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Pathophysiological effects of alpha-synuclein on SNARE complex proteins in models of alpha-synucleinopathies

EMMA BROLIN



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UPPSALA
2022

ISSN 1651-6206
ISBN 978-91-513-1648-2
URN urn:nbn:se:uu:diva-487286

Dissertation presented at Uppsala University to be publicly examined in Rudbecksalen, Rudbecklaboratoriet, Dag Hammarskjölds Väg 20, Uppsala, Thursday, 15 December 2022 at 09:15 for the degree of Doctor of Philosophy (Faculty of Medicine). The examination will be conducted in English. Faculty examiner: Associate professor Arianna Bellucci (Department of Molecular and Translational Medicine, University of Brescia, Italy).

Abstract

Brolin, E. 2022. Pathophysiological effects of alpha-synuclein on SNARE complex proteins in models of alpha-synucleinopathies. *Digital Comprehensive Summaries of Uppsala Dissertations from the Faculty of Medicine* 1882. 65 pp. Uppsala: Acta Universitatis Upsaliensis. ISBN 978-91-513-1648-2.

Accumulation and spread of alpha-synuclein (α -syn) aggregates are central to the disease pathogenesis of Parkinson's disease and dementia with Lewy bodies, collectively known as α -synucleinopathies. Native α -syn is a monomeric presynaptic protein that can act as a molecular chaperone for the soluble N-ethylmaleimide-sensitive factor attachment protein receptor (SNARE) complex assembly. The aim of this thesis was to investigate the pathophysiological effect of different α -syn species on SNARE protein distribution and to study the involvement of extracellular vesicles (EVs) in the propagation of α -syn pathology.

In **paper I**, the co-localization between α -syn and the SNARE proteins VAMP-2, SNAP-25 and syntaxin-1, was analyzed in primary cortical neurons from transgenic (tg) human A30P α -syn and wild type (wt) mice using proximity ligation assay (PLA). The results demonstrated that SNARE proteins co-localized with total α -syn mainly in neuronal processes, and with A30P α -syn predominantly in the cell soma.

In **paper II**, we investigated how altered molecular properties of α -syn could affect its cellular processing. Different α -syn constructs were expressed in SH-SY5Y cells and the culture medium was analyzed for free-floating α -syn, as well as for α -syn within the EV fraction. Modifications in the N-terminal increased the EV secretion and enhanced the cell-to-cell transfer of α -syn.

In **paper III**, the synaptic α -syn species of the A30P tg mouse brain were biochemically characterized and their effect on SNARE protein distribution was analyzed with western blot and PLA. We found that synaptosomal α -syn aggregates were mainly composed of non-phosphorylated human A30P α -syn. A decrease of intact SNARE complexes was observed in the tg A30P synaptosomes and in the prefrontal cortex, even though the total levels of SNARE proteins were unchanged in A30P compared to wt mice.

In **paper IV**, we studied the effect of α -syn monomers and α -syn preformed fibrils (PFFs) on SNARE protein distribution in wt primary neurons, using PLA. Both short- and long-term exposure to α -syn monomers or PFFs altered the co-localization of SNARE proteins. Promoting the long-term uptake of α -syn by using a protein delivery reagent, further increased SNARE protein redistribution. In contrast, a PFF-induced SNARE protein redistribution was not observed when lysosomal degradation was inhibited. Interestingly, addition of EVs from monomer- and PFF-treated astrocytes also affected SNARE protein distribution in recipient neurons.

Taken together, the results from this thesis indicate that synaptic α -syn aggregates and EV-associated α -syn could be promising therapeutic targets in the α -synucleinopathies.

Keywords: Alpha-synuclein, Parkinson's disease, synaptosome, SNARE complex, proximity ligation assay, extracellular vesicles

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ISSN 1651-6206

ISBN 978-91-513-1648-2

URN urn:nbn:se:uu:diva-487286 (<http://urn.kb.se/resolve?urn=urn:nbn:se:uu:diva-487286>)

*Till min familj för all
värme och glädje*

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List of Papers

This thesis is based on the following papers, which are referred to in the text by their Roman numerals.

- I. Almandoz-Gil, L., **Persson, E.**, Lindström, V., Ingelsson, M., Bergström, J. (2018) *In situ* proximity ligation assay reveals colocalization of alpha-synuclein and SNARE proteins in murine primary neurons. *Front Neurol*, 9:1–11
- II. Gustafsson, G., Lööv, C., **Persson, E.**, Lázaro, DF., Takeda, S., Bergström, J., Erlandsson, A., Sehlin, D., Balaj, L., György, B., Hallbeck, M., Outeiro, TF., Breakefield, XO., Hyman, BT., Ingelsson, M. (2018). Secretion and uptake of α -synuclein via extracellular vesicles in cultured cells. *Cell Mol Neurobiol*, 38(8), 1539–1550
- III. **Brolin, E.**, Almandoz-Gil, L., Rofo, F., Ekmark-Lewén, S., Hyman, BT., Ingelsson, M., Bergström, J. Alpha-synuclein aggregates affect the distribution of SNARE complex proteins in synapses of A30P alpha-synuclein transgenic mice and Parkinson's disease brain. *Submitted manuscript*.
- IV. **Brolin, E.**, Erlandsson, A., Bergström, J. Altered distribution of SNARE proteins in primary neurons exposed to different alpha-synuclein species. *Manuscript*.

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Abbreviations

Aa	Amino acid
AD	Alzheimer's disease
α -syn	Alpha-synuclein
bFGF	Basic fibroblast growth factor
BiFC	Bimolecular fluorescence complementation assay
CHQ	Chloroquine
CMA	Chaperone-mediated autophagy
CNTF	Ciliary neurotrophic factor
CSF	Cerebrospinal fluid
CSP α	Cysteine string protein- α
DIV	Days <i>in vitro</i>
DLB	Dementia with Lewy bodies
EGF	Epidermal growth factor
(T)EM	Transmission electron microscope
EV	Extracellular vesicle
EYFP	Enhanced yellow fluorescent protein
FFP	Free-floating protein
GCI	Glial cytoplasmic inclusion
ILV	Intraluminal vesicle
LAMP2A	Lysosome-associated membrane protein 2A
LB	Lewy body
LN	Lewy neurite
LRRK2	Leucine-rich kinase 2
MSA	Multiple system atrophy
MVB	Multivesicular body
NAC	Non-amyloid- β component
PBS	Phosphate buffered saline
PD	Parkinson's disease
PDD	Parkinson's disease dementia
PFC	Prefrontal cortex
PFF	Preformed fibrils
PLA	Proximity ligation assay
pS129	Alpha-synuclein phosphorylated at ser129
RCA	Rolling circle amplification
SDS-PAGE	Sodium dodecyl sulfate polyacrylamide gel electrophoresis

SNAP-25	Synaptosomal-associated protein of 25 kDa
SNARE	Soluble <i>N</i> -ethylmaleimide-sensitive factor attachment protein receptor
SV	Synaptic vesicle
Tg	Transgenic
VAMP-2	Vesicle associated membrane protein-2
Wt	Wild type

Introduction

Neurodegeneration and the presence of intracellular inclusions called Lewy bodies (LBs), Lewy neurites (LNs) or glial cytoplasmic inclusions (GCIs) are considered as the classic histopathological hallmarks of Parkinson's disease (PD), dementia with Lewy bodies (DLB) and multiple system atrophy (MSA) ¹⁻⁴. The main component of the inclusions is fibrillar aggregates of the protein alpha-synuclein (α -syn) ⁵, hence, the above mentioned disorders will be referred to as α -synucleinopathies.

Since degradation of synapses and axons occur before neuronal loss ⁶⁻⁸, and α -syn has a predominant presynaptic localization ⁹, the hypothesis that the pathogenesis may start with the aggregation of α -syn at the synapses is gaining attention ¹⁰⁻¹². The physiological function of α -syn at the synapse is unclear, but it is thought to be involved during the neurotransmitter release as a chaperone to the soluble *N*-ethylmaleimide-sensitive factor attachment protein receptor (SNARE) complex assembly ¹³.

The focus of this thesis was to investigate the pathophysiological effect of α -syn on the SNARE proteins in α -synucleinopathy models. Additionally, the potential role of extracellular vesicles (EVs) in the propagation of different forms of α -syn was also investigated.

Alpha-synuclein

Alpha-synuclein is a small (140 amino acids (aa), **Figure 1**) protein encoded by the *SNCA* gene ¹⁴. It is abundantly expressed in the brain but is also found throughout the body, including in muscles, kidneys, lung, heart and blood ¹⁴⁻¹⁶. Together with β - and γ -synuclein, it composes the synuclein family. The three proteins share a sequence homology in mainly the N-terminus, but only α -syn forms aggregates in the α -synucleinopathies ⁵.

Native α -syn exists in equilibrium between a cytosolic and a membrane-bound state. The main physiological species of α -syn is believed to be an unfolded monomer. However, a tetrameric cytosolic form of α -syn has been suggested to be found in human erythrocytes ^{17,18}. It has been reported that native membrane-bound α -syn assembles into multimers on synaptic vesicles (SVs)

^{19,20}. However, the existence of these physiological oligomers has been debated and they are suggested to be structurally distinct from pathogenic aggregates found in the α -synucleinopathies ¹⁹.

The N-terminal (aa 1-60) domain of α -syn has seven 11-residue repeats with a KTKEGV sequence and can form an amphipathic α -helical structure in the vicinity of lipid membranes ^{21,22}. The induced α -helical structure has a poor hydrophobic interface, which leads to the favored binding to membranes with high curvature and negatively charged lipids such as SV ²³⁻²⁶.

The middle part (aa 61-95) of α -syn is often referred to the non-amyloid- β component (NAC) region, as it was originally isolated from senile plaques in Alzheimer's disease (AD) ^{16,27}. It is hydrophobic and has a high propensity for aggregation. The NAC region is believed to constitute the β -sheet-rich core observed in pathologic aggregates ²⁸.

The hydrophilic and acidic C-terminus (aa 96-140) is flexible and can undergo several posttranslational modifications. It is important for protein-protein interactions and contributes to the function and chaperone-like activity of α -syn. It is the least conserved region among the synucleins ²⁹.

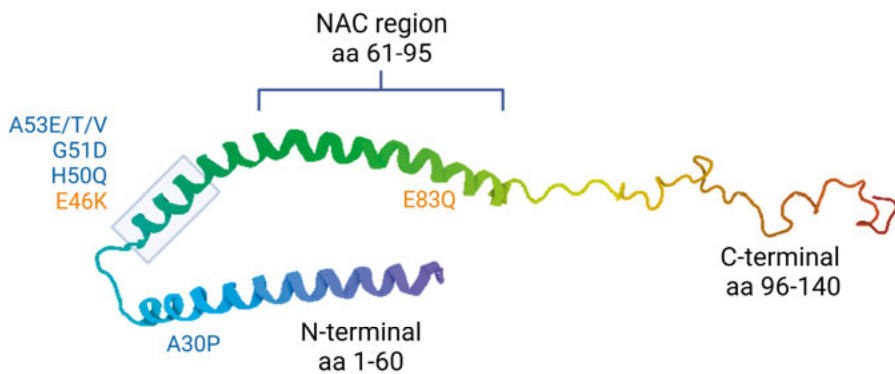


Figure 1. Nuclear magnetic resonance prediction of micelle-bound human α -syn structure (1xq8, from the protein data bank 30). N-terminal (aa 1-60) gain an α -helical structure while interacting with phospholipid membranes. The NAC region (aa 61-95) is hydrophobic and has a propensity for self-aggregation. The C-terminus (aa 96-140) is hydrophilic, flexible and prone to post-translational modifications, including phosphorylation of ser129 (pS129). Several missense mutations in the N-terminal and one mutation in the NAC-region causes PD (blue) and DLB (orange).

In the brain, α -syn is predominantly expressed in neocortex, hippocampus, striatum, thalamus and cerebellum ²⁷. It is mainly located at the nerve terminals, although it has also been observed in mitochondria, nucleus, endoplasmic reticulum, Golgi and by the cytoskeleton ^{9,14,15}. Both the favored binding to SVs and its involvement with protein interactions, are suggested to induce the synaptic targeting ¹⁵.

The SNARE complex

All cellular processes involving fusion between two membranes are dependent on SNARE complex assembly, composed of various SNARE proteins. In the presynapse, neurotransmitter release is enabled by the fusion between the SV membrane and the plasma membrane. The three core SNARE proteins involved in neurotransmitter release are vesicle-associated membrane protein-2 (VAMP-2), synaptosomal-associated protein of 25 kDa (SNAP-25) and syntaxin-1 (**Figure 2**)³⁰. Vesicle-associated membrane protein-2, also known as synaptobrevin-2, consists of 116 aa and is classified as v-SNARE, as it is associated to the vesicle membrane³¹. Both SNAP-25 (206 aa) and syntaxin-1 (288 aa) are classified as t-SNAREs since they are associated with the “target” or plasma membrane³². Whereas VAMP-2 and syntaxin-1 are associated to the membranes via its respective C-terminal transmembrane domain, SNAP-25 is bound to the plasma membrane via palmitoylation of several cysteine residues³³.

