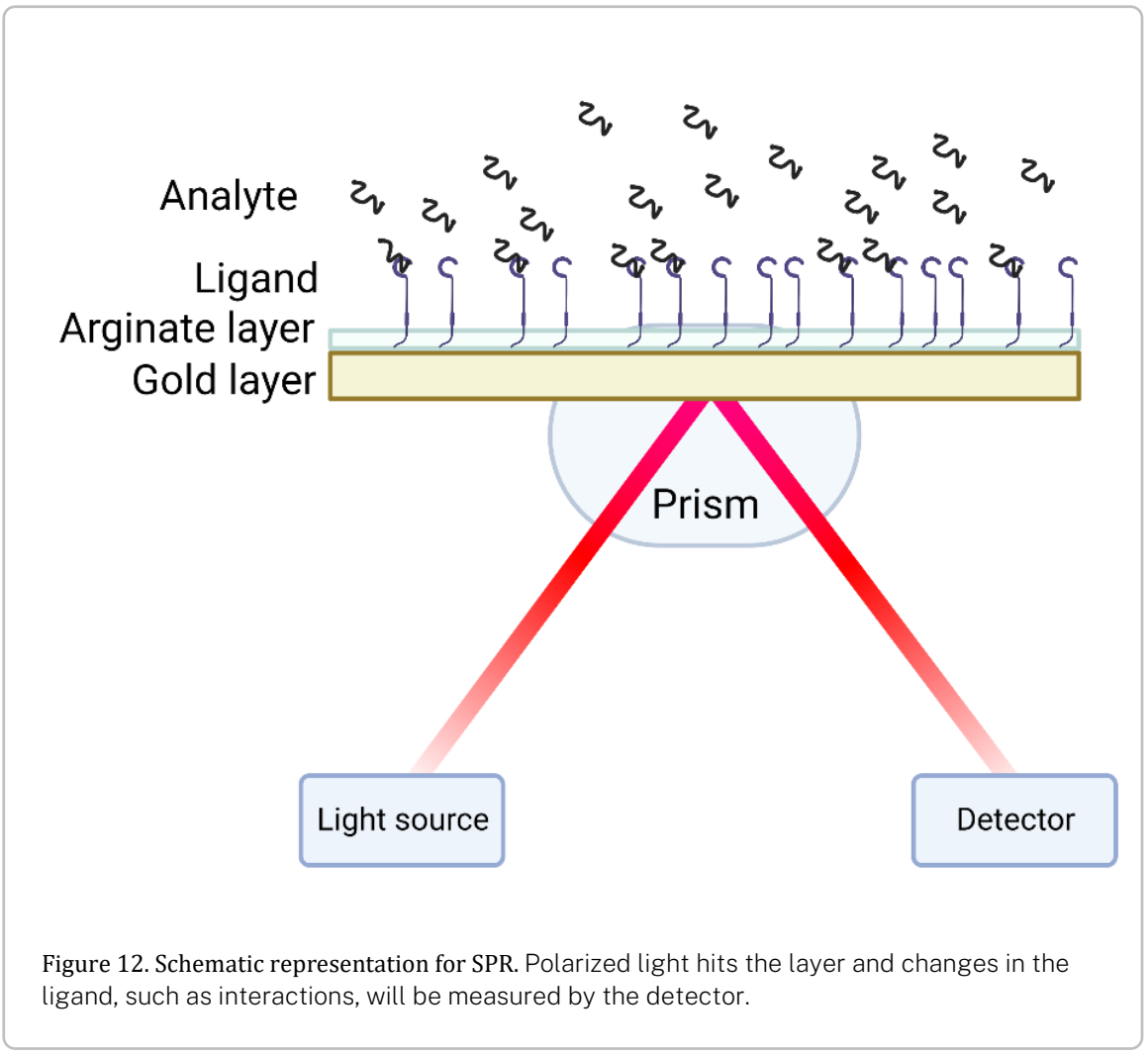


Figure 12. Schematic representation for SPR. Polarized light hits the layer and changes in the ligand, such as interactions, will be measured by the detector.

3.4.3. Nuclear magnetic resonance

All spectra were recorded on a Bruker 600 MHz Advance III spectrometer equipped with a cryogenically cooled triple resonance ^{1}H ($^{13}C/^{15}N$) TCI probe. ^{1}H-^{15}N heteronuclear single quantum coherence (HSQC) experiments were all recorded at 15°C using protein samples

dissolved in H_2O supplemented with 10% D_2O. Nuclear magnetic resonance (NMR) parameters used in each experiment are described next.

^{1}H-^{15}N HSQC experiments were done using 16 scans, 1042 complex points (sweep-width of 16 ppm in the ^{1}H dimensions), and 256 complex points (sweep-width of 26 ppm in the ^{15}N dimensions). The sequence-specific assignments for aSyn backbone were transcribed from previous literature (Maltsev, Ying and Bax, 2012; Miotto *et al.*, 2015). Only unambiguously assigned and well-resolved peaks were included for further analysis. The I/I_0 ratios obtained for aSyn, in the absence or presence of PrP^C, with different concentrations of NaCl were plotted as a function of the protein sequence to calculate the intensity perturbation profiles of the protein. The mean weighted chemical shifts displacements (MWΔCS) for ^{1}H-^{15}N were calculated as $[(\Delta\delta^{1}H)^2 + (\Delta\delta^{15}N/10)^2]^{1/2}$.

Acquisition and processing of NMR spectra were performed using TOPSPIN 3.2 (Bruker Biospin). 2D spectra analysis was performed using CCPN, and data were plotted in GraphPad PRISM 5 (GraphPad Software, San Diego, CA, USA).

3.4.4. Tryptophan fluorescence spectroscopy

Intrinsic tryptophan fluorescence was measured using a Cary Eclypse Fluorimeter (Agilent Technologies, Santa Clara, CA)]. Samples were excited with a wavelength of 280 nm, and spectra were collected from 300 to 450 nm. Samples were measured at 25°C. K_D was obtained using the function $Y = \frac{I*X}{K_{D+X}}$, where *I* is the maximum amplitude differential fluorescence and K_D is the affinity constant.

3.5. Software and statistics

Statistical analysis was done using GraphPad PRISM 5 (GraphPad Software, San Diego, CA, USA). Processing of the Figures was performed using ImageJ NIH, USA and/or Adobe Illustrator (Adobe Inc, San Jose, CA, USA). Colocalization measurements were obtained using the JaCoP plugin for ImageJ.

Figures were composed with BioRender.com or Microscoft PowerPoint (Microsoft Corporation).

Statistical tests were repeated-measures ANOVA for grouped analysis, followed by pos-hoc Dunnet´s tests for multiple comparisons and students-two-tailed t-test, one-way-Analysis of Variance (ANOVA).

Data were expressed as mean ± SD and significance levels as follows: *: $p < 0.05$; **: $p < 0.01$; ***: $p < 0.001$.

CHAPTER 4

Results

4.1. DIRECT INTERACTION BETWEEN RECOMBINANT α-SYNUCLEIN AND PRION PROTEIN IS DETECTED USING MULTIPLE BIOPHYSICAL METHODS

4.1.1. Production of recombinant prion protein and aSyn

To characterize the proteins produced, we conducted a preliminary analysis of the recombinant proteins. For that, we employed of multiple biophysical and molecular biology methods.

We verified the purity of recombinant aSyn and PrP^C by SDS-PAGE and size-exclusion chromatography (SEC) (Figure 13). For aSyn, SDS-PAGE showed its expected size, with no additional bands present when stained with Coomassie Blue (Figure 13a). Chromatography peak confirms that protein was expressed and purified from the soluble fraction (Figure 13b). For PrP^C we observed the expected size with no additional bands when stained with Coomassie blue (Figure 13c). The absence of any additional bands indicates a well-prepared and pure sample. Therefore, any potential discrepancies in future results cannot be attributed to impurities in the protein preparation.

In addition, a 1D proton NMR of 50 µM samples of aSyn (Figure 14b) and PrP^C (Figure 14c) in buffer B (MES 20 mM, NaCl 100 mM pH 6.5) was recorded at 15ºC. Well defined and resolved resonances were obtained for both proteins.

We then added both proteins at a 1:1 ratio in the conditions described previously (Figure 14a). We observed a decrease in peak intensity of tryptophan residues in PrP (indicated by an asterisk) and the disappearance of tyrosine residues in the aSyn signal (indicated by a double asterisk). However, the phenylalanine signal in the aSyn N-terminus and NAC regions was preserved (marked with a triple asterisk), indicating that the tyrosine residues whose resonances peak disappeared are likely located in the C-terminus. aSyn contains four methionine residues, two of which are present in the C-terminal region. In the right spectrum (marked with a plus sign), we observed the loss of one signal and a shift in another. These results suggest that there is a specific interaction between aSyn and PrP^C, with potential implications for understanding the mechanisms of neurodegenerative diseases.

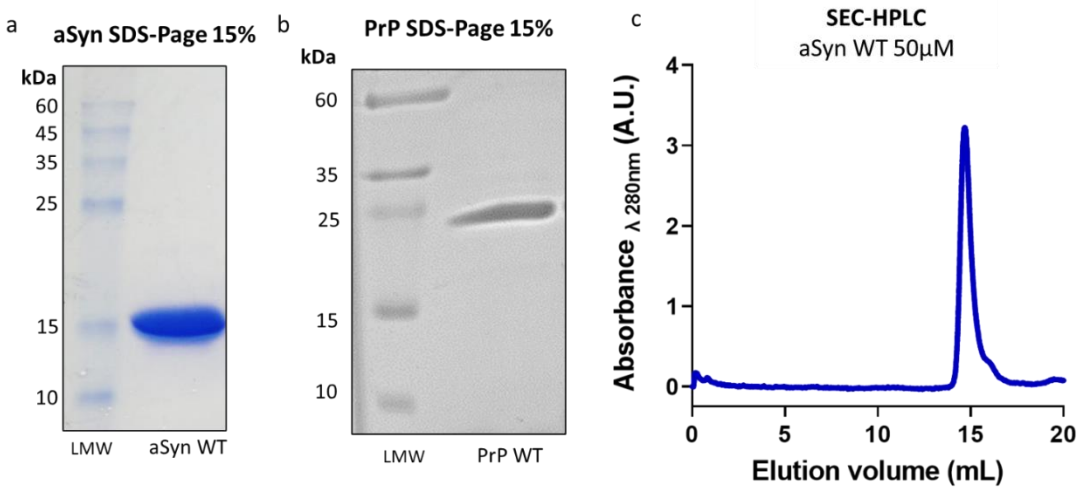


Figure 13. Evaluation of recombinant protein methods. Assessment of recombinant protein using SDS-PAGE 15% with Coomassie blue for aSyn (A) and PrP (B), and chromatography of aSyn (C). The absence of additional peaks or bands in the gel suggest a pure preparation with the proteins intact.

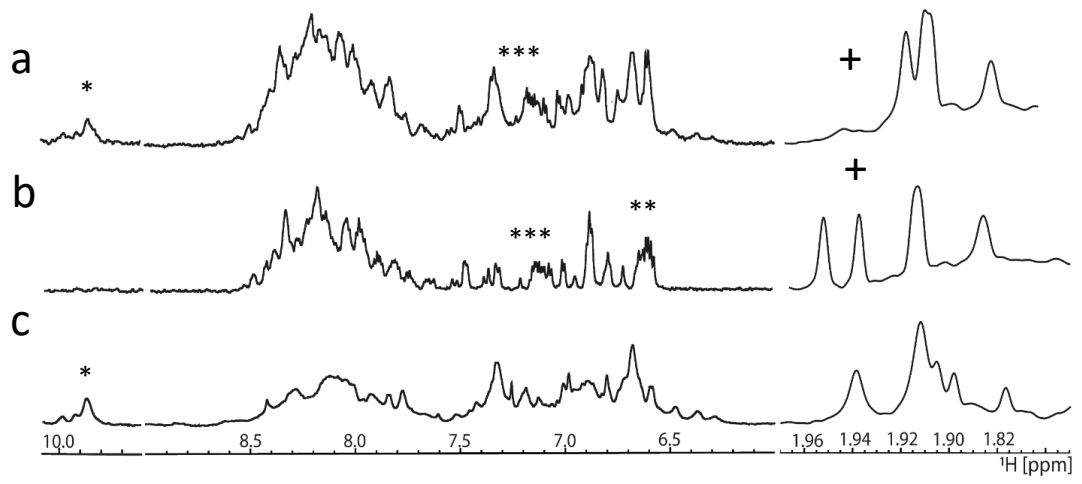


Figure 14. Interaction of WT aSyn and recombinant PrP detected by 1D proton NMR. We can observe in the left panel ^{1}H NMR spectra of side chains of ASyn + 1 equivalent of PrP (A), ASyn (B) and PrP (C); In the right panel between 2.0 and 1.8 ppm we can observe the methionine regions of (A), (B) and (C). Spectra were aquired at 15 °C in NMR Buffer of samples containing 50 µM of WT recombinant protein. Asterisk indicates a decrease in peak intensity of the tryptophan's present in PrP and the double asterisk sign the disappearance of tyrosine from the ASyn signal. The preservation of the ASyn phenylalanine signal (marked with a triple asterisk), from the N-terminus and NAC regions, suggests that the tyrosine residues were derived from the C-terminus. Out of the four methionine's present in aSyn, two are located in the C-terminal. In the right spectra, marked with a plus sign, we were able to see that one signal was loss and other was shifted.

4.1.2. Interaction between aSyn and PrP is mainly established in aSyn C-terminal through electrostatic bonds

We recorded a two-dimensional ^{1}H-^{15}N HSQC spectrum of ^{15}N-labeled aSyn. Next, we recorded a second spectrum of 50μM of ^{15}N-labeled aSyn in the presence of one molar equivalent of recombinant PrP.

The NMR spectrum of aSyn:PrP revealed significant changes in the 111-140 residues of aSyn located in the acidic C-terminal, including decreased signal intensity and a high difference in mean weight chemical shift displacements for these residues. This suggests that the interaction between aSyn and PrP takes place in the C-terminal of aSyn (Figure 15).

To further investigate the nature of the protein-protein interaction, we incubated aSyn and PrP with 200 mM of sodium chloride (NaCl), which specifically disrupts electrostatic bonds. We observed that the signal intensity and position of the shifter resonances were reconstituted after the addition of NaCl, indicating that it successfully disrupted the electrostatic interactions between the proteins, which were responsible for the formation of the protein-protein complex (Figure 15a).

These findings suggest that the interaction between aSyn and PrP is electrostatic in nature and provides valuable insights into the mechanisms underlying neurodegenerative diseases.

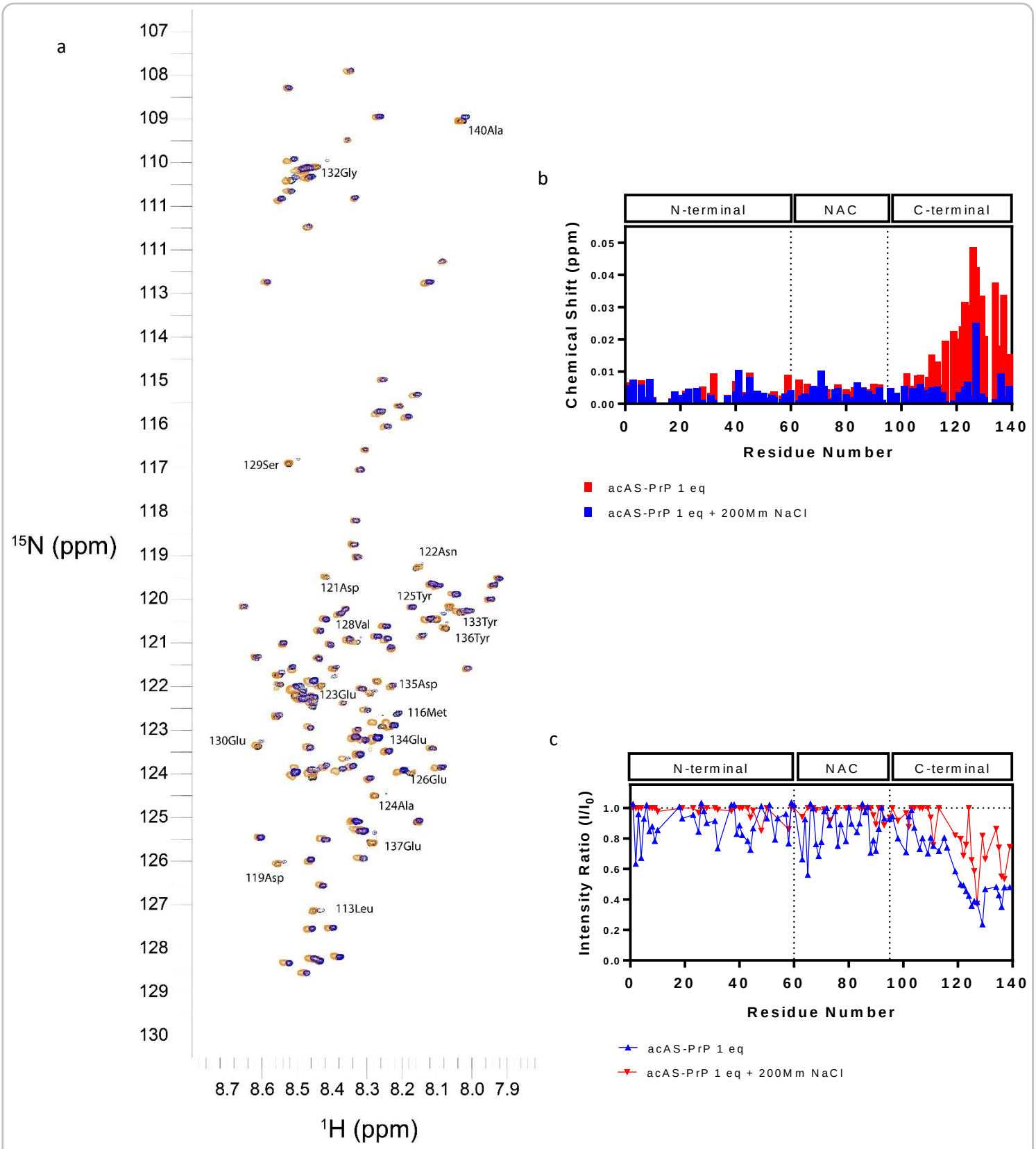


Figure 15. Interaction between aSyn and PrP is mainly established in aSyn C-terminal. A) Overlaid contour plots of 1H-15N heteronuclear single quantum correlation (HSQC) spectra of 50 μM WT aSyn(black), 50 μM WT aSyn in addition of one equivalent of PrP (blue) and aSyn, PrP and 200mM of NaCl (yellow). (B) Differences in the mean weight chemical shifts displacements between WT aSyn and WT aSyn+PrP, and in addition of NaCl. (C) Peak intensity changes between WT aSyn and WT aSyn+PrP and in addition of NaCl. All experiments werer ecorded at 15 °C using 15N isotopically labeled protein samples dissolved in NMR buffer.

4.1.3. N-terminal tryptophans from PrP play a role in the interaction

To investigate the interaction between aSyn and PrP, we examined changes in the fluorescence of tryptophan residues in PrP. Given that aSyn does not contain tryptophan, any changes in fluorescence would indicate an interaction between tyrosines from aSyn and PrP tryptophans, most notably from the octapeptide repeats and CC. We observed a decrease in tryptophan fluorescence in PrP with increasing amounts of aSyn, indicating an interaction, possibly of aromatic nature between aSyn and PrP (Figure 16).

To quantify this interaction, we calculated a K_D for the interaction between aSyn and PrP tryptophans. K_D is a measure of the strength of binding between two molecules, such as a protein and a ligand. It represents the concentration of ligand at which half of the binding sites on the protein are occupied. Mathematically, it is defined as the ratio of K_d to the K_a for the protein-ligand interaction. A lower K_D indicates a stronger binding affinity between the two molecules. The decrease in fluorescence suggests that the interaction is likely due to quenching of the aromatic residues in PrP, which could be caused by either energy transfer between residues or conformational changes induced by the interaction (Chen and Barkley, 1998).

This fluorescence-based approach provides complementary information to the 2D NMR spectroscopy used to study the interaction between aSyn and PrP. By combining these methods, we were able to gain a more complete understanding of the interaction between these two proteins.