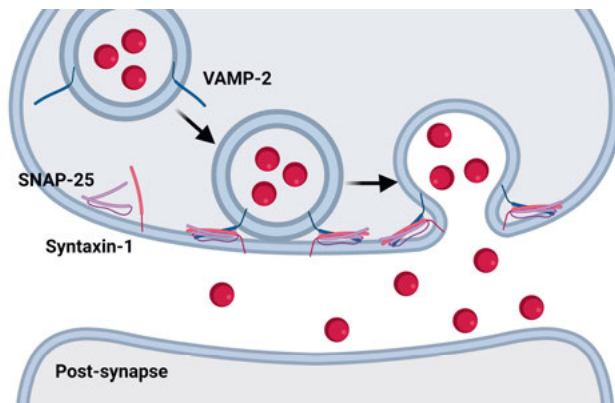


Figure 2. SNARE-mediated neurotransmitter release. VAMP-2 (blue) at the SV interacts with SNAP-25 (purple) and syntaxin-1 (pink) at the plasma membrane and causes fusion of the SV and plasma membrane and release of neurotransmitters (red).

A common feature of the SNARE proteins is a 60-70 residue SNARE motif, which is unstructured in each unbound individual protein, but forms a four-helix bundle when assembled into the SNARE complex³⁴. The helices are composed of four classes of SNARE motifs (R-, Qa-, Qb- and Qc-SNARE motif). The R-SNARE proteins are typically located at the vesicle membrane and have an arginine residue in the central SNARE motif, while Q-SNAREs have a glutamine residue and are usually located at the target membrane³⁵. As the R-SNARE (VAMP-2) and the three Q-SNAREs (two SNAP-25 and one syntaxin-1) form the four-helix bundle, this generates an inward force that pushes the two membranes tightly together and enables membrane fusion³⁴.

Apart from the SNARE proteins, several other proteins are involved in neurotransmitter release. The fast response to an action potential is enabled by

complexin-1 and synaptotagmin. Complexin-1 functions as a clamp around the SNARE complex, holding it in a frozen state^{33,35}. After an action potential, depolarization causes voltage-sensitive channels to open and allow influx of Ca^{2+} . The Ca^{2+} -sensor synaptotagmin then causes complexin-1 to release^{33,35}. Furthermore, the membrane fusion is dependent on the Sec1/Munc18-like proteins, which form a clasp around the SNARE complex and regulate the fusogenic action of the SNARE proteins³⁶.

Alpha-synuclein at the presynapse

The physiological function of α -syn is still unclear, but the presynaptic localization and the favored binding to SV, suggest involvement with endo- and exocytosis (**Figure 3**). The first evidence that α -syn could act as a chaperone during SNARE complex assembly, became evident in a knockout mouse model of cysteine string protein- α (CSP α), a chaperone for SNARE complex assembly. In this model, an overexpression of α -syn was shown to abolish neurodegeneration caused by the deletion of CSP α ³⁷. It is believed that the binding between α -syn and the SNARE complex occurs via an interaction between the C-terminal part of α -syn (aa 96-110) and the N-terminal of VAMP-2 (aa 1-28)^{13,38}. Alternatively, it has been suggested that α -syn either facilitates the SNARE complex assembly by cross-bridging VAMP-2 and the plasma-membrane^{39,40}, by clustering SVs by binding the SV membrane and VAMP-2 on separate SVs³⁸, or by expanding the fusion pore⁴¹.

The function of α -syn as a chaperone to the SNARE complex was questioned by another study claiming α -syn can inhibit SNARE-mediated membrane fusion by direct interaction with lipids, and not by binding VAMP-2⁴². Furthermore, SNARE complex function has been shown to be inhibited by increasing α -syn levels through a direct interaction with the lipid membranes⁴³, and that Ca^{2+} causes α -syn to release SV and thereby increase SNARE-mediated membrane fusion⁴⁴.

The N-terminal lipid-binding domain of α -syn has also been proposed to have a role in SV motility and maintenance of SV pools⁴⁵⁻⁴⁸. Moreover, physiological α -syn multimers could reduce the motility of SVs and attenuated recycling²⁰. The interaction with VAMP-2 and simultaneous binding to SVs with the N-terminal domain have also been suggested to cluster SVs at the active zone⁴⁹. In addition, a double-anchor mechanism have been described, where one α -syn molecule simultaneously binds two separate SVs⁵⁰.

Alpha-synuclein is also involved during endocytosis^{51,52}, and was recently found to co-localize with caveolin positive vesicles⁵³. The favored binding to high curvature membranes is also suggested to facilitate generation of membrane curvature during both endo- and exocytosis⁵⁴.

Alpha-synuclein is expressed after synapse development and is generally not believed to be essential during synapse formation⁴⁸. Yet, it has been

shown to promote early neurite outgrowth by facilitating the polymerization of tubulin in primary neurons⁵⁵. Triple knockout of all three synucleins resulted in impaired synapse structure and function and reduced survival in transgenic (tg) mice⁵⁶, whereas the deletion of α -syn caused reduced number of dopaminergic neurons⁵⁷. The synuclein-null mice also displayed alterations in the levels of synaptic proteins, where younger mice displayed increased levels of complexin-1/2, synapsin IIb and 14-3-3 β and ϵ . Interestingly, the same proteins were reduced in an older age mouse group in addition to a reduction of SNAP-25 and increased levels of VAMP-2⁵⁶.

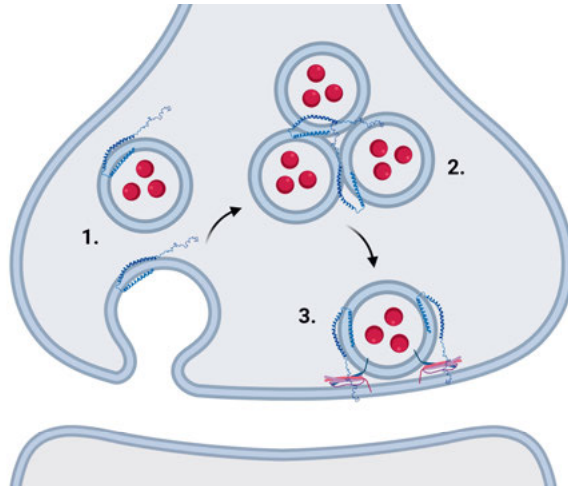


Figure 3. Function of α -syn at the presynapse. Alpha-synuclein stabilizing SV membrane and facilitating endocytosis (1). Clustering of SVs by double-anchoring of α -syn or by simultaneous binding to SV membrane and VAMP-2 (2). Alpha-synuclein as a co-chaperone to SNARE complex assembly (3).

Alpha-synucleinopathies

The symptoms of the α -synucleinopathies largely depends on in which brain region and cell type that is affected by α -syn aggregation. But due to the large overlap in symptoms, it is usually hard to set a definite diagnosis. In PD, the motor symptoms are caused by the degeneration of dopaminergic neurons in the substantia nigra. It is also affected in DLB, although the main pathological change in DLB is in the neocortex and limbic system⁵⁸ leading to symptoms like fluctuating cognition, recurrent visual hallucinations and Parkinsonism⁵⁹. Unlike PD and DLB, the main cell type affected in MSA is oligodendrocytes. Alpha-synuclein inclusions in oligodendrocytes are referred to as GCIs, which are related to autonomic failure, cerebellar ataxia, parkinsonism, urinary dysfunction and corticospinal dysfunction^{2,60}.

Parkinson's disease

Parkinson's disease is the second most common neurodegenerative disorder after AD with a prevalence of 0.3% of the population⁶¹. Age is the greatest risk factor and the prevalence increases to 1% in people >60 years of age⁶¹. It is a slowly progressing disorder and patients are typically diagnosed at a late stage of disease. The first symptoms arise when approximately 30% of the dopaminergic neurons in the substantia nigra, and 50 to 60% of axon terminals, have already been lost⁶.

The loss of dopaminergic neurons in the substantia nigra causes the classical motor symptoms in PD. The clinical diagnosis is typically based on the presence of at least two of the symptoms resting tremor, bradykinesia, postural instability or rigidity^{62,63}. Furthermore, any secondary causes of Parkinsonism need to be excluded. The diagnosis can be strengthened by an asymmetric onset of symptoms, a good response to levodopa treatment and by a long-term follow-up. However, a definite diagnosis can only be made upon a *post mortem* neuropathological assessment showing the concomitant degeneration of substantia nigra and the presence of LBs in the surviving neurons^{63,64}.

In contrast to other neurodegenerative disorders, there are efficient treatments that considerably attenuate the motor symptoms in PD. The most successful treatment is based on substituting the loss of dopamine with levodopa⁶². However, levodopa administration only treats the symptoms and not the underlying cause of the disease. The neurodegeneration will continue to progress and the efficiency of the levodopa diminish over time. Up to 40% of the patients have reported adverse effects of levodopa after 5 years of treatment⁶⁵. The most common side effects are motor fluctuations and dyskinesia, which appears as the therapeutic window becomes smaller. In many cases this can be counteracted with adjustments in the dose and with smaller time windows between intakes, although over time the symptoms will worsen as the disease progresses. Moreover, recent studies that have aimed to explain the selective vulnerability of the dopaminergic neurons in PD, have suggested that the dopamine itself might cause the aggregation of α -syn and thus cause further degeneration⁶⁶.

Apart from the characteristic motor symptoms, PD patients are often affected by several non-motor symptoms. These can include loss of smell, constipation, depression, sleep disorders and cognitive impairment^{67,68}. About 30% of the PD patients will also develop dementia⁶⁹. The onset of the non-motor symptoms can arise decades before the onset of the motor symptoms during a so called prodromal stage⁶³. During the prodromal stage the neurodegeneration has started, but has not yet started to cause motor symptoms. Hence, the diagnosis is challenging during this time, due to the risk of alternative diagnoses. However, in the future it will be crucial to set an early correct diagnosis, since efforts are being made to develop disease modifying drugs, which would be most effective before extensive neurodegeneration.

Dementia with Lewy bodies

Dementia with Lewy bodies is the second most common form of dementia after AD. It is often under-diagnosed and taken for AD, due to the similarities in the disease phenotype. In a study from the UK only 4.6% of all dementia cases were diagnosed with DLB⁷⁰, despite autopsy reports revealing LB pathology in 20% of the brains⁵⁸.

A clinical diagnosis is often based on the core clinical features, which includes fluctuating cognitive decline, recurrent visual hallucinations, spontaneous extrapyramidal motor features and rapid eye movement sleep behavior disorder⁷¹. Both the symptoms and the pattern of cerebral atrophy is similar between DLB and PD dementia (PDD)⁷². A PDD diagnosis is typically given if the motor deficits appear before the onset of dementia, whereas the diagnosis DLB is used if dementia appear before or concurrently (within the first year) with parkinsonian motor deficits^{72,73}.

Voxel-based morphometry of the whole brain revealed grey matter atrophy in the frontal, temporal, occipital and subcortical areas of PDD and DLB⁷². Alpha-synuclein inclusions in the form of LBs and LNs are found in the brainstem, neocortical and limbic areas in DLB^{71,74}. Reduced levels of SNAP-25 in the prefrontal and cingulate cortex were associated with a more rapid cognitive decline in DLB brains⁷⁵. Furthermore, it is common with mixed protein pathologies. For example, about one third to half of patients diagnosed with AD have some degree of mixed pathology with both amyloid- β and tau pathology, which are the characteristics of AD, together with LBs and LNs^{76,77}. Mixed pathology with hyperphosphorylated tau also leads to shortened life expectancy of α -synucleinopathy patients⁷⁸. Moreover, about half of DLB patients have cerebrovascular pathology⁷⁹.

To date, the treatment options for DLB is limited, which makes it essential to identify molecular mechanisms, which could be targeted to prevent degeneration.

Genetic links

Although about 90% of PD cases are sporadic, α -syn is further linked to disease based on findings of mutations and multiplications of the *SNCA* gene, which causes autosomal dominant forms of α -synucleinopathies. For example, several point-mutations (*A30P*, *H50Q*, *G51D*, *A53E*, *A53T* and *A53V*) are associated with PD, whereas the *E46K* and *E83Q* mutations are linked to DLB^{80,81,90,91,82–89}. Interestingly, most mutations are situated in the lipid-binding N-terminal region of α -syn²⁹, apart from *E83Q*, which is situated in the mid-region^{86,92}. The mutations and multiplications of the α -syn gene tend to cause an increased propensity to aggregate and in most cases cause early onset PD or DLB^{93–95}. The mutations located in N-terminal affect the dimer interface

of fibrils and lead to the formation of fibrils with distinct morphologies⁹⁶. The mid-region mutation (*E83Q*) also accelerate fibrillization and further increase toxicity and seeding activity⁹².

The A30P mutation promotes the formation of pathological oligomers, but also reduces the affinity for lipid membrane of the monomeric forms of A30P α -syn^{21,95,97–99}. The A30P mutation has also been suggested to cause reduced presynaptic targeting, as well as impaired SNARE complex assembly and SV clustering at the active zone^{49,100}. Contradictory, it has also been suggested to inhibit SNARE-mediated membrane fusion less effectively due to reduced binding to phospholipids, and not through direct binding to VAMP-2^{42,43}.