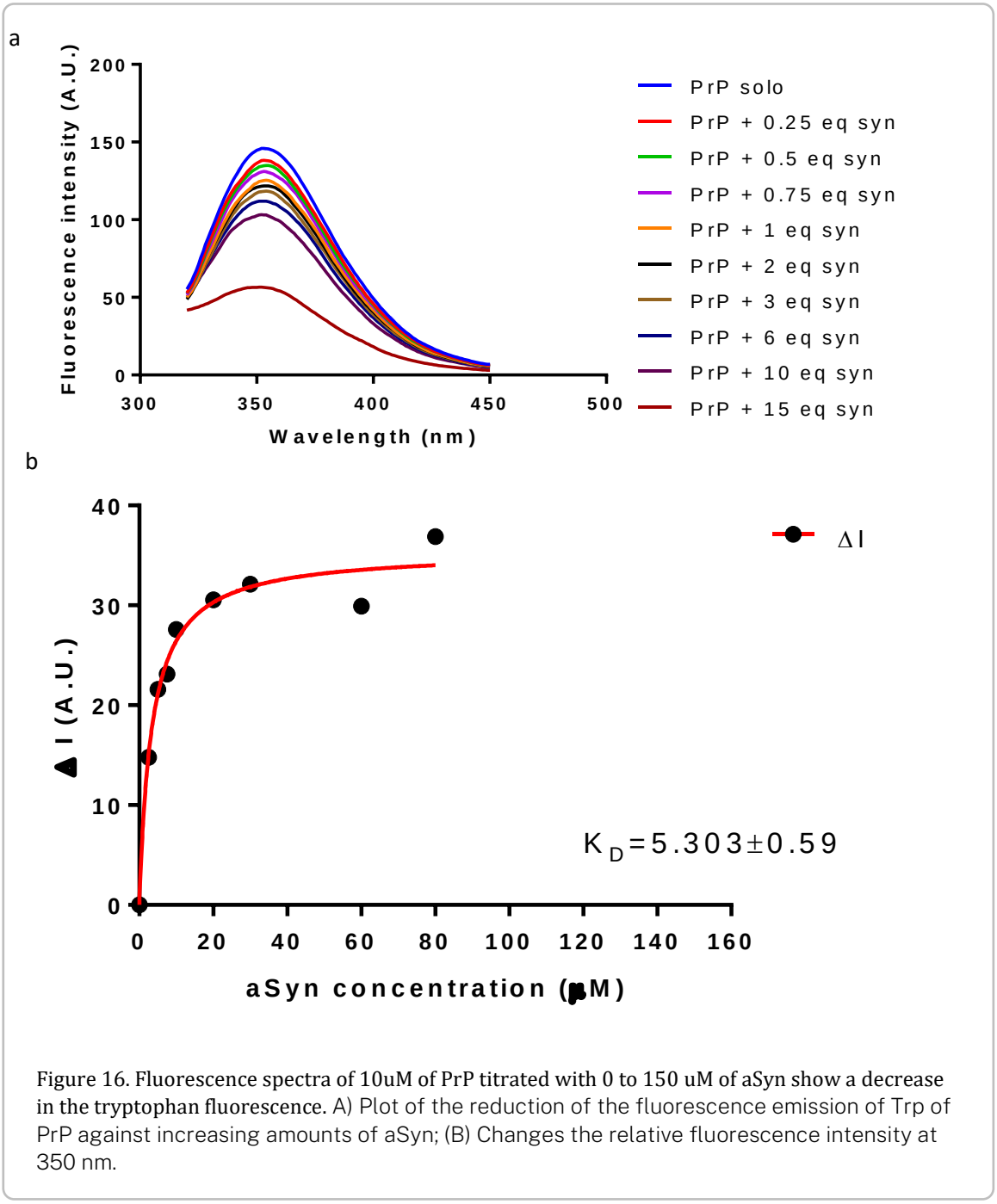
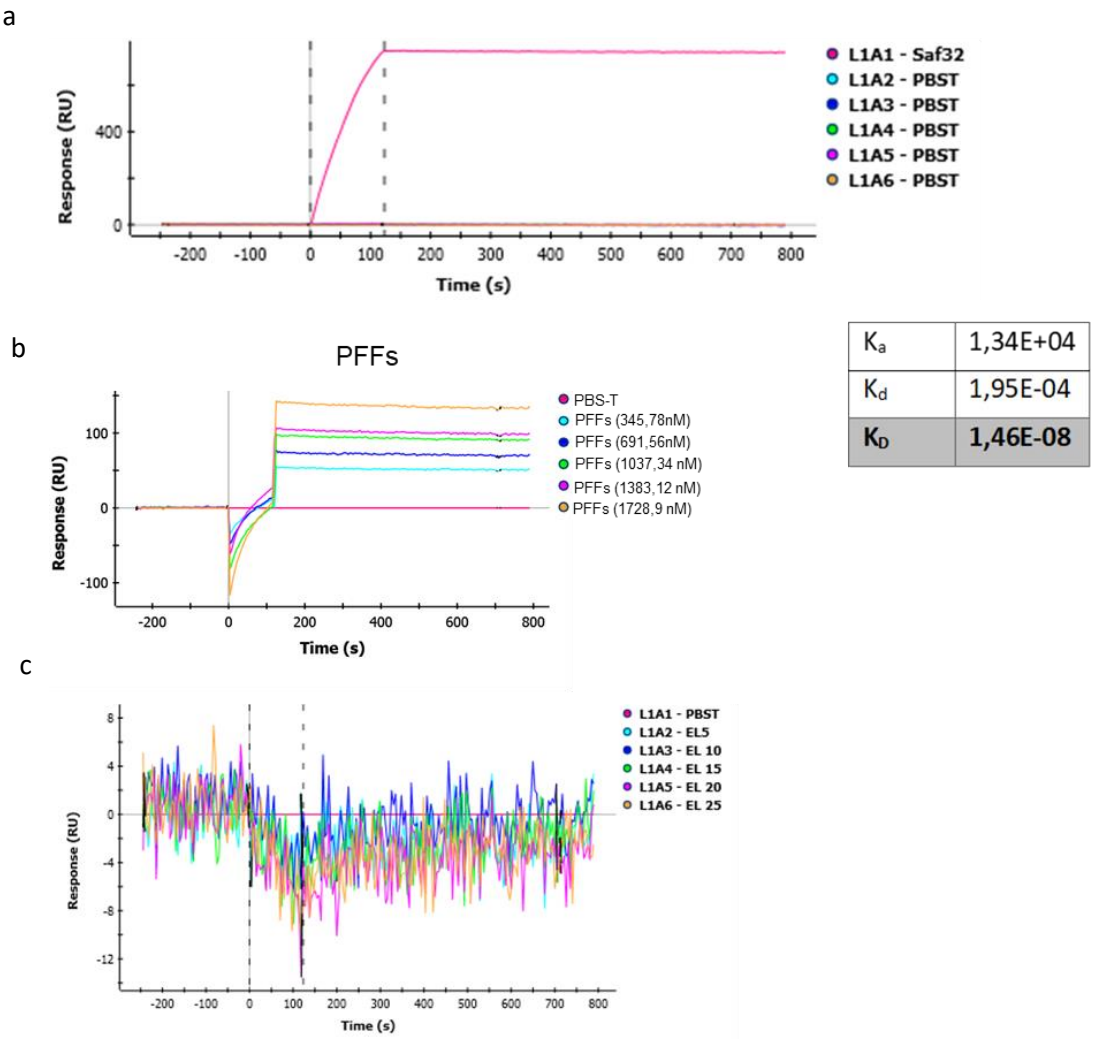


Figure 16. Fluorescence spectra of 10uM of PrP titrated with 0 to 150 uM of aSyn show a decrease in the tryptophan fluorescence. A) Plot of the reduction of the fluorescence emission of Trp of PrP against increasing amounts of aSyn; (B) Changes the relative fluorescence intensity at 350 nm.

4.1.4. aSyn PFFs show direct interaction with PrP^C in SPR

To further study the equilibrium between association and dissociation of the interaction between aSyn and PrP, we performed SPR spectrometry. We used a Biacore T200 instrument and immobilized PrP onto a GLC chip using standard amine-coupling chemistry. To verify successful anchoring of PrP, we ran a positive control using the SAF32 antibody, which is specific to PrP.

PFFs of aSyn were added at different concentrations and the SPR signal was monitored in real-time. The interaction between aSyn and PrP was detected, and a Langmuir model was used to calculate the K_D for the interaction. The K_D was found to be within the range



K_a	1,34E+04
K_d	1,95E-04
K_D	**1,46E-08**

Figure 17. Detection of direct interaction between PrP and aSyn by surface plasmon resonance. Recombinant PrP (rPrP; 20 ng/mL) was coupled to a GLC chip. A) Binding of anti-PrP mAb SAF32 indicates that the PrP ligand is bound to the GLC chip. B) SPR sensogram of the recombinant PrP and PFFs of aSyn interaction. PFFs aSyn (345.78 nM; 691.56 nM; 1037.34 nM; 1383.12 nM and 1728.9 nM) was injected as an analyte. For fitting, the 1:1 Langmuir model was used. The table shows the measured binding values C) Reference sensogram without any ligand.

of other reported protein-protein interactions, indicating a strong interaction between aSyn and PrP (Figure 17).The use of SPR allowed us to not only confirm the interaction between aSyn and PrP, but also provided kinetic information about the interaction, such as the K_a and K_d. This method can be particularly useful for identifying potential drugs or inhibitors that may interfere with the interaction between aSyn and PrP, as it allows for the screening of large numbers of compounds in a relatively short amount of time.

4.2. EFFECT OF THE INTERACTION ON THE AGGREGATION PROPENSITY OF aSYN

4.2.1. PrP^C potentiates aSyn seeded-aggregation *in vitro*

As seen in the previous subchapter, PrP interacts with the C-terminal of aSyn, which has been implicated in aSyn oligomerization and fibrillation. To investigate the potential modulatory effect of PrP on aSyn aggregation, we employed the real-time quaking-induced conversion (RT-QuIC) assay. This assay allows for the detection of amyloid fibril formation through the incorporation of a seeding agent (PFFs in our case) into monomeric aSyn, which leads to the amplification of the fibrillar signal over time.

We incubated recombinant aSyn with or without PrP in the presence of PFF seeds and used BSA as a molecular crowding control, as previous studies have shown that molecular crowding can accelerate aSyn aggregation(Biswas *et al.*, 2021). Notably, samples containing PrP alone or a mixture of PrP with PFF seeds did not show any increase in ThT fluorescence at any time during the experiment, suggesting that only aSyn aggregation was detected.

By quantifying the monomer incorporation rate and analyzing the area under the curve, we determined that the presence of PrP increased aSyn aggregation and made the reaction more efficient (Figure 18). These results suggest that PrP may play a role in aSyn aggregation and highlight the potential for modulatory effects of PrP in neurodegenerative diseases.

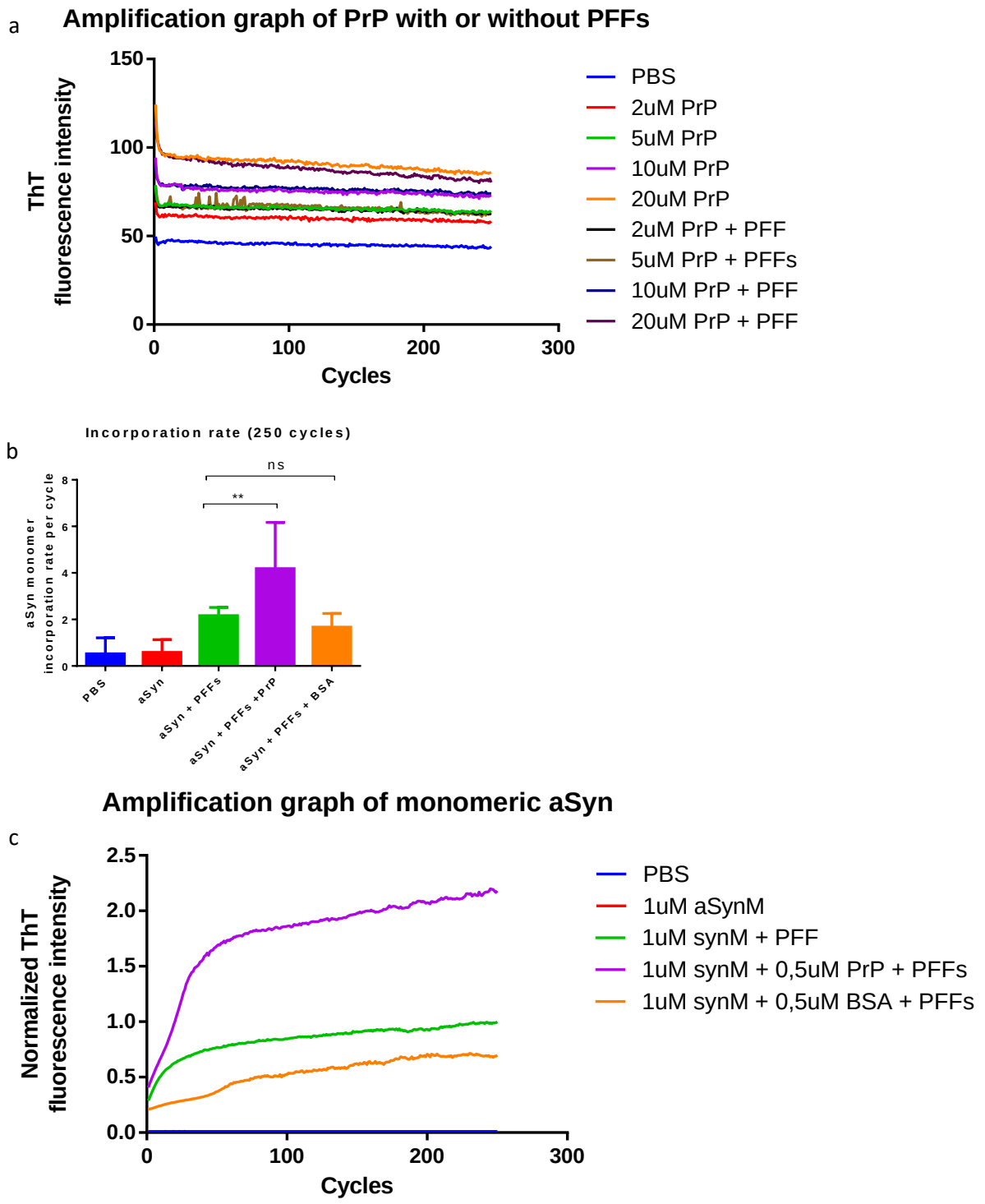


Figure 18. PrP increases aSyn aggregation when seeded. a) Graph of amplification of different PrP concentrations with increasing amounts of PFFs. Fluorescence increase was observed in any of the conditions b) RT-QuIC results show a significant increase in monomer incorporation rate per cycle in the presence of 0.5 μM PrP. . Blue: Only PBS was inserted into the mix; red: 1 μM of recombinant aSyn seeded with 0.7 μM of PFFs; green: 1 μM of recombinant aSyn seeded with 0.7 μM of PFFs and 0.5 μM of recombinant PrP; purple: 1 μM of recombinant aSyn seeded with 0.7 μM of PFFs and 0.5 μM of BSA. Statistical analysis: unpaired t-test and multiple comparison test between conditions in the presence of PrP or BSA with untreated condition with aSyn c) RT-QuiC graph of the monomeric aSyn (1 μM) amplification in the presence or absence of PrP (0.5 μM) (representative out of N=7). BSA was using as a molecular crowding control, being present in the same molarity as PrP. Protein amplified in presence of 0.5 μM of recombinant PrP shows higher ThT fluorescence intensity than in its absence. Blue: Only PBS was inserted into the mix; red: 1 μM of recombinant aSyn seeded with 0.7 μM of PFFs; green: 1 μM of recombinant aSyn seeded with 0.7 μM of PFFs and 0.5 μM of recombinant PrP; purple: 1 μM of recombinant aSyn seeded with 0.7 μM of PFFs and 0.5 μM of BSA. All data are expressed as mean ± SEM; Student's t-test; * p-value < 0.05, ** p-value < 0.01, ***p-value < 0.00);

4.2.2. PrP^C expression does not increase number of aSyn aggregates but colocalizes with pSer129 aSyn

We used HEK293 cells stably expressing aSyn-GFP to investigate the seeding properties of exogenous PFFs of aSyn in endogenous aSyn in relation to PrP expression. In addition, we stained the cells for phosphorylated aSyn (p-aSyn) to verify the presence of p-aSyn-positive exogenous aggregates. ICC was performed on aSyn-GFP-expressing HEK293 cells transfected with PrP-Kozak or an empty vector, as a control, and treated with recombinant aSyn. Staining was performed with p-aSyn (red), PrP (magenta), and DAPI (blue) (Figure 19). The number and size of aSyn accumulations were consistent across all conditions, independent of treatment or PrP expression. Accumulations were visible in all conditions.

As shown in Figure 20, O/E PrP did not affect the number and size of endogenous aSyn accumulations, as well as the number, intensity, and size of p-aSyn accumulations. The results were consistent with those obtained in cells transfected with an empty vector.

We also conducted Pearson's colocalization coefficient analysis for all conditions to examine the colocalization between p-aSyn accumulations and PrP. Strong colocalization was observed between aSyn and PrP, as analyzed by staining of PrP and endogenous aSyn, PrP and p-aSyn, and endogenous aSyn and p-aSyn (Figure 21). We observed increased colocalization of p-aSyn accumulations with PrP, as compared to most aSyn accumulation. The results are consistent with our hypothesis that PrP plays a role in the propagation of aSyn pathology.

In summary, our findings suggest that PrP expression does not influence the number and size of aSyn accumulations, but may enhance the colocalization of p-aSyn accumulations with PrP (see Figures 19, 20, and 21). These results contribute to our understanding of the role of PrP in aSyn pathology and may provide insights into potential therapeutic targets for PD.

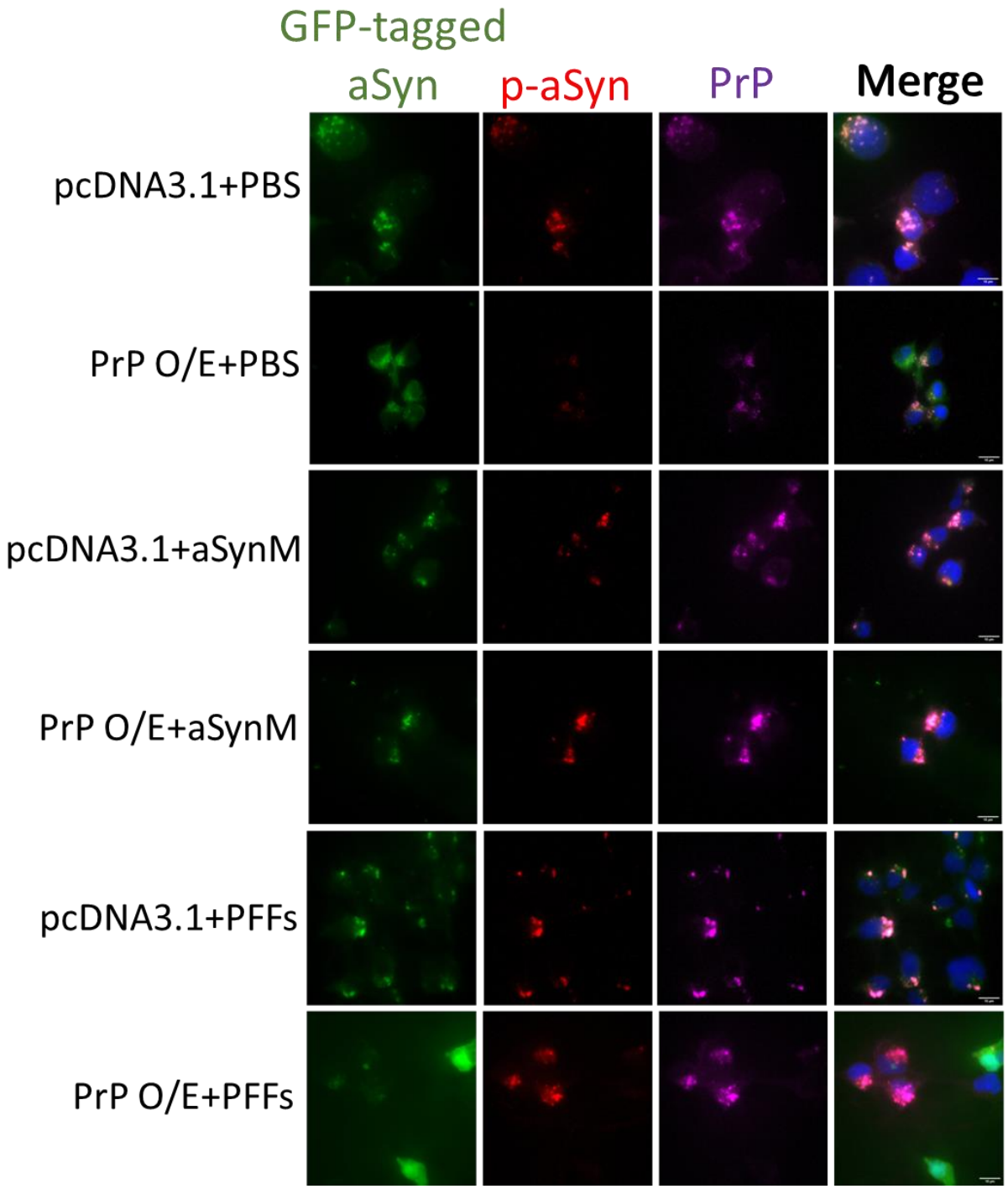


Figure 19. ICC of PrP transfected and treated aSyn-GFP HEK293 cells. aSyn-GFP-expressing HEK293 cells transfected with PrP-Kozak or empty vector were treated with recombinant aSyn and staining was performed with p-aSyn (red), PrP (magenta), and DAPI (blue). Scale bar: 10 μm