Genome wide association studies have also linked the *SNCA* gene to sporadic cases of PD^{101,102}. Moreover, familial PD can also be caused by mutations in the genes encoding leucine-rich kinase 2 (LRRK2), parkin, PINK1 and DJ-1^{103–106}.

Alpha-synuclein aggregation

The presence of α -syn aggregates in various parts of the brain in PD, DLB and MSA suggests a common pathological mechanism, with selective vulnerability in certain populations of neurons and glia. The aggregation of α -syn follows a nucleation polymerization pathway (**Figure 4**)¹⁰⁷. The start of pathological aggregation occurs when the conformation of α -syn changes from a random coil and/or α -helical structure into a β -sheet rich conformation¹⁰⁸. This leads to a continuous self-association of α -syn with the formation of oligomers, protofibrils and fibrils with an increasing molecular weight and hydrophobicity. The β -sheet structure observed in α -syn oligomers and protofibrils exhibit an anti-parallel orientation, which changes into a parallel β -sheet structure with the formation of fibrils, which finally deposit as LBs and LNs¹⁰⁹.

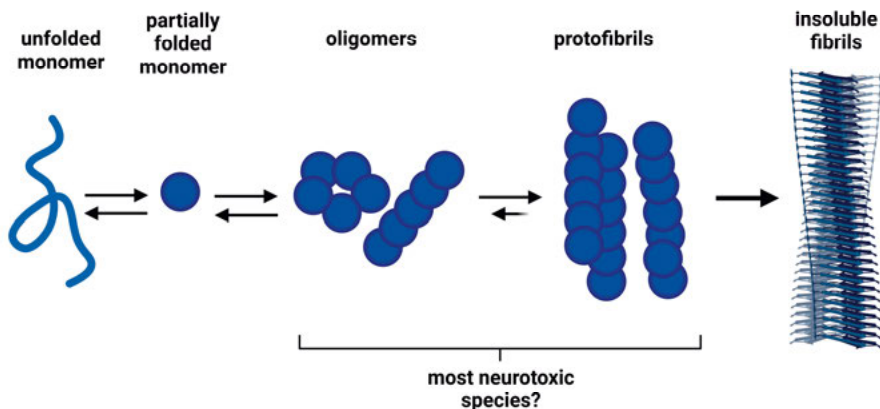


Figure 4. Aggregation cascade of α -syn. When the natively unstructured protein has a conformational shift into a β -sheet formation it increases its aggregation propensity and start to self-assemble. First the oligomeric species are soluble, but as the aggregation continues into larger protofibrils they become more compact. Finally, they aggregate into insoluble fibrils which are the major constituent of LBs, LNs and GCIs. The soluble oligomers and protofibrils are thought to be the most neurotoxic species.

The α -syn in LBs and LNs is phosphorylated at serine 129^{110,111} and resistant to digestion with proteinase K, a broad spectrum serine protease^{10,111–115}. Apart from α -syn, the LBs also contain ubiquitin, neurofilaments and approximately 550 of other proteins, including kinases, chaperones and other synaptic proteins such as synapsin III^{5,116–118}.

The number of Lewy bodies is poorly correlated with disease severity and insoluble aggregates have even been suggested to have a neuroprotective function^{119,120}. Instead, increasing evidence points towards smaller α -syn aggregates (oligomers or protofibrils) as the main neurotoxic agent in α -synucleinopathies¹²¹. Studies have suggested several potential neurotoxic mechanisms of oligomers, including altered neuronal structure, permeabilization of membranes and inflammation^{121–124}. Furthermore, the small size and surface hydrophobicity of oligomers have been correlated to neurotoxicity¹²⁵. As the composition and structure of the oligomers are very heterogenous, the neurotoxicity could be caused by a combination of several processes¹²⁶. At the start of aggregation, the majority of α -syn oligomers are thought to be situated in the synapse, and the fact that synaptic degeneration precedes neuronal death^{8,127}, suggest that synaptic α -syn oligomers would be an interesting therapeutic target.

Synaptic pathology

Due to the presynaptic localization of α -syn, and the observed degeneration of synapses in the pathogenesis of PD and DLB, α -syn pathology has been suggested to start at the synapses. Brain imaging techniques have shown a specific loss of excitatory synapses in striatum of PD brains^{7,8}. Furthermore, the concentration of dopamine is reduced by 80 or 98% in the caudate or putamen, respectively¹²⁸. The loss of dendritic spines in the brain of DLB patients has been shown to be correlated to the presence of small aggregated α -syn species in the synapses¹²⁷. Similar small synaptic α -syn aggregates are also found in other α -synucleinopathies, as well as in tg α -syn mouse models^{10,12,129}.

Alterations in physiological α -syn levels, as well as α -syn aggregation have been correlated with reduced levels of several presynaptic proteins in cells and brain tissue from tg mice and α -synucleinopathy patients^{118,127,130–132}. Recently, anomalies were found in both the central and peripheral PD-transcrip-

tomes involving exocytosis, including the SNARE proteins VAMP-2 and syntaxin-1¹³³. Furthermore, increased concentration of SNAP-25 in the cerebrospinal fluid (CSF) of PD patients is related to the severity of cognitive and motor symptoms¹³⁴. Interestingly, synaptic dysfunction with reduced levels of both α -syn and VAMP-2 were also found in a DYT1 dystonia model¹³⁵.

The formation of α -syn aggregates, or increased levels of physiological α -syn, can also alter the function of the SNARE complex. Even low concentrations of α -syn oligomers reduced exocytosis by inhibiting SNARE-mediated lipid mixing^{40,136}. Furthermore, overexpression of α -syn caused reduced vesicle docking and neurotransmitter release^{43,46}. Synaptic vesicle clustering can also be disrupted by the lipid-binding deficiency of the A30P α -syn mutation⁴⁹.

Alpha-synuclein can also influence the distribution of the SNARE proteins and other synaptic proteins. For instance, both tg mice expressing truncated (1-120) or A30P α -syn, and adenovirus-mediated overexpression of human α -syn in non-tg mice, displayed a redistribution of SNARE proteins^{137,138}. Moreover, α -syn has also been shown to influence the distribution of synapsin III¹³⁹ and the dopamine transporter¹⁴⁰.

Interestingly, synaptic proteins can also influence the aggregation propensity of α -syn. For example, the introduction of a single knock-in SNAP-25 mutation in mice, resulted in SNARE complex dysfunction and also led to a redistribution and aggregation of presynaptic endogenous mouse α -syn¹⁴¹. In addition, removal of synapsin III has been shown to protect against the formation of α -syn pathology¹³⁸. Also the SNARE-associated protein Munc18-1 is suggested to be a chaperone for α -syn and could reduce the aggregation propensity of α -syn¹⁴². Taken together, increasing evidence indicate a molecular link between SNARE protein dysfunction and α -syn aggregation.

Propagation of pathology

The progression of α -syn pathology in PD is typically divided into Braak stages, based on the Braak or the ascending theory¹⁴³. The direction of the spreading of α -syn pathology is upwards and forward; from the brainstem, into the midbrain and towards the prefrontal cortex (PFC). However, the theory have been criticized as too simplified, due to the great phenotypic heterogeneity observed in PD¹⁴⁴. Furthermore, the model cannot be applied to other α -synucleinopathies, such as DLB. It has also been suggested that during the prodromal stage of PD, the α -syn aggregation starts in peripheral organs such as the gut, causing some of the non-motor symptoms before spreading via the vagus nerve into the brainstem¹⁴⁵. For example, α -syn aggregates have been found in the vagus nerve¹⁴⁶.

A great effort has been made to understand how the propagation of disease may occur. The first compelling evidence of the spread of α -syn pathology

was made in grafted dopaminergic neurons, which over time displayed the presence of LB pathology^{147,148}. Moreover, extracellular α -syn aggregates have also been detected in blood and CSF^{149,150}. It has been shown, both in various cell and mouse models, that *in vitro* generated preformed fibrils (PFFs) of recombinant α -syn can be used to induce α -syn pathology¹⁵¹. A debate is still ongoing if the spread of α -syn pathology should be classified as a prion-like disorder. Interestingly, the SNARE proteins have been implicated in the propagation of α -syn pathology, as the transmission of α -syn aggregates were blocked by the targeted degradation of VAMP-2¹⁵².

Extracellular vesicles

The intracellular burden of α -syn pathology can cause exocytosis of extracellular vesicles (EVs), which in turn potentially could contribute to cell-to-cell transfer of α -syn aggregates¹⁵³. Extracellular vesicles are a collective term, which describes both microvesicles and exosomes. Whereas microvesicles (50-1000 nm in diameter) buds directly from the plasma membrane, exosomes (50-150 nm in diameter) are instead formed as intraluminal vesicles (ILV) inside late endosomes/multivesicular bodies (MVBs)^{154,155}. The ILVs are then released as exosomes to the extracellular space when the MVB fuses with the plasma membrane^{154,155}.

Recently, EVs have been suggested as a potential biomarker for PD as concentration of α -syn oligomer associated with EVs are correlated with disease duration and severity^{156,157}. Moreover, uptake of α -syn aggregates were facilitated by association with EV¹⁵⁸ and EVs derived from CSF or brain of α -synucleinopathy patients induced α -syn aggregation when seeded on cells or injected into the brain of wt mice^{159,160}.

Degradation of α -syn

The degradation of α -syn both depends on the form of α -syn and the localization. The proteasomes and lysosomes play complementary roles of intracellular α -syn clearance^{153,161–163}. In addition, proteolytic enzymes or endocytosis by neighboring astrocytes and microglia may also contribute to the degradation of extracellular α -syn^{153,164–166}. Furthermore, the extracellular α -syn form affects the clearance. Whereas fibrils are endocytosed and degraded in lysosomes, monomeric α -syn is directly translocated across the plasma membrane and degraded by cellular proteolytic systems¹⁶⁷.

Different disease modifications alter not only the degradation efficiency of α -syn, but also the overall clearance mechanism. For instance, both the A53T mutation and methionine oxidation of α -syn reduce degradation of α -syn by the proteasome^{161,168}. Moreover, the disease mutants A53T and A30P α -syn

bind to the chaperone-mediated autophagy (CMA) receptor lysosome-associated membrane protein 2A (LAMP2A) with high affinity, causing reduced lysosomal clearance of both α -syn and other substrates¹⁶⁹. Likewise, a general reduction of autophagosomal clearance was observed after failed degradation of α -syn aggregates¹⁷⁰.

Failed clearance, and obstruction of degradation pathways also contribute to α -syn aggregation. For example, inhibition of CMA causes α -syn aggregation¹⁶³, and accumulation of α -syn aggregates can be found in models of lysosomal storage disorders¹⁷¹. Interestingly, LRRK2 mutations, which are the most common genetic cause of PD, have been found to regulate autophagic activity^{172–175}, indicating a possible disease causing mechanism.

Role of glia and neuroinflammation

There are almost as many glial cells, such as astrocytes and microglia, in the brain as neurons¹⁷⁶. They are vital for a wide array of functions, including structural integrity and supply of nutrients and oxygen. Astrocytes have been given its name due to the star-shaped appearance, which provides multiple contacts with neurons and other cell types. They have several functions, including maintenance of the blood brain barrier, provision of nutrients to neurons and controlling the balance of extracellular ions and neurotransmitters at the synapses¹⁷⁷.

When astrocytes recognize pathogens or injury, it triggers a defense mechanism referred to as reactive gliosis¹⁷⁸. This condition leads to an increased size of astrocytes and to the secretion of cytokines and chemokines, which ultimately causes neuroinflammation. They also remove pathogens and cell debris through phagocytosis¹⁷⁹.

Astrocytes can recognize the β -sheet structure of aggregated α -syn as a pathogen through pattern-recognition receptors, similar to the innate immune system outside the central nervous system^{180–182}. However, in the α -synucleinopathies, aggregated α -syn continues to accumulate for years, which causes a chronic activation of reactive gliosis and the physiological function of astrocytes gets compromised. Furthermore, the continued release of inflammatory mediators could become neurotoxic over time.

Astrocytes can also engulf extracellular α -syn aggregates¹⁸¹. The phagocytosed α -syn aggregates get transferred to the lysosomes for degradation¹⁸³. However, the compact β -sheet structure is resistant to degeneration and gets accumulated in the astrocytes^{184,185}. Moreover, hyperactivation of glial phagocytosis have been suggested to be deleterious in the α -synucleinopathies, as the normal glial function gets compromised¹⁸⁶. Astrocytes have also been shown to spread α -syn aggregates through tunneling nanotubes and through the release of EVs^{184,187,188}.

Aims

The overall aim of this thesis was to investigate the effect of different α -syn species on SNARE protein distribution and SNARE complex formation. In addition, we sought to investigate the role of EVs in the spreading of α -syn pathology. In order to study the pathophysiological effect of α -syn on SNARE proteins and spread of α -syn pathology, we used different cell culture models and analyzed brain material from a tg mouse model as well as from PD and DLB patients.