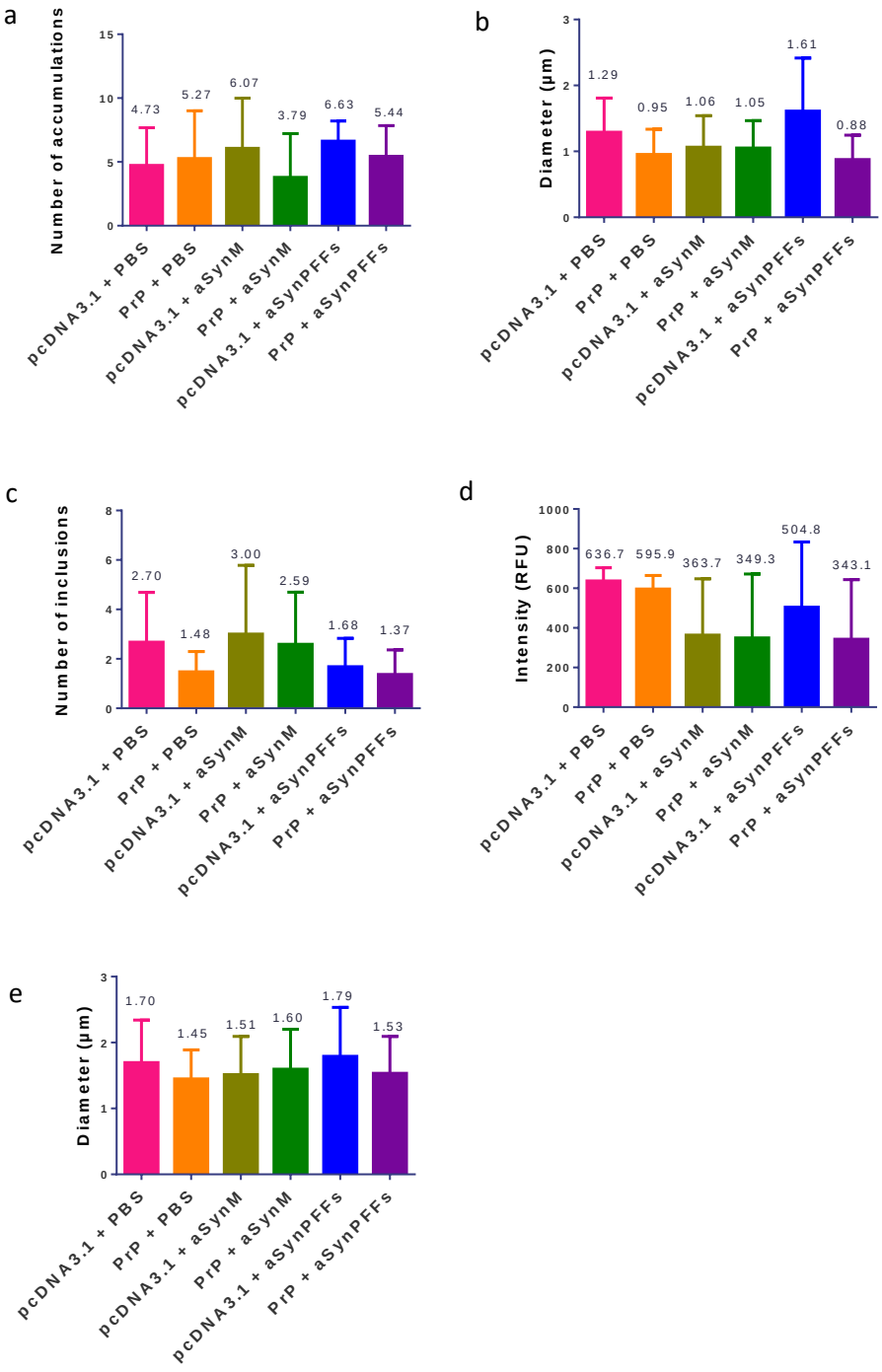


Figure 20. Overexpressed PrP does not influence aSyn accumulations. For endogenous aSyn number (a) and size (b) of accumulations do not differ between conditions. Likewise, there are no differences in number (c), intensity (d) or size (e) of phosphorylated aSyn accumulations.

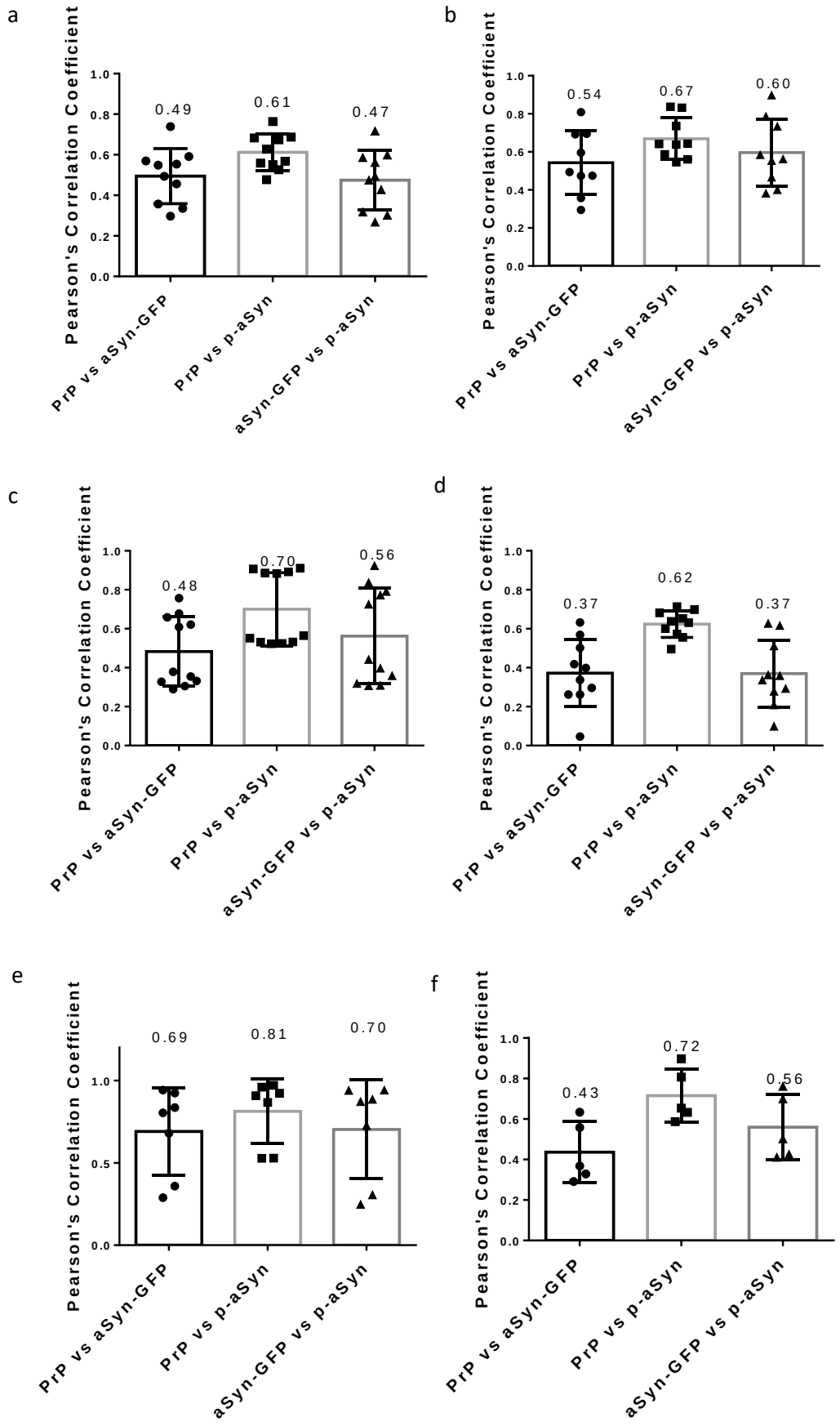


Figure 21. Strong colocalization between aSyn and PrP was detected. Analysed staining of PrP and endogenous aSyn, PrP and phosphorylated aSyn, and endogenous aSyn and phosphorylated aSyn does show higher colocalization between PrP and p-aSyn compared with PrP and aSyn-GFP. Colocalization of cells transfected empty vector (graph a, b and c) compared with cells O/E PrP (graph d, e and f), treated with PBS (a and d), aSyn monomers (b and e) or aSyn PFFs (c and f).

4.3. UPTAKE OF RECOMBINANT aSYN IN CELLULAR MODELS

4.3.1. PrP^C does not increase internalization of aSyn species, suggesting a role as a sensor

We first confirmed that PrP^C expression was increased in cells transfected with a plasmid encoding PrP^C with a Kozak sequence compared to cells transfected with an empty pcDNA3.1. vector, as shown by a representative western blot stained against aSyn, PrP, and β-actin (Figure 22a). Quantification of the relative expression levels of aSyn and PrP showed that aSyn expression was significantly higher in cells O/E PrP and treated with aSyn PFFs (Figure 22b).

When cells were treated with monomeric aSyn or aSyn PFFs or PBS, the relative aSyn expression did not differ between WT and PrP O/E cells (Figure 22c). However, there was a tendency of higher aSyn uptake in PrP O/E cells after both treatments. When cells were treated with aSyn PFFs, aSyn uptake was significantly higher in both WT and PrP O/E cells (Figure 22d). These results suggest that the presence of PrP may enhance aSyn uptake, especially when aSyn is in its aggregated form. This could potentially lead to increased aSyn pathology in the presence of PrP. This data supports the hypothesis that PrP may play a role in the progression of synucleinopathies such as PD.

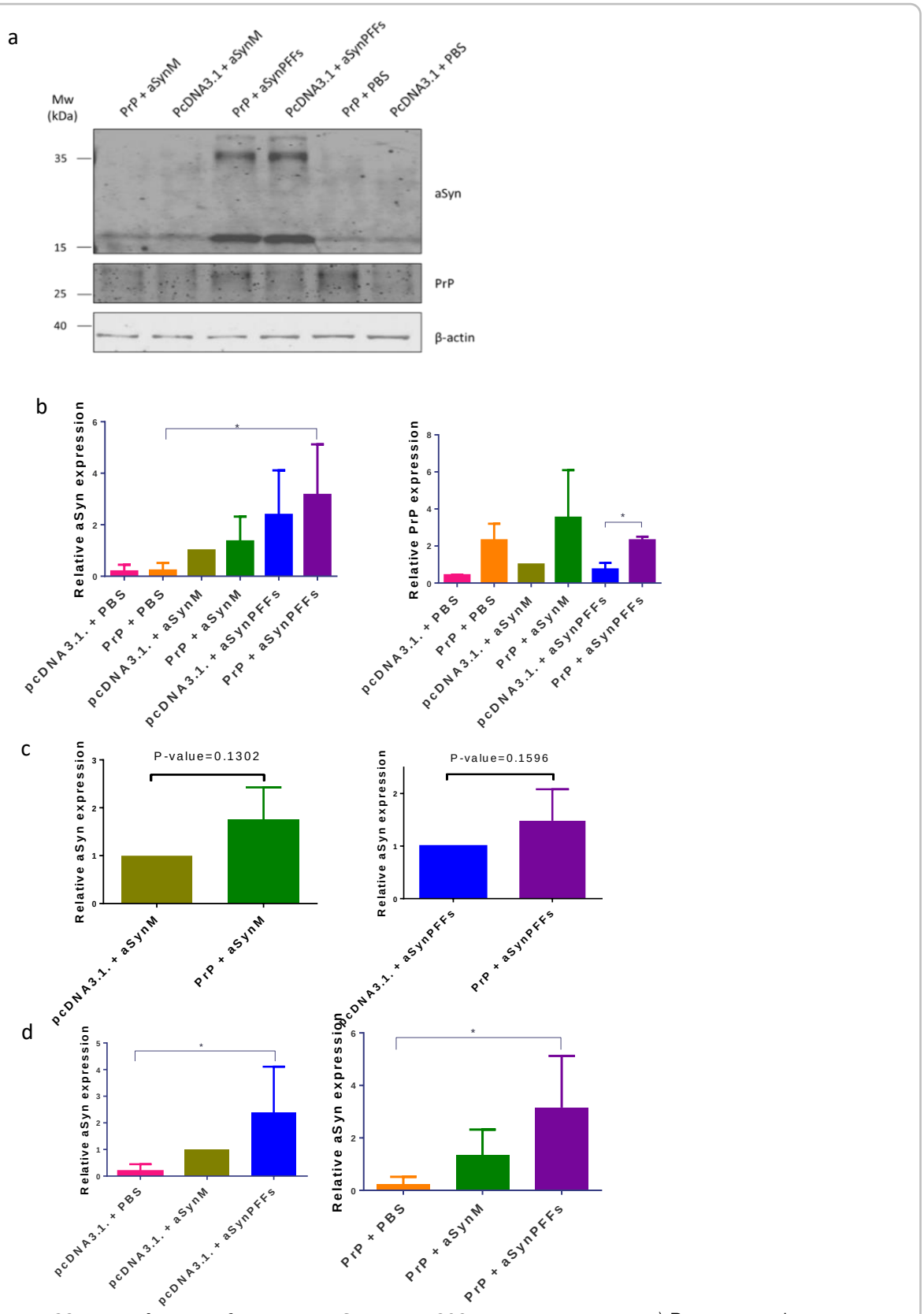


Figure 22. Internalization of exogenous aSyn in HEK293 overexpressing PrP. a) Representative western blot stained against aSyn, PrP and β-actin. (b) Quantification of relative aSyn expression and relative PrP expression. Cells without aSyn treatment show low levels of endogenous aSyn. Expression levels of aSyn was significantly higher in PrP transfected cells after treatment with aSyn PFFs. PrP expression is higher in PrP overexpressing cells. (c) Relative aSyn expression does not differ WT and PrP over-expressing cells after treatment with monomeric aSyn or with aSyn PFFs. Data shows tendency of higher aSyn uptake in PrP overexpressing cells after both treatments. (d) aSyn uptake is significantly higher after PFF treatment in WT and PrP overexpressing cells. N=4, *p<0.05

4.4. UPTAKE OF RECOMBINANT aSYN IN CELLULAR MODELS

4.4.1. Interaction between PrP^C and aSyn is conformation specific in cellular models

BiFC is a commonly used technique for validating protein-protein interactions within cells. In this technique, two proteins of interest are fused to complementary fragments of a fluorescent protein, allowing visualization of their interaction within cells. In our study, we used BiFC to investigate the interaction between aSyn and PrP^C.

To achieve this, we cloned a venus fragment 1 fused to the N-terminal of aSyn (VN-aSyn) and PrP^C (VN-PrP), and a venus fragment 2 fused to the C-terminal of aSyn (aSyn-VC) and PrP^C (PrP-VC). This approach has been widely used to investigate aSyn oligomerization and the interaction between aSyn monomers (Outeiro *et al.*, 2008).

We first co-transfected HEK293 cells with aSyn to confirm aSyn oligomerization, which resulted in fluorescence as expected. We then co-transfected cells with VN-aSyn and PrP-VC, aSyn-VC and VN-PrP, and VN-PrP and PrP-VC. Expression was confirmed using western blot and ICC (Figure 23 and 24). We observed Venus fluorescence only when the fragments were in the N-terminal of PrP^C and C-terminal of aSyn. Interestingly, we did not observe fluorescence when tagging the C-terminal of PrP and N-terminal of aSyn (Figure 24), although the proteins were still being expressed as confirmed by SDS-PAGE and immunolabeling. This suggests that the interaction between aSyn and PrP^C in cell models is orientation-specific.

Furthermore, we observed that the interaction between aSyn and PrP^C was only visible in the perinuclear region, indicating that it may be occurring in the ER-Golgi network. These findings provide insight into the mechanism of interaction between aSyn and PrP^C and may help in developing therapeutic interventions for synucleinopathies.

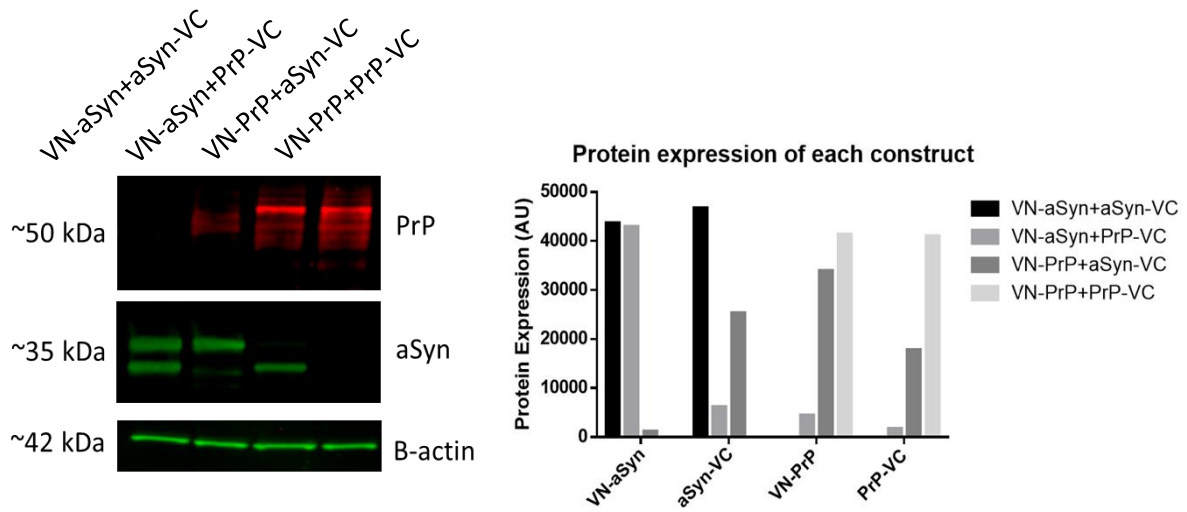


Figure 23. Expression of the co-transfected vectors in HEK293 cells. Both vectors are being stably expressed in HEK293, as seen by western blot. Protein basally expressed is not detectable.