Specific aims

- I To study the co-localization between α -syn and SNARE proteins in primary neurons from A30P tg and non-tg mice.
- II To investigate whether altered molecular properties of α -syn can affect the secretion and cell-to-cell transfer of α -syn in SH-SY5Y cells.
- III To characterize synaptic α -syn in A30P tg mice and investigate changes in the levels and distribution of SNARE proteins in A30P tg mice, as well as in PD and DLB brain tissue.
- IV To investigate changes in the distribution of SNARE proteins in primary neurons exposed to different α -syn species.

Materials and methods

Material and sample preparations

In this thesis, we have utilized several models and techniques to study molecular alterations which are caused by α -synucleinopathies.

Ethical statement

The experiments involving human tissue in **paper III** were approved by the regional ethical committee (2005-103; 2005-06-29 and 2009/089: 2009-04-22, respectively). All animal experiments were carried out in accordance with the EU Directive 2010/63/EU for animal experiments and approved by the animal ethics committee of Uppsala, Sweden (C75/13 and C92/14 for **paper I**, 2013-06-30, N245/10, N246/10, C92/14 and 5.8.18-08038/2018 for **paper III** and 5.8.18-08472/18 for **paper IV**). The mice were housed in a 12 h dark-light cycle, in an enriched environment and food and water were provided *ad libitum*.

Human samples

In order to investigate the molecular changes, which appear in the brain of α -synucleinopathy patients, we have utilized brains from patients with PD and DLB, as well as non-neurodegenerative controls. In **paper III**, brain material from putamen and Brodmann area 24, corresponding to the anterior cingulate cortex (hereafter referred to as cingulate cortex), were used. In PD, there is a substantial loss of dopamine in the putamen^{128,189}. Lewy body pathology in the cingulate cortex is correlated with cognitive decline in PD¹⁹⁰ and a reduction of SNAP-25 in the same area predicts cognitive decline in DLB and PDD⁷⁵. Furthermore, small synaptic α -syn aggregates have also been found in the cingulate cortex of both PD and DLB brain^{127,146}.

The brain materials were retrieved from the brain bank at Uppsala University, Sweden, and from Massachusetts General Hospital, Boston, USA.

Animal models

We have utilized a mouse model overexpressing human α -syn with the A30P mutation under the Thy-1 promoter, in order to study the consequences of α -

syn pathology on molecular mechanisms. The A30P tg mice express human α -syn two-fold relative to the endogenous mouse α -syn¹⁹¹. Despite reports of reduced synaptic targeting of α -syn with the A30P mutation in cultured cells¹⁰⁰, anterograde transport of A30P α -syn to the synapses has been demonstrated in the mouse line¹⁹¹. The mice develop extensive pS129 positive somal inclusions and neurites in the brain stem, olfactory bulb, cortex, and hippocampus^{111,192,193}.

The A30P tg mice undergo a cognitive decline at 12 mo, whereas they develop an impaired locomotor behavior at 8 mo and fine motor impairment already at 2 mo^{194–196}. The mice also experience dysfunction and molecular changes, including reduced protein expression of VAMP-2, in the gut at a young age before onset of motor impairments¹⁹⁷.

We used the mouse model in **paper I** to investigate the molecular changes on the SNARE proteins in neurons overexpressing α -syn and human α -syn with the A30P mutation. In **paper III**, it was used to biochemically characterize synaptic α -syn aggregates and investigate alterations of SNARE protein expression and distribution in synaptosomes and brain tissue sections.

Cell models

Primary cortical neurons

In **papers I and IV**, primary cortical neurons were used to study the molecular changes, which appear while inducing α -syn pathology. The neurons were dissected from E14 mice. After dissection, the neurons were plated at a concentration of 90 000 cells/ml on poly-L-ornithine and laminin coated coverslips. The cells were grown in neurobasal medium supplemented with B27, L-glutamine, penicillin and streptomycin and kept at 37° C with 5% CO₂.

In **paper I**, neurons were prepared from both A30P tg (described above) and wt mouse brains of the same background strain, C57BL/6J, whereas in **paper IV**, only wt mouse neurons were used. The cells in **paper IV** were exposed to α -syn monomers, PFFs or as control PBS (described in the section “Seeding”).

The neurons were fixed with 4% paraformaldehyde at 12 days *in vitro* (DIV) for **paper I** and at 19 or 20 DIV for **paper IV**, and kept in PBS until further analyses.

Cell lines

In **paper II**, we used the SH-SY5Y cell line derived from human neuroblastoma cells. The cell model is popular in PD research for its human origin and resemblance to dopaminergic neurons^{198,199}. The cells were used to study the secretion and uptake of different forms of α -syn via EVs or direct secretion as free-floating protein (FFP). The SH-SY5Y cells were transiently transfected to express different forms of α -syn and the cell medium was collected and

analyzed for presence of extracellular α -syn as associated to EV or FFP (further described below).

Astrocytes

The molecular consequences of glia-to-neuron transfer of EV-associated α -syn on SNARE protein distribution were analyzed in **paper IV**. Mouse astrocytes were differentiated from neurospheres. Briefly, cerebral cortices were dissected from E14 mice and suspended and cultured to neurospheres in DMEM/F12 GlutaMax medium supplemented with B27, penicillin, streptomycin, HEPES buffer, basic fibroblast growth factor (bFGF) and epidermal growth factor (EGF). Astrocytes were then seeded at a concentration of 183 000 cells/ml in medium containing ciliary neurotrophic factor (CNTF).

Synaptosomes

In **project III**, we used synaptosome enrichment to specifically study the synaptic changes during α -syn pathology. Synaptosomes are isolated nerve terminals, which are separated from axons and dendrites through homogenization in a glass-Teflon tissue grinder^{200,201}. The membrane, which is pinched off during the homogenization, reseals into synaptosomal spherical structures. The synaptosomes can then be enriched by subcellular fractionation²⁰²⁻²⁰⁴. The synaptosomes have a diameter of 0.5-1 μ m and contain presynaptic proteins, SVs, mitochondria and parts of the post-synaptic density²⁰¹.

We utilized synaptosomal fractions from A30P tg and wt control mice, as well as PD, DLB and non-neurodegenerative control brains to characterize the effect of α -syn pathology on the SNARE proteins. Briefly, the mice were anesthetized with isoflurane and transcardially perfused with 0.9% saline and the left-brain hemispheres were snap frozen with dry ice and stored at -70° C. The human brain tissues were fresh frozen in -70° C without fixatives. After tissue homogenization with a glass-Teflon tissue grinder, the brain tissue homogenates were first centrifuged at low speed to remove nuclei and cell debris. The supernatant was then centrifuged at higher speed to separate the cytosolic soluble fraction from a crude synaptosome fraction. The crude synaptosome pellet was then resuspended and further fractionated via ultracentrifugation over a discontinuous Ficoll gradient (6, 9 and 13% Ficoll). The enriched synaptosome fraction was then recovered in the 9-13% interface.

Brain sections

In **paper III**, brain sections from A30P tg and wt control brains were used to investigate the regional changes in SNARE protein levels and distribution. The right hemisphere was fixed in formaldehyde, embedded in paraffin, sagittally sectioned (5 μ m) and mounted on glass slides.

Preformed fibrils

We generated PFFs from recombinant mouse α -syn monomers from Proteos Inc. (Kalamazoo, MI) to use for seeding experiments in **paper IV** (described in the section “Seeding”). First, samples were centrifuged at 15 000 x g for 10 min at 4° C and the concentration was determined using Nanodrop (ϵ for mouse α -syn: 7450 M⁻¹cm⁻¹, DeNovix). After diluting the sample in 2x PBS to 5 mg/ml, the sample was sterile filtered through a 0.45 μ m cellulose acetate filter (Costar spin-x) at 16 000 x g for 5 min. A small aliquot was stored at -70° C, to be used as monomeric α -syn in the experiments. The rest of the sample was vortexed at high speed for 3 sec before being placed on 1000 rpm nutation for 7 d at 37° C for generation of PFFs and then stored at -70° C. The PFFs were diluted to 2 mg/ml and sonicated (Sonics) at 20% amplitude with a 1 sec on, 1 sec off cycle for 1 min before addition to cells. The monomeric sample was not sonicated.

Isolation of extracellular vesicles

In **paper II and IV**, we studied the propensity of cell-to-cell transfer of α -syn via EV secretion. Extracellular vesicles were isolated from condition medium of transfected SH-SY5Y cells (**paper II**) and α -syn monomer- or PFF-treated astrocytes (**paper IV**) using ultracentrifugation. First dead cells and debris were removed by filtering the medium through a 0.45 μ m syringe filter (**paper II**) or by centrifugation at 300 xg, followed by an additional centrifugation at 2000 xg (**paper IV**). Then, the supernatant was ultracentrifuged at >100,000 xg. In **paper II**, we collected the FFP fraction and the EV pellet, which was resuspended in PBS, filtered through 0.22 μ m Millex syringe filter and recentrifuged. The EVs were then resuspended in either PBS (0.1% bovine serum albumin) or radioimmunoprecipitation assay (RIPA) buffer with protease inhibitor cocktail to study either external α -syn only or external + internal EV-associated α -syn. In **paper IV**, the EV pellet was resuspended in neurobasal medium and added to primary neurons.

In **paper II**, the EVs were characterized, by analyzing the expression of exosomal markers using western blot analysis and by measuring the EV size with EM and NanoSight. The results indicated that the EVs were a mixture of exosomes and larger EVs, since we detected Flotillin-1 expression, along with a size distribution of 100–300 nm.

Methods

Immunological techniques

When the body detects a novel pathogen or foreign antigen, the adaptive immune system creates an immunological memory which enhances the response to future encounters. One such mechanism is the production of antibodies that recognize the antigen with high specificity. Several techniques have been developed utilizing the antigen recognition. The antibodies for laboratory purposes are typically generated in mice and rabbits, but can also be produced in other animals such as donkeys or goats.

Immunocytochemistry/Immunofluorescence

In immunocytochemistry, antibody-antigen recognition is used to detect proteins in fixed cells. It can be used to visualize and measure both endogenous protein levels (**paper I and IV**) and transgenic protein expression (**paper II**). Before addition of the antibody, the cells are usually permeabilized with surfactants such as Triton X-100, to enable the antibodies to access intracellular protein targets. A serum (normal goat serum) is usually added to block non-specific binding.

Primary antibodies are selected to detect the protein of interest. Several different primary antibodies can be used simultaneously, as long as they are raised in different animals. Secondary antibodies are selected to bind the primary antibodies and they are conjugated to a reporter molecule which enables visualization in a microscope. In immunocytochemistry, the reporter molecule is most often a fluorophore, hence the name immunofluorescence. Secondary antibodies need to be selected with fluorophores of different emission and absorption spectra with no spectral overlap, in order to avoid bleed-through and unspecific signals.

Finally, a mounting medium is typically combined with DAPI, which counterstains DNA. The mounting medium protects the cells and preserves the fluorescence during imaging.

Immunohistochemistry

Immunohistochemistry is a well-established technique to visualize proteins in tissue. It is the same concept and has the same origin as immunocytochemistry, although the tissue preparation and detection method vary slightly. We used mouse brains, which were formalin-fixed and embedded in paraffin in **paper III**, even though frozen tissue can also be used. The tissue was sagittally sectioned on glass slides with a thickness of 5 μm .

Before staining, the paraffin needs to be removed and the tissue rehydrated, and this is done in a series of baths containing first xylene and then ethanol of decreasing concentration. Heat-induced epitope retrieval is used to enable the recovery of antigen reactivity in the formalin-fixed paraffin-embedded tissue.

Similar to immunocytochemistry, the tissue is permeabilized and blocked. Hydrogen peroxidase (H₂O₂) is used to block endogenous peroxidase activity, which otherwise could react with the chromogen and cause unspecific staining.

Just as in immunocytochemistry, fluorescently labelled secondary antibodies can be used for visualization. However, brain tissue emits extensive autofluorescence. Instead we used biotinylated secondary antibodies and streptavidin-HRP. The signal was visualized with NovaRed and hematoxylin was used for counterstain. The chromogenic detection enables visualization in a bright field microscope, which makes it generally easier to evaluate the tissue than with fluorescence microscopy.

SDS-PAGE western blot

Western blot can be used to detect proteins in a homogenate. The proteins are first separated by size with a sodium dodecyl sulfate (SDS)-polyacrylamide gel electrophoresis (PAGE). The SDS denatures the proteins and gives them a negative charge. The proteins are then separated through electrophoresis over a polyacrylamide gel, where smaller proteins migrate faster through the gel mesh. The proteins on the gel are then transferred to a membrane, which is stained with antibodies. Similar to IHC and IF, primary antibodies are selected to bind the proteins of interest and secondary antibodies are conjugated to chromogens or fluorophores for detection.

We utilized western blot for detection of α -syn and SNARE proteins in **paper III**. In the same paper, we also measured intact SNARE complexes in the synaptosomes. Since SNARE complexes are heat-sensitive, but SDS resistant^{100,141,205}, intact SNARE complexes can be measured by eliminating the boiling-step of the protocol. Western blot was also used in **paper II** to measure markers in the lysed EVs and the corresponding FFP fraction.