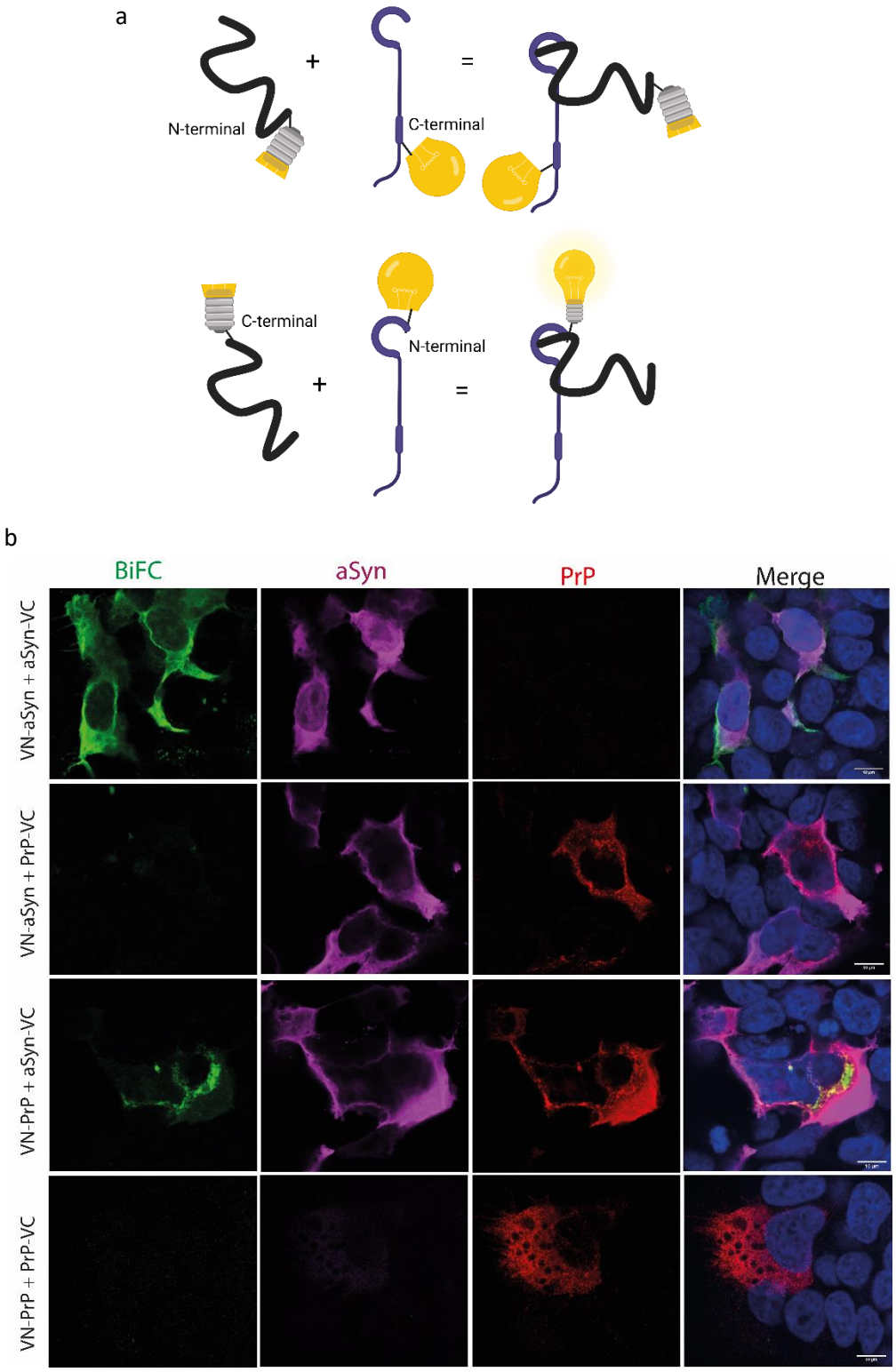


Figure 24. Interaction betweeen aSyn and PrP is detected and orientation specific. a) Schematic representation of BiFC. b) fluorescence from the BiFC signal was only seen with both aSyn constructs due to oligomerization and with N-terminal tagged PrP co-transfected with C-terminal tagged aSyn, suggesting that the interaction in cells is orientation specific.

4.4.2. PrP strongly colocalizes with aSyn in HEK293 cells

To further verify the interaction between aSyn and PrP and the specificity of the BiFC signal, we performed different colocalization analyses. Pearson's correlation coefficient was used to measure the correlation of the intensity distribution between channels. We observed stronger colocalization between PrP and the BiFC signal, suggesting a strong relationship between the signal intensity and localization between these two proteins. However, the lower correlation values between aSyn and the BiFC signal were due to the contrasting strong signal of aSyn, which covered most of the cytoplasm, compared to the BiFC signal, which was mostly visible in the perinuclear area.

This observation was further corroborated by the Mander's correlation coefficient, which describes the contribution of each channel to the pixels of interest. We found a strong colocalization between the PrP signal with BiFC, indicating that most of the PrP present intracellularly is interacting with aSyn. However, we did not observe a strong colocalization between aSyn and PrP with the BiFC signal, suggesting that not all aSyn is interacting with PrP intracellularly, or that the interaction is not specific to the region of interest (Figure 25).

Overall, these colocalization analyses further support the specificity of the BiFC signal and confirm the interaction between aSyn and PrP in intracellular regions.

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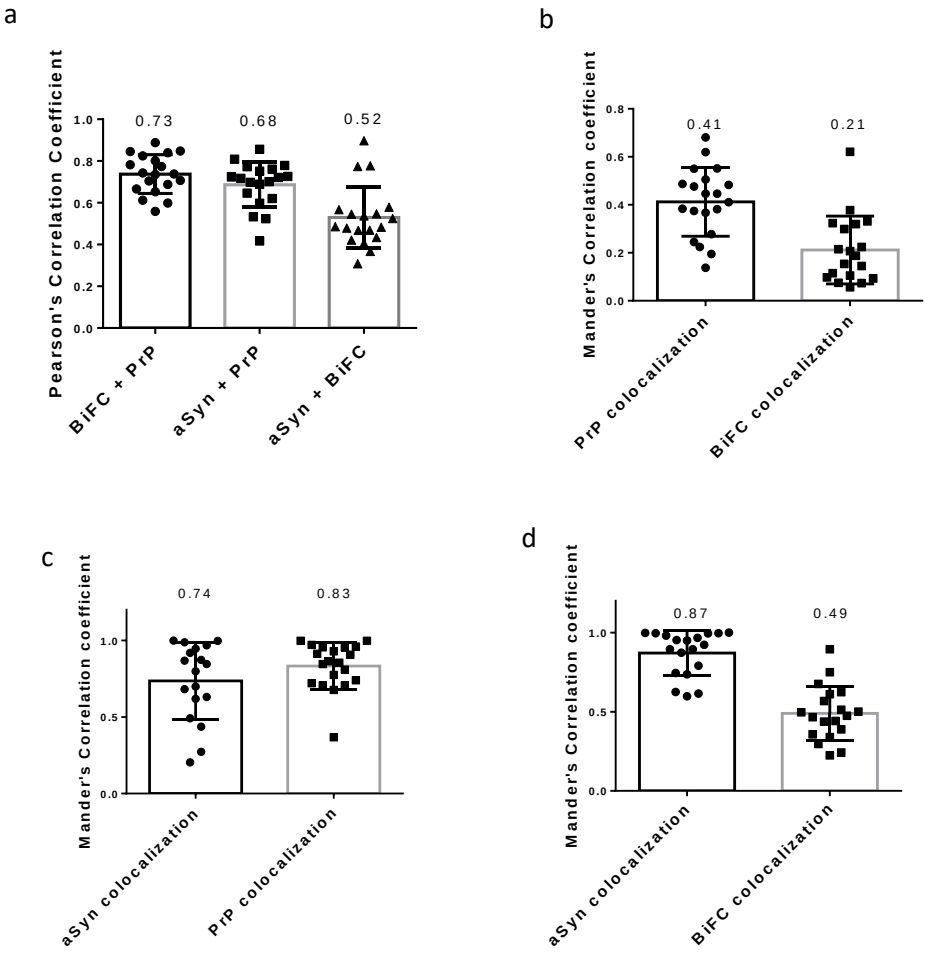

Figure 25. Colocalization between the BiFC, aSyn and PrP signals. On the top left we can observe the values for pearson's correlation coefficient. Top right, the Mander's correlation coefficient of the aSyn signal with different signals, bottom left the MCC for BiFC with different signals and lower right, the MCC for the PrP signal with the other signals.

CHAPTER 5

Discussion

5.1. aSyn interacts directly with PrP in an orientation specific way

Previous research has produced conflicting findings regarding the interaction between aSyn and PrP (Ferreira *et al.*, 2017; Brás, Lopes and Outeiro, 2018; La Vitola *et al.*, 2019; Domingues *et al.*, 2022). While some studies suggest that a direct interaction is possible, others have proposed that a macromolecular complex, not involving direct interaction between these two proteins, may be involved (Domingues *et al.*, 2022). The oligomeric state of aSyn is also a point of contention, with some studies indicating that only oligomeric aSyn interacts with PrP while others suggest that monomeric aSyn could also interact (Ferreira *et al.*, 2017; Urrea *et al.*, 2017). Nonetheless, prior work has identified the interacting region of PrP and demonstrated the inhibition of PrP as a means of reversing synuclein pathology, aSyn spreading, and calcium cytotoxicity (Ferreira *et al.*, 2017; Thom *et al.*, 2021). Therefore, characterizing the interacting region and nature of the interaction between aSyn and PrP is crucial for developing strategies to address problems associated with this interaction.

Previous research has shown that the internalization of aSyn and its detrimental effects could be modulated by PrP, however by understanding the nature of this interaction, we can further understand if the N-terminal effects could be playing a role simultaneously, such as the interaction with DJ-1 (Burbulla *et al.*, 2017). In addition to that, it is known that a big part of aSyn present in pathology can be truncated, which could modulate this interaction in strength.

Our study shows that the acidic C-terminal region of aSyn primarily interacts with PrP through electrostatic interactions. We hypothesize that tryptophan residues on PrP may also bind with the C-terminal tyrosine residues of aSyn through aromatic interactions. Moreover, our results suggest that the specific orientation of the interaction is essential for intracellular interactions between aSyn and PrP. Other factors, such as PTMs and mutations of aSyn and the conformational states of aSyn, may also impact the interaction with PrP. Therefore, future studies could investigate the role of these additional factors in the interaction between aSyn and PrP.

In addition to that, mutations that have already shown to affect the interaction of aSyn with the membranes could also provide different interacting strengths with PrP. Analyzing and comparing the different K_D using the different methods described in this work could further the idea that that different species of aSyn can cause neurodegeneration using different

methods, and supporting the idea that conflicting results may be cause by differences in the aSyn production methodologies.

Our study provides valuable insights into the nature of the interaction and may aid in the development of more targeted therapeutic approaches for synucleinopathies. Targeting the C-terminal region of aSyn or the tryptophans of PrP may be a promising strategy for preventing or reversing the effects of aSyn aggregation and spreading. Additionally, residue-specific mutations and biophysical techniques could be used to further explore the effects of this interaction.

Furthermore, our study opens up new avenues for exploring the role of PrP in neurodegeneration beyond its well-established involvement in prion diseases. The specific identification of the aSyn-PrP interaction could have important therapeutic implications. Developing compounds that specifically target the identified interaction between aSyn and PrP, such as those targeting the C-terminal of aSyn or the N-terminal of PrP, could lead to more specific and effective therapies. Additionally, the identification of the key residues involved in the interaction could guide the development of immunotherapies targeting these residues, providing a more targeted approach to treating synucleinopathies.