Sandwich ELISA

Enzyme-linked immunosorbent assay (ELISA) is another method to measure specific proteins in a complex homogenate. In contrast to SDS-PAGE western blot, proteins are not separated by size, but instead the protein concentration is determined by using a recombinant protein standard.

We have used sandwich ELISA in **paper I and III**. The name refers to the protein or antigen being “sandwiched” between a capture antibody, which is coated on the plate, and a reporter antibody. The capture antibody binds the protein of interest to the plate, which allows for the other proteins and materials in the homogenate to be washed away, leaving only what you intend to detect. A reporter antibody, which is specific for the protein of interest, but which targets a different epitope (unless the purpose is to measure multimeric forms of a specific protein) is then added. Horseradish peroxidase conjugated secondary antibodies are typically used for detection by measuring absorbance with a spectrophotometer.

Proximity ligation assay

In **project I, II and IV**, we used proximity ligation assay (PLA) for detection of protein-protein co-localization in primary neurons. Proximity ligation assay is an antibody-based system that can be used to assess protein co-localization (**Figure 5**). The antibodies used in the PLA are linked to an oligonucleotide sequence, which forms a template for a rolling circle amplification (RCA) if the proteins are within 40 nm proximity from each other. The product from the RCA is tagged with fluorescently labelled nucleotides, which can be monitored in a standard fluorescence microscope ²⁰⁶.

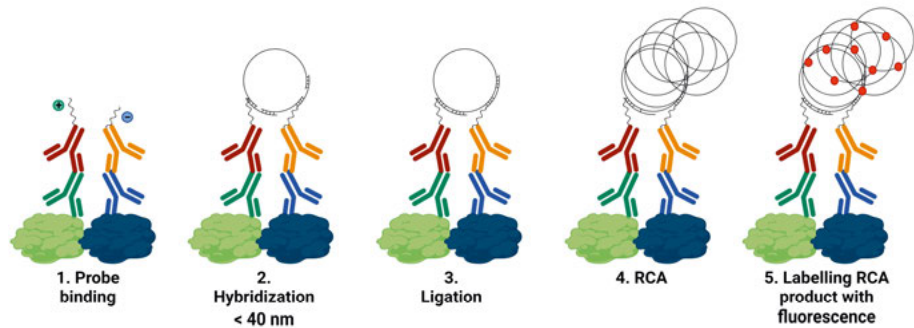


Figure 5. Proximity ligation assay for detection of co-localizing proteins. The proteins of in-terest are targeted with primary antibodies of different species. Secondary antibodies fused to oligonucleotide sequences (PLA probes) detects the primary antibodies (1). If the antigen is located <40 nm proximity, the PLA probes will hybridize (2) and ligate (3) to form a template for RCA (4). The RCA product is labelled with fluorescence (5) forming a PLA punctum which can be visualized in a microscope.

Even though PLA does not show protein-protein interactions *per se*, but rather proteins in close proximity, the technique has several advantages compared to similar methods. Standard immunocytochemistry or immunofluorescence staining are widely used to show co-localization between proteins as the tags from different antibodies overlap. However, this method does not indicate how much of the proteins that co-localize. Even in combination with super-resolution microscopy, PLA has an advantage. In PLA the fluorescently labelled RCA product will appear as an individual punctum. Hence, the number of PLA puncta will provide an estimate of the frequency of proteins co-localization, which is then easily quantifiable.

Other methods such as FRAP/FRET or bimolecular fluorescence complementation assay (BiFC), are considered to be more specific for direct protein-protein interactions and have the advantage of enabling live cell monitoring ^{207,208}. However, these methods rely on the transfection of proteins into the cell or the use of tg animal models. With PLA, we are instead able to study endogenous protein-protein co-localizations directly.

Bimolecular Fluorescence Complementation assay

The BiFC assay, is a useful method for studying protein-protein interactions. In order to detect interactions, the two proteins of interest are fused with respective half of a fluorescent protein and expressed in cells (**Figure 6**). If the fusion-proteins interact it results in reconstitution of the fluorescent tag. In **paper II**, α -syn was fused to either the N-terminal or C-terminal half of enhanced yellow fluorescent protein (EYFP) Venus and expressed via Lipofectamine 2000 transfection of pcDNA3.1+ vectors.

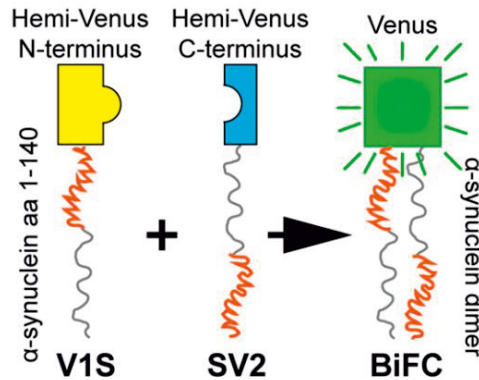


Figure 6. Bimolecular fluorescence complementation assay. The N-terminal half of Venus (yellow) was fused to the N-terminus of α -syn (red, V1S) and the C-terminal half of Venus (blue) was fused to the C-terminus of α -syn (grey, SV2). As α -syn assembles into dimers, the two Venus halves are reconstituted into a functional fluorophore (green).

Seeding

The aggregation cascade of α -syn can be accelerated (*seeded*) by addition of α -syn aggregates. This has been exploited to generate α -syn pathology cell and mouse models. For example, injection of oligomers or PFFs into the striatum of mice or addition of α -syn aggregates to primary neuronal cultures causes recruitment of endogenous α -syn and cell-to-cell transfer of protein aggregates, similar to what is observed in PD^{122,209–213}

In **paper IV**, we exposed wt primary neurons to α -syn monomers and PFFs to investigate the effect on SNARE protein distribution. Both short-term and long-term exposure were analyzed to investigate the direct effect of the α -syn species, as well as their potential seeding effect on endogenous α -syn. To potentially increase the seeding efficacy in the long-term experiments, we also included a protein delivery reagent, which increased the uptake of the seeds. Furthermore, we blocked the clearance of α -syn monomers and PFFs by inhibiting lysosomal degradation.

Lastly, we investigated potential glial-to-neuron transfer of α -syn by exposing astrocytes to α -syn monomers or PFFs, prior to isolation of EVs from the culture medium. The astrocytic EVs were then added to primary neurons.

Imaging

Throughout this thesis, we have used four different microscopy techniques; confocal microscopy, fluorescence microscopy, bright field microscopy and electron microscopy (EM, further described below).

The difference between a standard fluorescence microscope and confocal microscope is that during imaging with a confocal microscope the focus of the illumination and detection lies in a single spot which is moved over the specimen²¹⁴. It substantially reduces background signal and makes it possible to discriminate structures in different focal planes. However, even though the resolution becomes greater, it is a time-consuming process which makes it unpractical for quantifications. In **paper I**, confocal microscopy was used for generation of example images but imaging for quantification was used with a standard fluorescence microscope.

Since we were interested in detecting proteins in the whole cell in **paper I**, we used z-stack imaging to create a three dimensional view of the cells. However, in **paper IV** we concluded that single plane images would suffice since neuronal processes on glass slides are relatively flat and can get captured in a single image.

While capturing IF images for quantitative measurements, which was done in all the papers of this thesis, it is important to keep the light intensity and exposure time set constant to enable direct comparisons between the different images.

In **paper III**, we used bright field microscopy for imaging of SNARE protein expression in brain sections of A30P tg and wt mice.

Electron microscopy

EM imaging was performed to analyze the EV and FFP fractions from the SH-SY5Y culture medium (**paper II**), as well as the size of PFF before and after sonication (**paper IV**). Prior to isolation, the EVs were fixed in 2.5% glutaraldehyde. All samples were contrasted with uranyl acetate. In **paper II**, the samples were imaged with a H-7100 transmission EM (Hitachi, Chiyoda, Japan). In **paper IV**, the samples were imaged with TecnaiTM G2 Spirit BioTwin transmission EM (Thermo Fisher/FEI) at 80 kV with an ORIUS SC200 CCD camera and analyzed with Gatan Digital Micrograph software (both from Gatan Inc.).

Image analysis

To increase the resolution for analysis, we performed image deconvolution in **paper I**. When light falls over different media, such as air, glass or liquid, it causes diffraction or convolution as the light course is scattered. Furthermore, objects that falls outside the focal plane becomes blurry in an image taken with a fluorescence microscope. The “true” signal can be restored by image deconvolution by adjusting for the optical properties of the microscope, glass slides, mounting medium and fluorescent tag.

ImageJ-win64 was used for image analysis in all projects. For counting PLA puncta, the background was first subtracted, followed by selection of a threshold to determine positive signals. The threshold was kept constant within the same experiment to allow direct comparison between the groups. The PLA puncta was separated with the watershed tool and counted with the analyze particle tool. In **paper I**, the number of PLA puncta was quantified with the ImageJ 3D Objects Counter to avoid counting the same puncta twice in the different z-stack layers. The number of PLA puncta in neurons was normalized to number of cells in **paper I** and to the area of F-actin in **paper IV**.

Results and discussion

Assessment of α -syn species at synapses

A central hypothesis in this thesis is that α -syn pathology can be found in the synapses. This idea is supported by previous findings of small synaptic α -syn aggregates in both patient and α -syn tg mouse brains^{10,12,127,129}. In order to investigate the nature of synaptic α -syn species in A30P tg mice, in **paper III**, synaptosomal preparations were subjected to western blotting. The synaptosomes were shown to contain both human and mouse α -syn (**Figure 7**). The A30P mutation makes α -syn less prone to bind to lipid membranes^{21,97,98}, thereby potentially reducing the synaptic targeting¹⁰⁰. However, similar to previous reports of α -syn in the synapses of the same mouse model¹⁹¹, we detected human A30P α -syn in the synaptosomes, confirming the synaptic localization of A30P α -syn. Furthermore, as previously reported¹⁹¹ we detected more than a two-fold increase of total α -syn in the A30P tg mouse synaptosomes. Thus, not only does the mouse model contain human α -syn with a disease-causing mutation, but also overall increased levels of α -syn, which in itself can contribute to pathogenesis. For instance, *SNCA* gene duplications/triplications and alterations in α -syn clearance can cause PD^{82,83,163,171}.

Human α -syn was more resistant to digestion with proteinase K compared to mouse α -syn, indicating that synaptic aggregates were comprised mainly of human α -syn. Cross-seeding between human and mouse α -syn is less efficient than homologous seeding²¹⁵, which could be a potential explanation to why the mouse α -syn was not part of the aggregates.

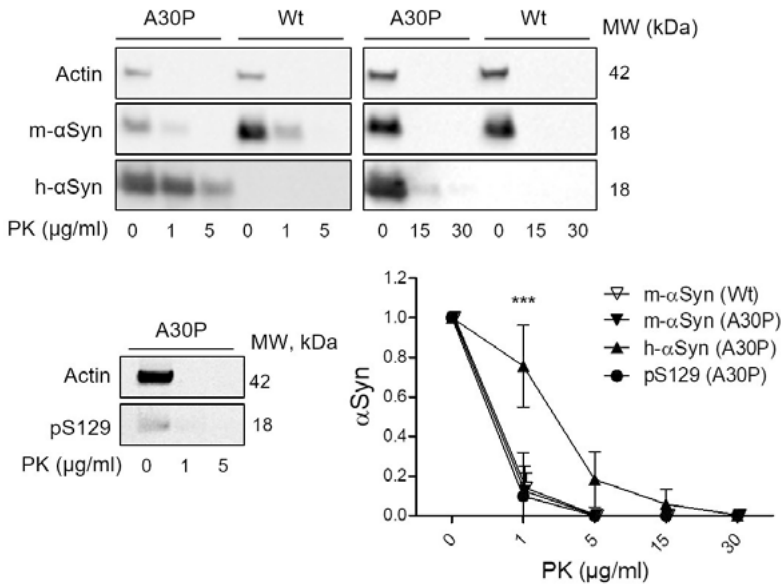


Figure 7. Synaptic α -syn aggregates were mainly composed of non-phosphorylated human A30P α -syn in the A30P tg mouse brains. Representative images of western blot analysis of synaptosomal human, mouse and pS129 α -syn after proteinase K treatment at 0, 1, 5, 15 and 30 μ g/ml. Actin was used as loading control. Data presented as mean \pm SD.