Overall, our study represents an important step forward in our understanding of the complex interactions between aSyn and PrP and provides a foundation for future research aimed at developing more effective treatments for neurodegenerative diseases. The potential biophysical effects of the aSyn-PrP interaction may also have therapeutic implications, such as the potential role of PTMs in protein conformation or dynamics. Finally, the use of computational methods to predict the specific amino acids involved in protein-protein interactions may have wider applications in the field of drug discovery and protein engineering.

5.2. PrP may potentiate aSyn oligomerization

The role of membrane receptors in synucleinopathies is mostly focused on the effect in spreading and activation of pathological and cytotoxic pathways. In the case of PrP, due to the interaction with aSyn C-terminal we wanted to assess if there was any potential role in aSyn aggregation kinetics.

Previous research has demonstrated that aSyn could cluster with PrP into micron-sized condensates (Rösener *et al.*, 2020; Agarwal *et al.*, 2022), however it was unclear the effect of PrP in the seeded aggregation of aSyn.

Our results showed that the presence of PrP significantly enhanced seeded aSyn aggregation, as evidenced by a significant increase in monomer incorporation rate per cycle in the RT-QuIC assay. However, interestingly, we found that these results were not consistent in our cellular models, suggesting either a possible sensor role in the cells or a survivorship bias in H4 cells (Tanaka *et al.*, 2004; Gonçalves, Matos and Outeiro, 2010; Markopoulou *et al.*, 2014; Brás, Lopes and Outeiro, 2018). Therefore, further studies are warranted to decipher the underlying mechanisms of PrP-aSyn interaction.

To gain insight into the role of PrP in aSyn aggregation, we propose future studies to investigate the aggregation kinetics in cellular models using different time points, in order to evaluate the early stages of aggregate formation, or their potential toxic/cytoprotective roles. Additionally, other biophysical methods such as transmission electron microscopy or Fourier-transform infrared could be used to analyze the aggregates derived from this interaction to provide more information regarding possible structural differences, seeding properties, and the potential role that these aggregates could play in the pathology.

In addition to the potential role of PrP in aSyn aggregation kinetics, our observation of colocalization between PrP and p-aSyn suggests that PrP could play a role in aSyn phosphorylation. PrP has been previously reported to interact with different proteins, triggering various downstream signaling pathways, including those related to phosphorylation. This suggests the possibility of a feedback loop where PrP-aSyn interaction may increase aSyn phosphorylation levels in the cell, further exacerbating the disease pathology. Investigating the precise mechanisms underlying PrP-aSyn interaction and its effect on aSyn phosphorylation will be crucial in developing targeted therapeutic interventions for synucleinopathies.

Furthermore, our study highlights the need for a more comprehensive understanding of aSyn pathology, which may involve complex interplay between different cellular components and membrane receptors. In this context, exploring the role of other membrane receptors and their interaction with aSyn may provide additional insights into the disease mechanisms. Additionally, investigating the potential impact of aSyn aggregates formed via PrP-aSyn interaction on cellular toxicity and spreading could reveal new potential for therapeutic intervention. Potential therapeutic targets could include the inhibition of PrP-

aSyn interaction or the modulation of PrP expression. Inhibiting the PrP-aSyn interaction could potentially prevent or slow down the process of aSyn aggregation, thereby reducing the burden of protein aggregates in the brain. Additionally, targeting the phosphorylation pathways of aSyn could also be a promising approach, as it has been suggested that the accumulation of phosphorylated aSyn is a key factor in the development of synucleinopathies. Ultimately, more research is needed to fully understand the mechanisms of PrP-aSyn interaction and the role of PrP in aSyn pathology, in order to develop effective therapies targeting these processes.

In conclusion, our study provides evidence for the potential role of PrP in aSyn aggregation kinetics and phosphorylation, with implications for the development of targeted interventions for synucleinopathies. Further investigation into the mechanisms underlying PrP-aSyn interaction, as well as exploration of other membrane receptors and their interaction with aSyn, could provide a more comprehensive understanding of the disease pathology and identify novel therapeutic targets.

5.3. Intracellular potential effects of PrP interaction with aSyn

Using BiFC method, we verified that the fluorescence is detected intracellularly in the perinuclear region. This suggests that not only this interaction can happen extracellularly, where the mature PrP is mostly found, but also intracellularly, possibly during the protein production of PrP.

This could suggest further potential roles in not only excitotoxicity and spreading, but it could also potentially affect the overall efficiency and accuracy of protein synthesis in the cell, leading to downstream effects on cellular homeostasis and function. However, further research is needed to fully elucidate the potential impacts of this interaction on cellular processes. The interaction between PrP and aSyn during PrP protein production could potentially affect protein synthesis mechanisms. A previous study has shown that PrP interacts with ribosomal proteins and is associated with polysomes, suggesting that PrP may play a role in translation regulation. The presence of aSyn during this process may modulate the translation of specific proteins and could impact overall cellular protein synthesis. Additionally, aSyn has been shown to interact with ribosomes and affect their function, suggesting that the interaction between aSyn and PrP during protein synthesis could have a significant impact on ribosomal function and, in turn, on cellular homeostasis.

The interaction between PrP and aSyn could also potentially impact aSyn protein folding. A previous study has shown that PrP interacts with other proteins involved in protein folding, such as Hsp70, suggesting that PrP may play a role in the folding of misfolded proteins. The interaction between aSyn and PrP during protein production may affect the folding of aSyn and contribute to its misfolding and aggregation. Additionally, aSyn has been shown to interact with chaperone proteins, and the interaction between aSyn and PrP could potentially impact the function of these chaperones, further contributing to aSyn aggregation.

The interaction between PrP and aSyn during protein production could potentially impact the membrane binding properties of aSyn. It has been shown that PrP interacts with lipid membranes, and aSyn has a high affinity for lipid membranes, which is thought to play a key role in aSyn aggregation and toxicity. The interaction between PrP and aSyn during protein production may modulate the membrane binding properties of aSyn and affect its aggregation and toxicity. Additionally, a previous study has shown that aSyn affects membrane permeability and could impact calcium signaling and mitochondrial function, which could further contribute to neurodegeneration. The interaction between PrP and aSyn during protein production may impact aSyn-mediated alterations in calcium signaling and mitochondrial function and contribute to neurodegeneration.

The intracellular interaction between PrP and aSyn may also have implications for cellular energy production and oxidative stress. It has been suggested that aSyn accumulation in neurons can disrupt mitochondrial function, leading to decreased ATP production and increased oxidative stress. In addition, studies have shown that PrP can play a role in mitochondrial function and calcium homeostasis. Therefore, it is possible that the interaction between PrP and aSyn could exacerbate the effects of aSyn accumulation on mitochondria and calcium signaling, leading to increased oxidative stress and energy deficits in affected cells. Further studies are needed to determine the exact mechanisms underlying these potential effects.

Finally, the interaction between PrP and aSyn may have implications for the immune response in the brain. Recent studies have shown that microglia, the resident immune cells in the brain, play a crucial role in clearing aSyn aggregates and preventing their spread. In addition, studies have suggested that PrP may play a role in the activation of microglia and the immune response. Therefore, it is possible that the interaction between PrP and aSyn could impact microglial function and immune response in the brain. Further studies are

needed to determine the exact nature of this interaction and its implications for the immune response in synucleinopathies.

In conclusion, the potential impact on aSyn aggregation kinetics and membrane binding properties could have important implications for the progression of synucleinopathies. Furthermore, the possibility of PrP playing a role in aSyn phosphorylation via a feedback loop could have implications for the development of therapeutics targeting the PrP-aSyn interaction. Overall, these findings highlight the importance of studying the intricate molecular mechanisms involved in the pathogenesis of synucleinopathies, and suggest potential avenues for the development of new therapies to combat these devastating diseases.

CHAPTER 6

Conclusion

6. SUMMARY AND CONCLUSION

The study of the interaction between aSyn and PrP has been a subject of significant research interest due to their involvement in the pathology of neurodegenerative diseases. The findings of previous studies on the interaction between these two proteins have been conflicting, and the oligomeric state of aSyn has been a point of contention. This study contributes to our understanding of the nature of the interaction between aSyn and PrP by identifying the interacting region of PrP and demonstrating the importance of the specific orientation of the interaction.

The study shows that the acidic C-terminal region of aSyn primarily interacts with PrP through electrostatic interactions, and the tryptophan residues on PrP may also bind with the C-terminal tyrosine residues of aSyn through aromatic interactions. The identification of the key residues involved in this interaction could guide the development of immunotherapies targeting these residues, thus providing a more targeted approach to treating synucleinopathies. Furthermore, the use of computational methods to predict the specific amino acids involved in protein-protein interactions may have wider applications in the field of drug discovery and protein engineering.

The study also highlights the potential role of PrP in aSyn aggregation kinetics, as evidenced by a significant increase in monomer incorporation rate per cycle in the RT-QuIC assay. However, the results were not consistent in cellular models, suggesting a possible sensor role in the cells or survivorship bias in H4 cells. Further studies are needed to understand the underlying mechanisms of PrP-aSyn interaction and its effect on aSyn phosphorylation.

Future studies could investigate the role of other membrane receptors and their interaction with aSyn, which may provide additional insights into the disease mechanisms. Additionally, investigating the potential impact of aSyn aggregates formed via PrP-aSyn interaction on cellular toxicity and spreading could reveal new key targets for therapeutic intervention.

Targeting the C-terminal region of aSyn or the tryptophans of PrP may be a promising strategy for preventing or reversing the effects of aSyn aggregation and spreading. Additionally, residue-specific mutations and biophysical techniques could be used to further explore the effects of this interaction. Developing compounds that specifically target the identified effects between aSyn and PrP, such as those targeting the C-terminal of aSyn or the N-terminal of PrP, could lead to more specific and effective therapies.

Investigating the precise mechanisms underlying PrP-aSyn interaction and its effect on aSyn phosphorylation will be crucial in developing targeted therapeutic interventions for synucleinopathies. Inhibiting the PrP-aSyn interaction could potentially prevent or slow down the process of aSyn aggregation, thereby reducing the burden of protein aggregates in the brain. Additionally, targeting the phosphorylation pathways of aSyn could be a promising therapeutic approach.

Overall, the study represents an important step forward in our understanding of the complex interactions between aSyn and PrP and provides a foundation for future research aimed at developing more effective treatments for neurodegenerative diseases. The potential biophysical effects of the aSyn-PrP interaction may also have therapeutic implications, such as the potential role of PTMs in protein conformation or dynamics. This study highlights the need for a more comprehensive understanding of aSyn pathology, which may involve complex interplay between different cellular components and membrane receptors.

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