The synaptosomes also contained α -syn phosphorylated at ser129 (pS129). Although the majority of α -syn in LBs are pS129¹¹⁰ positive, the pS129 α -syn measured in the synaptosomes were easily degraded by proteinase K. In addition, we also detected synaptosomal pS129 α -syn in 11 mo A30P tg mice, but similar to the older age group, the pS129 α -syn in the younger mice were readily degraded by proteinase K. Similarly, pS129 α -syn positive areas were negative for thioflavin S in A30P tg mouse brain¹⁹³. This could indicate that pS129 α -syn in A30P tg mouse brains are less compact than in other α -syn mouse models. Thus, it appears that the synaptic pS129 α -syn in A30P tg mouse brain exist as a monomeric form or is part of smaller and less compact aggregates. A hypothesis is that pS129 α -syn might have a neuroprotective function with reduced propensity of α -syn aggregation and increased clearance of pathogenic species^{216,217}. The idea is supported by the fact that pS129 α -syn appear to be located at the outer boarder of LBs and the phosphorylation has been suggested to be a late event of disease^{216,218}. Furthermore, C-terminal truncations of α -syn, where the aa 129 is removed, causes enhanced α -syn aggregation^{219,220}. This could potentially explain why pS129 α -syn is not aggregated in the synaptosomes. However, further studies need to be done in order to strengthen the theory.

Challenges in modeling α -synucleinopathies in primary neurons

In order to study molecular changes caused by α -syn aggregation, we have utilized two different approaches to mimic α -synucleinopathy-like pathology in primary neurons. In **paper I**, we cultured primary cortical neurons derived from embryonic A30P tg mouse brains. The A30P tg mice overexpress human α -syn with the A30P mutation which causes early onset PD in humans. However, the expression of human A30P α -syn was low in the A30P tg neurons. The human A30P α -syn was mainly found in the soma and not to same extent in the synapses. This may be explained by the late expression of the Thy-1 promoter^{221,222} and the fact that the neurons were fixed at an early stage (12 DIV). On the contrary, the brain material used for the synaptosome experiments were collected from adult mice, where A30P α -syn is expected to be found at the synapse.

In **paper IV**, we exposed the neurons to α -syn PFFs to induce α -syn pathology. The wt neurons in **paper IV** were cultured for 19 or 20 DIV to allow maturation of the neurons and synapses. Whereas previous seeding-studies have claimed to generate a robust response to exposure of α -syn PFFs with substantial pS129 α -syn levels²¹², we were only able to induce low levels of pS129 α -syn (**Figure 8**). One possible explanation could be the size of the PFFs utilized. The mean size of the sonicated PFFs used for seeding in our experiments were 72.2 nm (SD = 34.0 nm), whereas the optimal length of PFFs for seeding have been shown be less than 50 nm²²³.

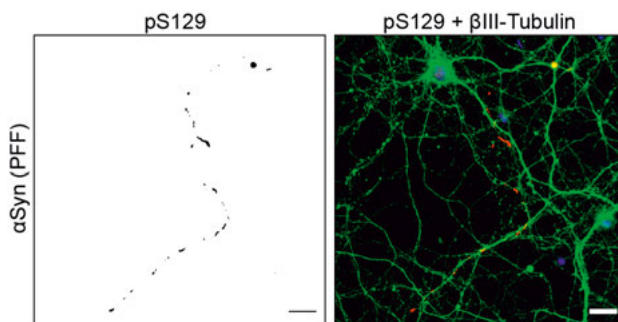


Figure 8. Long-term PFF exposure in primary neurons generated a modest pS129 α -syn signal. Immunofluorescence image of pS129 α -syn in primary cortical neurons (19 DIV) following 7 d of exposure to PFFs. The black and white image is an inversion of the pS129 α -syn signal, which is red in the RGB image to the right. β III-tubulin (green) and DAPI (blue). Scale bar 20 μ m.

Only about 24.4% of the PFFs used in our experiments had a length shorter than 50 nm, which could potentially explain the low amount of formed pS129 α -syn pathology. To further promote the formation of endogenous α -syn pathology, we utilized a couple of different strategies by either using the protein

delivery reagent Chariot™ to increase the uptake of recombinant α -syn, or by blocking the degradation by the lysosomal inhibitor chloroquine (CHQ). Even though lysosomal inhibition with CHQ induced higher pS129 α -syn levels than the other seeding experiments, we were still not able to achieve the same level of α -syn pathology as previously reported²¹².

Although, we were not able to induce robust α -syn pathology in the primary neurons, our data indicate that even a low amount of pathology could affect SNARE protein distribution in our seeding model (described below).

Differential co-localization of SNARE proteins with physiological and pathological forms of α -syn

Since α -syn has been suggested to alter SNARE protein levels, distribution and ultimately function, we wanted to investigate if both physiological and pathological α -syn co-localized with the SNARE proteins in our models. In **paper I**, we used PLA to study the interaction between α -syn and the SNARE proteins in both wt and A30P tg primary cortical neurons. First, we used an antibody which recognized both mouse and human α -syn to measure total α -syn in combination with antibodies against VAMP-2, SNAP-25 and syntaxin-1, respectively. We were able to visualize co-localization between α -syn and all three SNARE proteins. The PLA signals were both situated in the processes and in the soma. Quantification of 3D images captured by z-stacks spanning the whole neurons indicated that the majority of the PLA puncta were found in the neuronal processes within 1-2 image layers of the neurons. Thus, for the quantifications performed on primary neurons in **paper IV**, we concluded that single images in the neuronal process-layer would suffice.

In **paper I**, all three SNARE proteins also co-localized with A30P α -syn while using a human α -syn specific antibody (**Figure 9A**). The PLA puncta were mainly found in the neuronal soma, and thus differed from the PLA with a total α -syn antibody, and instead matched the expression of human α -syn. As described earlier, this is most likely due to the late expression of the Thy-1 promoter and the earlier fixation at 12 DIV.

We also detected a co-localization between VAMP-2 and pS129 α -syn in the A30P tg mouse brain using *in situ* PLA in **paper III**. We measured the co-localization in the area CA1 and CA3 of hippocampus as well as in the prefrontal cortex (PFC, **Figure 9B**). In comparison, wt mouse brain did not show any co-localization between pS129 α -syn and VAMP-2, which is expected since pS129 is not normally detected in wt mouse brain.

As previously shown in the synaptosomes, and earlier studies in the A30P tg mice¹⁹³, the presence of pS129 α -syn does not necessarily mean that the α -syn is in an aggregated form. Thus, the PLA signal could display co-localization between VAMP-2 and both monomeric and aggregated pS129 α -syn. A

method to determine the compactness of the aggregated α -syn is to pre-treat the tissue with proteinase K before PLA staining. Although, VAMP-2 would likely be degraded, it would be interesting to see if some of the VAMP-2 immunoreactivity would be retained, which would indicate that VAMP-2 is incorporated in pS129 α -syn aggregates.

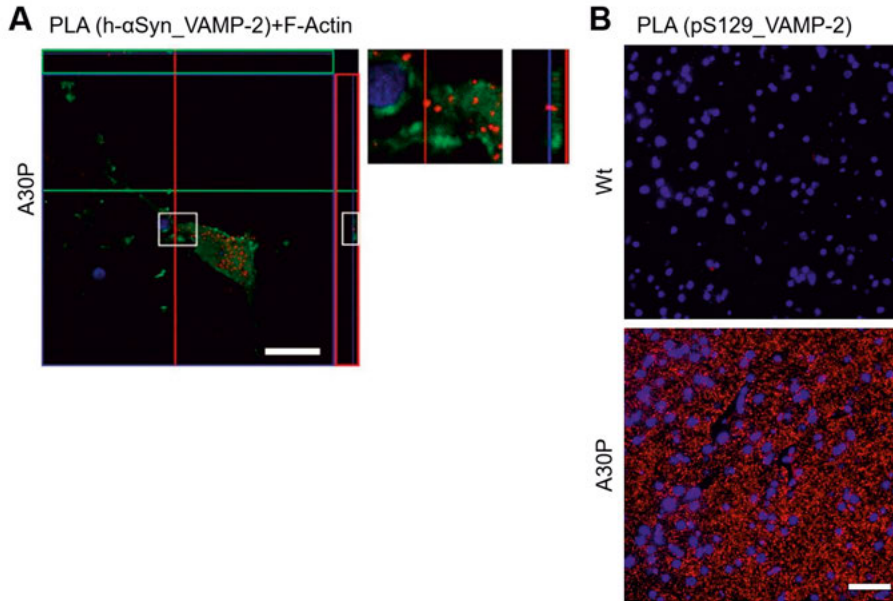


Figure 9. Co-localization between pathogenic α -syn and VAMP-2 in primary neurons and in the A30P tg mouse brain. (A) Confocal image of PLA between human α -syn and VAMP-2 (red) in A30P tg neurons. F-actin (green) and DAPI (blue). Scale bar 20 μ m. (B) *In situ* PLA between pS129 α -syn and VAMP-2 (red) in PFC of A30P tg and wt mouse brains. DAPI (blue). Scale bar 50 μ m.

Altered distribution of SNARE proteins in α -synucleinopathy models

Overexpression of A30P α -syn induced changes in SNARE complex formation and protein distribution *ex vivo* but not *in vitro*

In **paper I**, there was no significant difference in PLA puncta between A30P tg and wt neurons for either of the antibody combinations with total α -syn/SNARE protein PLA. However, as previously mentioned, this is most likely due to the low expression of the human A30P α -syn in the primary neurons.

On the contrary, the A30P tg mice displayed changes in both formation of SNARE complexes and distribution of SNARE proteins in synaptosomes and brain sections, compared to wt controls in **paper III**. Interestingly, we detected a reduction of intact SNARE complexes while analyzing unboiled samples by western blot (**Figure 10**), even though the total levels of the SNARE proteins were unchanged in the A30P tg mouse synaptosomes. Furthermore, PLA analysis revealed a reduced co-localization of SNAP-25 and syntaxin-1 in the PFC. The same area displayed a slight reduction of VAMP-2 levels with immunohistochemistry, however the levels of SNAP-25 was not altered. Interestingly, the same type of reduction of intact SNARE complexes with unaltered SNARE protein levels have previously been described in frontal lobe cerebral cortices of PD brain¹³¹.

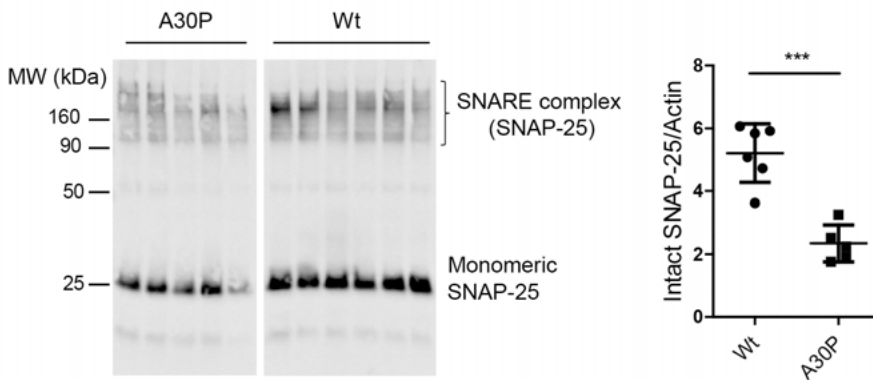


Figure 10. Reduction of intact SNARE complexes in synaptosomes from A30P tg mice. Representative images of western blot of SNAP-25 in unboiled synaptosomal fractions of A30P tg and wt mouse brain. Quantification of high molecular weight bands (intact SNARE complexes) relative to loading control β -actin (n = 5-6 per group). Two-tailed student's t-test (***) P < 0.001, data presented as mean \pm SD.

However, whether the altered distribution of SNARE proteins is caused by an increased level of total α -syn or the formation of A30P α -syn aggregates cannot be concluded definitely. For example, the reduced co-localization could be caused by α -syn aggregation, as we could show that human A30P α -syn forms aggregates in the synapses of the A30P tg mice (**paper III**). Furthermore, human A30P α -syn co-localized with the SNARE proteins in the primary neurons (**paper I**). Thus, human α -syn aggregates could potentially affect the SNARE protein distribution and SNARE complex formation. However, there is also an overexpression of human A30P α -syn in the mouse model, leading to increased total levels of α -syn (**paper III**). Hence, the reduction of intact SNARE complexed could also be a consequence of physiological α -syn overexpression, which previously has been shown to affect the motility and docking of the SVs^{43,45-47}.

Changes in the SNARE protein distribution upon direct exposure to different α -syn species

In **paper IV**, we studied the direct effect of different α -syn species on the SNARE protein distribution in wt primary neurons. The benefit, compared to working with the A30P tg mouse model, is that there is no general overexpression of α -syn and the observed effects are only generated by the exogenously added α -syn species. We exposed neurons to α -syn monomers or PFFs for 2 or 24 h before fixation to study a direct effect of the individual α -syn species. The time of exposure was chosen to give the neurons time to internalize the recombinant proteins, but not enough time to induce endogenous α -syn seeding. It has previously been demonstrated that α -syn PFFs can be endocytosed after 1 h and then transferred to lysosomes after 24-48 h in hippocampal neurons²²⁴.

Interestingly, with PLA we observed an increase of VAMP-2/syntaxin-1 co-localization, whereas the co-localization between SNAP-25 and syntaxin-1 was reduced after 24 h exposure to both monomeric α -syn and PFFs. The reduced co-localization of SNAP-25 and syntaxin-1 could be explained by the clustering of either exogenous added monomeric or fibrillar α -syn at the plasma membrane, since both SNAP-25 and syntaxin-1 is localized there²²⁵.

As α -syn has been reported to be a chaperone in SNARE complex assembly^{13,37}, the increased co-localization between VAMP-2 and syntaxin-1, observed with both monomeric and fibrillar α -syn, could potentially be explained by that the different α -syn species bind to VAMP-2 and promotes its interaction with syntaxin-1.

Another aspect is that the increased VAMP-2/syntaxin-1 co-localization seen with either monomeric α -syn or PFFs could potentially represent different functional outcomes. Since we have not performed any functional studies or EM to analyze the SV pools, we cannot conclude that the increased co-localization would actually increase (or even decrease) neurotransmitter release or affect the SV motility. Thus, further studies are needed to elucidate how the observed redistributions would affect the function of the SNARE complexes and neurotransmitter release.

Changes in the SNARE protein distribution upon long-term exposure to different α -syn species

To investigate the effects of endogenous seeding of α -syn on SNARE protein distribution, we exposed wt neurons to α -syn PFFs for 7 d in **paper IV**. As discussed earlier, we were not able to induce a robust pS129 α -syn pathology in these set of experiments. However, despite the low levels of induced pS129 α -syn, the long-term exposure to α -syn PFFs caused a reduced co-localization of VAMP-2 and SNAP-25 in wt neurons. When we attempted to further induce uptake of α -syn PFFs with the protein delivery reagent ChariotTM, it re-

sulted in an even more substantial decrease in VAMP-2/SNAP-25 co-localization (**Figure 11**). Interestingly, also long-term exposure of α -syn monomers reduced the co-localization between VAMP-2 and SNAP-25. This was somewhat surprising, since we expected the monomeric α -syn to be degraded by the neurons, since previous studies have shown that α -syn is transferred to and degraded by lysosomes within 48 h after endocytosis²¹¹. However, it is possible that the degradation was slower in the presence of Chariot™, or that monomeric α -syn induced other long-term effects in the neurons.

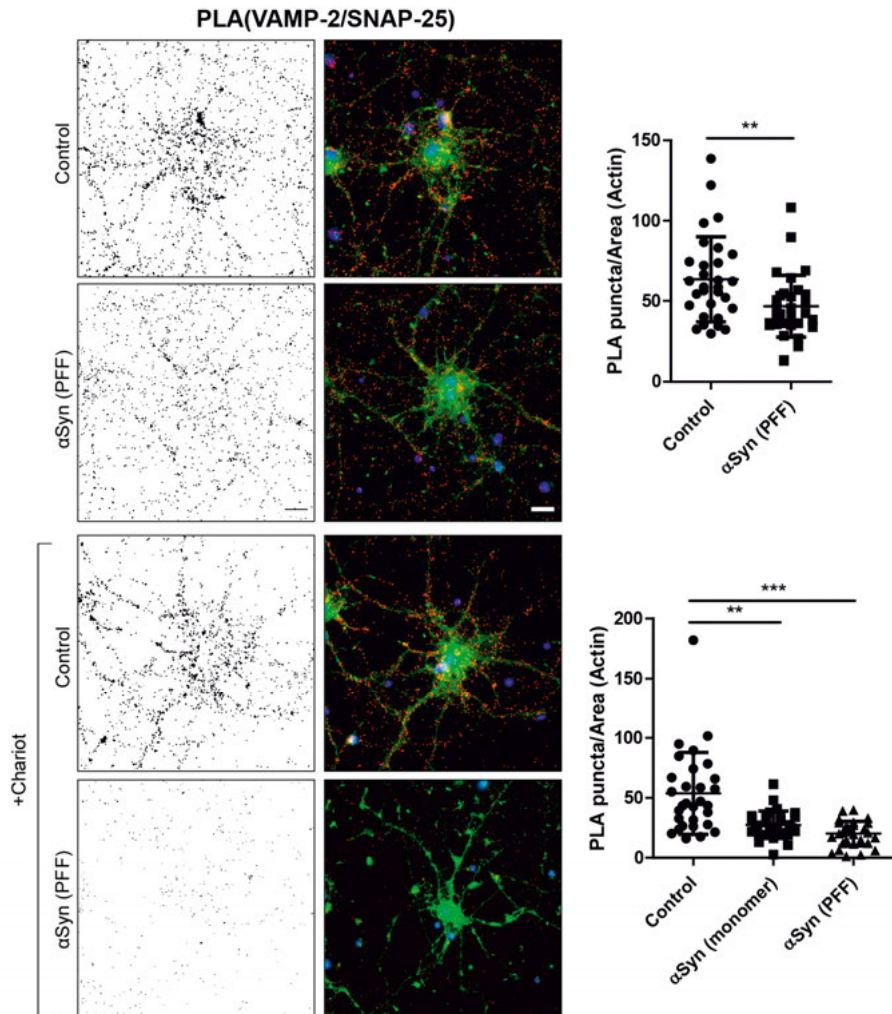


Figure 11. Long-term exposure (7 d) of α -syn PFFs caused reduced co-localization between VAMP-2 and SNAP-25 in primary cortical neurons. Representative images of PLA between VAMP-2 and SNAP-25 in neurons seeded with α -syn PFFs (70 nM) or PBS with or without the protein delivery reagent Chariot™. The black and white image depicts the PLA puncta, reflecting the red signal in the RGB image. F-actin (green) and DAPI (blue). Scale bar 20 μ m. Quantification of PLA puncta per area of F-actin. Mann Whitney's test (** P<0.01, *** P<0.001), data presented as mean \pm SD.

Lysosomal inhibition with CHQ in combination with α -syn monomers exposure also caused reduced co-localization between VAMP-2 and syntaxin-1. In the same experiment, we also observed a general reduction of neuronal processes while staining for F-actin. This could indicate that the reduced co-localization is not a specific effect on the SNARE proteins. However, reduced lysosomal degradation might also have caused an increased accumulation of monomers in the neurons. Thus, we might have generated a variant of an over-expression model, which previously has been demonstrated to reduce neurotransmitter release⁴⁶. Interestingly, in a mouse model of lysosomal storage disorders α -syn was shown to accumulate into insoluble aggregates and this resulted in lower presynaptic α -syn levels and impaired SNARE complex assembly¹⁷¹. This signifies the importance of further investigations into the exact molecular mechanisms, which resulted in altered SNARE protein co-localization.

Interestingly, the PFF-exposure together with CHQ treatment did not affect the SNARE protein distribution of VAMP-2 and syntaxin-1, even though the levels of pS129 α -syn increased. Inhibition of lysosomes with CHQ has previously been shown to increase pS129 α -syn in hippocampal neurons after PFF-exposure²²⁴. Thus, the hypothesis of a neuroprotective function of pS129 is again brought to mind: It is possible that increased levels of pS129 α -syn could reduce the recruitment of monomeric α -syn to the aggregates, and thus prevent SNARE protein redistribution.

Another explanation could be that partial lysosomal degradation of PFFs by the lysosomes actually makes the fibrillar species more neurotoxic. If lysosomes partially degrade PFFs, it could potentially make them more similar to oligomeric α -syn. Thereby, the SNARE protein redistribution seen after PFF-exposure could be inhibited by addition of CHQ.

Transfected overexpression of N-terminal modified α -syn in SH-SY5Y cells stimulated the secretion of α -syn via EVs

Since EV secretion have been proposed as a route of cell-to-cell transfer of α -syn species, and thereby spreading of disease, in **paper II** we wanted to see if

the molecular properties of α -syn redirect the cellular processing. We overexpressed different α -syn constructs in SH-SY5Y cells and measured the α -syn levels in the FFP and EV fractions of the condition medium by ELISA and WB. Likewise to previous reports, we found that only a small fraction (0.1-2%) of α -syn were associated to EVs^{158,226}. Furthermore, we observed that the EV-associated α -syn were mainly situated on the membrane of the EVs, but also to some extent resided inside the vesicles.

Whereas wt α -syn was predominantly secreted into the FFP fraction, N-terminal modifications of the protein redirected the cellular processing towards EV secretion. There was a shift towards EV secretion especially when the N-terminal part of Venus (aa 1-157) were fused to the N-terminus of α -syn (V1S). Also, the PD-linked mutation A53T α -syn was redirected towards EV secretion to a higher extent than wt α -syn. The A53T mutation is known to have increased propensity for aggregation^{227,228}. Thus, it appears that the molecular properties of α -syn could affect the cellular processing pathways. Interestingly, the decreased lipid-binding potential of A30P mutated α -syn did not appear to affect the degree of EV secretion. Yet, the mutation has a stronger effect on the binding potential of larger vesicle and less on membranes with higher curvature²⁴, thus, the effect might not be seen with the small EVs.

Uptake of EV-associated α -syn by the recipient cells

To study if the increased α -syn secretion via EVs could result in enhanced cell-to-cell transfer, **in paper II**, we transferred the EV and FFP fractions of SH-SY5Y cells transfected to overexpress V1S or V1S + SV2 (BiFC) to non-transfected recipient cells (**Figure 12**). We found that EV-associated V1S had markedly increased uptake compared to the other fractions. So, the redirection of cellular processing towards EV secretion also increased the propagation propensity. However, with this experiment we cannot determine in which cellular compartment the internalized EV-associated α -syn ended up.

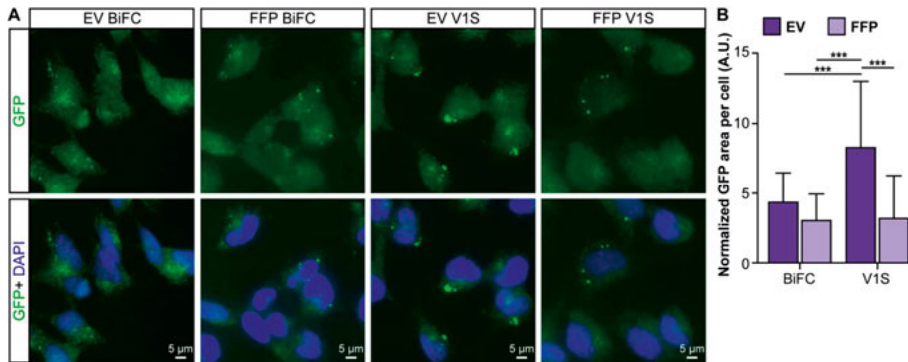


Figure 12. EV-associated, V1S-tagged α -syn is readily taken up by recipient cells. (A) Representative images of recipient SH-SY5Y cells which received EVs and FFPs from transfected SH-SY5Y cells expressing V1S + SV2 (BiFC) or V1S only. Immunocytochemistry against the hemi-Venus tag was performed to allow visualization of both constructs independently of dimerization (green). (B) Quantification of GFP signal in relation to number of cells (n=4). One-way ANOVA with Tukey's post hoc test (***) $p < 0.001$, data presented as mean \pm SD.

Astrocyte-derived EVs alter SNARE protein distribution

The laboratory has previously shown that astrocytes can accumulate and spread both aggregated α -syn and amyloid- β ^{184,185,187,229,230}. Therefore, in **paper IV** we additionally investigated if glial-to-neuron transmission via EV secretion could affect the distribution of SNARE proteins. Even though we only expected low amount of α -syn to be associated with the EVs, PLA between VAMP-2 and SNAP-25 revealed an increase in co-localization in primary neurons exposed to EVs from PFF-treated astrocytes compared to monomer-treated astrocytes. The distribution of SNARE proteins were significantly altered, even though the EVs isolated from PFF-treated astrocytes only generated very low levels of pS129 α -syn. The astrocytes have previously been shown to effectively degrade monomeric α -syn, but not PFFs¹⁸⁷. However, as discussed earlier, when we seeded primary neurons with α -syn monomer together with the protein delivery reagent ChariotTM, we could also see long-term effects on the SNARE proteins. Thus, α -syn monomer treated cells should not be regarded as a negative control *per se*. This contributes to the difficulty of interpreting the results of the PLA; are we actually visualizing an increased co-localization in the PFF-treated group, or a decreased PLA signal in the monomer-treated group?

Conclusions and future perspectives

In this thesis, the pathophysiological effects of different α -syn species on SNARE protein distribution and SNARE complex formation has been demonstrated. With both *in vitro* cell cultures and *ex vivo* brain tissue from A30P tg mice and α -synucleinopathy patients, it has been shown that monomeric and aggregated forms of α -syn can cause a redistribution of SNARE proteins. Furthermore, we have demonstrated that EVs secreted from both neurons and astrocytes can contribute to the propagation of pathogenic α -syn. Even though it has been challenging to find a perfect model, various models and different methods have been utilized to test our hypotheses. However, there are still several unanswered questions and new ideas to be investigated.

The results of our experiments could potentially be interpreted either as a loss-of-function, as the SNARE protein redistribution could have detrimental effects on the cells, or provide insights into a possible gain-of-toxic-function, with the accumulation of synaptic α -syn aggregates. Regardless, further studies need to elucidate the functional consequences of SNARE protein redistribution. For example, we do not know how the redistribution of SNARE protein affect neurotransmitter release. An interesting experiment to investigate this further would be to perform electrophysiology on the neurons. Additionally, EM imaging of the neuronal synapses would gain basic knowledge of how various α -syn species affect SV pools.

Furthermore, it would be interesting to perform similar experiments in a group of younger mice to investigate when the formation of synaptic α -syn aggregates and SNARE protein redistribution starts, and how this relates to the development of more mature LB- and LN-like pathology. Thus, we could potentially provide novel insights if synaptic changes are indeed early events of disease.

Additional experiments also need to be performed to in detail biochemically characterize the synaptic α -syn aggregates. It would be worth to investigate if there are different strains of synaptic α -syn aggregates and if the characteristics of the aggregates vary between different α -syn mouse models, or between different α -synucleinopathy patients.

Furthermore, we still do not know if the same effects on SNARE protein distribution can be detected in other tg α -syn mouse models. Since the A30P mutation has been shown to cause reduced lipid binding, and could thus alter the physiological function of the protein itself^{49,100}, it would be interesting to

investigate if there is a redistribution of SNARE proteins in mice overexpressing wt human α -syn. Additionally, it would be interesting to test whether α -syn (e.g., 1-95) PFFs that are lacking the proposed SNARE binding part of the C-terminal would induce the same short-term effect on SNARE protein distribution in primary neurons as full length α -syn PFFs.

It would also be informative to explore if targeting synaptic α -syn aggregates could reverse the effects on SNARE protein redistribution. This could potentially be performed by generating specific antibodies recognizing synaptic α -syn aggregates. Isolated synaptic α -syn aggregates from synaptosomes could be used for inoculation of mice to generate such antibodies. Synaptic α -syn aggregates would be a very interesting treatment target since that might both help save the synapses and neurons at early disease stages, as well as inhibiting/slowing down the propagation of α -syn pathology. Furthermore, the smaller aggregates, whether it may be oligomers or protofibrils, are more loosely packed than LBs and LNs and would be more easily degradable. Thus, these could also be a promising drug target, if we can identify strategies for neurons and/or glial cells to increase the clearance of the most toxic α -syn species.

Lastly, our experiments have shown that EVs are involved as a vehicle of cell-to-cell transfer of pathogenic α -syn. Hence, EV-associated α -syn could be a potential therapeutic target to reduce the spread of α -syn pathology.

The conclusion of this thesis further strengthens the notion of synaptic and EV-associated α -syn aggregates as valid targets for disease-modifying treatments of the α -synucleinopathies.

Populärvetenskaplig sammanfattning

Neurodegenerativa sjukdomar är tillstånd där nedbrytning och död av nervceller leder till försämrad hjärnfunktion. Detta leder till olika symptom, beroende på vilken del av hjärnan som är påverkad. Exempel på neurodegenerativa sjukdomar är Parkinsons sjukdom, Lewykroppsdemens, Alzheimers sjukdom och Huntingtons sjukdom. Gemensamt för dessa sjukdomar är onormala ansamlingar av aggregerade proteiner som tros ligga bakom förlusten av nervcellerna. Parkinsons sjukdom och Lewykroppsdemens räknas båda som α -synukleinopatier eftersom det i dessa fall är proteinet α -synuklein (α -syn) som ansamlas i hjärnan. Vid Parkinsons sjukdom leder α -syn ansamlingarna till nedbrytning av de dopaminproducerande cellerna i hjärnregionen substantia nigra, vilket orsakar de karakteristiska skakningarna, stela muskler och sämre rörelseförmåga.

Hjärnans nervceller kommunicerar via elektiska och kemiska signaler. Vid nervändsluten (synapserna) leder en elektisk impuls till frisättning av kemiska signalsubstanser (t.ex. dopamin) som registreras av en närliggande nervcell, som då i sin tur skapar en ny elektisk impuls och för informationen vidare. Signalsubstanserna är lagrade i synapsblåsor som måste öppnas upp vid cellmembranet efter en nervimpuls. Detta möjliggörs av ett specifikt proteinmaskineri som kallas SNARE komplexet, som nyper ihop synapsblåsan med cellmembranet.

Alfa-synuklein binder normalt till synapsblåsorna och tros interagera med ett av proteinerna i SNARE komplexet och därmed påverka dess funktion och signalsubstansfrisättningen. Men vad som händer med denna funktion vid sjukdom och aggregering av α -syn är fortfarande oklart.

I den här avhandlingen har jag undersökt hur SNARE proteinernas distribution påverkas av α -syn. I delarbete I och III, använde jag en musmodell som uttrycker humant α -syn med en Parkinson-mutation (A30P). I delarbete I kunde jag m.h.a. den antikroppsbaseerade tekniken ”proximity ligation assay” visa att både mus α -syn och humant A30P α -syn existerar i direkt kontakt (<40 nm) med SNARE komplexet i nervceller från A30P möss. Jag detekterade kontakt mellan alla tre SNARE proteiner som är involverade i signalsubstansfrisättning. Kontakten mellan mus alfa-synuklein och SNARE proteinerna var tydligast i nervcellernas utskott medan kontakten med humant A30P α -syn främst förekom i nervcellskroppen. I delarbete III visar jag att SNARE proteiner är omfördelade i hjärnan hos A30P möss och att det finns färre intakta

SNARE komplex. Detta kan vara ett tecken på försämrad synapsfunktion vilket kan utgöra en tidig händelse i sjukdomsutvecklingen hos människor.

I delarbete IV undersökte jag om omfördelning av SNARE proteinerna även kan ske i nervceller när de exponeras för α -syn aggregat. Denna typ av cellmodell, där man ”sår” patologi har tidigare visats härma sjukdomsutvecklingen och leda till ackumulering av nervcellernas eget α -syn. Jag noterade att SNARE proteinerna påverkades vid både korttidsexponering och över tid. Det intressanta är att förändringen var tydlig trots att vi endast mätte låga nivåer av sjukligt α -syn. Detta tyder på att omfördelningen av SNARE proteinerna kan ske väldigt tidigt i sjukdomsutvecklingen.

Jag undersökte även med vilken mekanism protein aggregat kan spridas i hjärnan. Hjärnans celler kan skicka blåsor (s.k. vesiklar) mellan varandra. Då celler har svårt att bryta ned beståndsdelar kan dessa i stället skickas ut för att tas om hand av friska celler. Problemet är att aggregerade proteiner är så kompakta att de är svåra att bryta ned även för friska celler och utsöndringen av vesiklar kan istället leda till att vesiklarna ”sår” sjukdom på samma sätt som jag gjorde i delarbete IV.

I delarbete II undersökte jag hur olika modifieringar av α -syn påverkar den cellulära omdirigeringen till ökat utsläpp via vesiklar. Jag kunde se att vissa typer av modifieringar ökade utsläppet av α -syn vesiklar och att detta ledde till ökat upptag i andra celler. Även i delarbete IV testade vi denna funktion. Då såg vi att α -syn vesiklar från en typ av stödjecell som kallas astrocyter kan leda till förändringar i SNARE komplexen hos nervceller.

Sammanfattningsvis, har jag i den här avhandlingen visat att aggregering av α -syn kan påverka SNARE komplexen redan vid till synes tidiga sjukdomsstadier. Detta kan vara en mekanism som driver på nedbrytningen av synapser och nervcellsöd. Men mer forskning krävs för att undersöka exakt hur omfördelningen av SNARE proteinerna påverkar deras funktion och signalsubstansfrisättningen.

Acknowledgements

The work was supported financially by grants from Swedish Research Council, Marianne and Marcus Wallenberg Foundation, The Swedish Brain Foundation, Parkinson Research Foundation, Swedish Alzheimer Foundation, Swedish Parkinson Foundation, Hans-Gabriel and Alice Trolle Wachtmeister's Foundation for Medical Research, Lennart and Christina Kalén, Stohne's Foundation, Swedish Dementia Foundation, Magnus Bergwall Foundation, Thore Nilsson Foundation, Åhlén Foundation, Loo and Hans Osterman's Foundation, King Gustaf V's and Queen Victoria's Freemason Foundation, Torsten Söderberg Foundation, and Sigurd and Elsa Golje's Foundation. I would like to thank the BioVis core at Uppsala University for their help with the TEM imaging and for teaching me ImageJ. Images in the introduction and materials and methods were created with BioRender.com.

This thesis was made possible with the help and guidance of many talented scientists and incredible human beings.

First of all I want to thank my main supervisor **Joakim**, who have guided me through both my master thesis and PhD journey. You have made me grow into more confident researcher and person. I thank you for your optimism and for encouraging me to try new things. Thanks to you I even enjoyed the final stretch and the writing process – which I never thought I would!

My co-supervisor, **Anna**, thank you for all the support and for giving me a new perspective whenever I have gotten stuck. It has been a comfort to be able to fall back on a solid support. I would like to thank my co-supervisor **Martin**, for all the help along the years. Both for the deep knowledge and scientific drive, as well as for ultra-fast and reliable help with the writing process.

Next, I would like to thank my dear friend **Leire**. Thank you for recruiting me to the amazing MolGer lab! You are also the one who convinced me to continue as PhD student. Without your encouragement, I would not have dared to aim for a PhD and would have missed out on an amazing journey. But most of all I thank you for all the fun times, both the laid back movie and game nights, as well as the incredible trip to the Basque country and to yours and Tsongs wedding.

Thank you **Anish**. I have said that a lot lately because of all the support you have been giving me, especially during the most stressful times. Thank you for helping me with the final images for the kappa and for staying with me

when I have struggled. It has been very fun to share the PhD journey with you and I will miss our lunches and fikas. You are a great friend.

A special thanks to my fellow molgerians, old and present. We have shared so many great experiences, everything from pub nights, game nights and exciting conferences or just bumping shoulders in a too small cell lab (too bad I didn't get to try the new one).

To the old crew, **Elisabeth**, **Tsong**, **Gabriel** who taught me the ropes and welcomed me to MolGer. Thank you for all the lovely dinners and great memories, like Chinese New Year at Tsongs closet-bedroom apartment. **Elisabeth**, you are such a positive person, thank you for all the laughs.

Thank you to the fellow PhD students who have walked the path along with me. **María**, thank you for your cheerful ways, **Silvio**, for the great raclette and **Tobias**, for the early lunches. **Jinar**, I cherish your friendship and admire your drive, you are going to go a long way. Good luck to you and **Dardan** and I hope you'll come back and visit often. I'll make sure to come and visit you too. To my dear friend **Evangelos**, thank you for all the support. I wish the best for you and **Maria** and the new up and coming molgerian. It will be so nice to see you as a father, you will do great! I love that you and Maria have welcomed Robin and I to the Uppsala Greek community and for inviting us to Thessaloniki. **Rebecca**, it has been wonderful to share the office with a friend and thank you for the great tips on writing. **Chiara** and **Daniel**, enjoy the parental leave! I hope we'll meet up for many play dates and lovely dinners.

Thank you **Eva**, **Gillian**, **Mengfei**, **Tobbe**, for the fun times and **Elin**, **Sara**, **Amelia**, and **Abdul** for continuing to keep the molger lab the best workplace ever.

Thank you **Ximena** for all the help and for the Japanese sweets. I hope you know how much everyone at MolGer appreciates you. **Sara** and **Agata**, for all the great discussions and the synaptic synuclein evening parties. **Agnieszka**, **Sahar**, **Linn**, **Johanna**, **Ulrika**, **Greta**, **Dag** and **Vilmantas** for the warm and inviting workplace. Thank you **Fadi**, **Mirjam**, **Ioannis**, **Nancy** and **Ross** for the help with the projects. Thank you **Camilla** for teaching me how to make macros with ImageJ, it helped a lot.

Thank you to **Lars**, **Martin** and **Stina** for the great leadership of the group.

To our extended Uppsala family, **Jonas**, **Erica**, **Jessica**, **Ivan**, **Tor**, **Malin** and **Emil** (and **the small ones** of course). Thank you for all the lovely memories, and ongoing traditions. Thank you **Jonas** for being the glue of the group and for adopting Robin as a BFF. Thank you **Erica** for the help with wedding dress shopping, the tons of baby clothes and mommy support-line! And thank you also, along with **Malin** and **Jessica** for coming with me to the spa day, let's make the boys regret introducing us to a new child-free tradition! It feels amazing to know that our kids will grow up surrounded by a network of support and playmates.

Tack **mamma** och **pappa** för allt stöd, och för att ni alltid har peppat mig när mitt självförtroende gjort att jag vacklat. Ni har alltid fått mig att känna att

oavsett hur det går så kommer det bli bra. Tack för ett liv fyllt med fantastiska minnen och erfarenheter. Jag ser fram emot alla kommande resor, spelkvällar och GTs.

Älskade **system**. Tack för att jag alltid kan ringa dig. Jag uppskattar verkligen vår relation. Stort lycka till med ditt nya företag. Jag kommer utnyttja dig till fullo!

Tack **Robin** för allt ditt stöd och för att du alltid har trott på mig. Tack också för att du har stått ut med mig den sista perioden och för att du har hållit liv i barnen och hushållet. Tack för vårt liv tillsammans (13 år nu), och vi har precis börjat vår mest spännande resa. **Saga** och **Alfred**, ni gör våra liv bättre varje dag. Det finns inget jag älskar mer än att komma hem till er!

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