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Alpha-Synuclein Oligomers

*Cellular Mechanisms and Aspects of Antibody
Treatment*

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Abstract

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In Parkinson's disease (PD) and dementia with Lewy bodies (DLB), aggregated α -synuclein deposit inside cells within the brain. Smaller soluble α -synuclein aggregates, oligomers, are present both intra- and extracellularly. The α -synuclein oligomers are known to be particularly harmful, although the underlying neurotoxic mechanisms are not fully understood. The aim of this thesis was to investigate the pathogenic roles of α -synuclein oligomers and the possibility to target such species with antibody treatment.

Passive immunotherapy with α -synuclein antibodies can lead to reduced pathology and ameliorated symptoms in transgenic mice. However, it remains unknown whether the antibodies are taken up by cells or whether they act extracellularly. In Paper I, we assessed cellular internalization of various α -synuclein monoclonal antibodies. The oligomer selective mAb47 displayed the highest uptake, which was promoted by the extracellular presence of α -synuclein.

Alpha-synuclein aggregates can be found in both neurons and glial cells, but the pathogenic role of glial deposits has only been sparsely investigated. In Paper II, co-cultures of neurons and glia were exposed to α -synuclein oligomers. The astrocytes in the cultures rapidly accumulated oligomers, which were only partially degraded by lysosomes. The sustained intracellular α -synuclein deposits were associated with mitochondrial stress reactions in the astrocytes.

In Paper III, we sought to explore whether the astrocytic pathology induced by α -synuclein oligomers could be ameliorated by antibody treatment. Pre-incubation of oligomers with mAb47 promoted α -synuclein clearance, reduced astrocytic accumulation and rescued cells from mitochondrial stress. We could demonstrate that binding of the antibody to its antigen in the extracellular space was crucial for these effects to occur.

The progressive pathology in PD is believed to be driven by cell-to-cell spreading of α -synuclein aggregates, potentially via exosomes and other extracellular vesicles (EVs). In Paper IV, we found that either fusing α -synuclein to a non-physiological protein tag or introducing the PD-causing A53T mutation directed α -synuclein towards EV secretion. Also, EV-associated α -synuclein was particularly prone to induce toxicity in recipient cells.

In conclusion, this thesis sheds new light on the cellular dysfunction related to α -synuclein pathology and on how the underlying pathogenic processes may be targeted by antibody treatment.

Keywords: Parkinson's Disease, Alpha-synuclein, Aggregation, Oligomers, Monoclonal Antibody, Glia, Astrocyte, Immunofluorescence

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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 **Gustafsson G**, Eriksson F, Möller C, Lopes da Fonseca T, Outeiro TF, Lannfelt L, Bergström J, Ingelsson M (2017) Cellular Uptake of α -Synuclein Oligomer Selective Antibodies is Enhanced by the Extracellular Presence of α -Synuclein and Mediated via Fc γ Receptors. *Cell Mol Neurobiol.* 37:121
- II Lindström V, **Gustafsson G**, Sanders LH, Howlett EH, Sigvardson J, Kasrayan A, Ingelsson M, Bergström J, Erlandsson A (2017) Extensive uptake of α -synuclein oligomers in astrocytes results in sustained intracellular deposits and mitochondrial damage. *Mol Cell Neurosci.* 82: 143-156
- III **Gustafsson G**, Lindström V, Rostami J, Nordström E, Lannfelt L, Bergström J, Ingelsson M, Erlandsson A. Alpha-synuclein oligomer-selective antibodies reduce intracellular accumulation and mitochondrial impairment in alpha-synuclein exposed astrocytes. *Submitted to J Neuroinflamm.*
- IV **Gustafsson G***, Lööv C*, Lázaro DF, Takeda S, Bergström J, Erlandsson A, Sehlin D, Balaj L, György B, Hallbeck M, Outeiro TF, Breakefield XO, Hyman BT, Ingelsson M. Secretion and uptake of α -synuclein via extracellular vesicles in cultured cells. *Submitted to PLOS ONE.*

* These authors have contributed equally to the work.

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Abbreviations

A β	Amyloid beta peptide
AD	Alzheimer's disease
α -syn	Alpha-synuclein
ATP13A2	ATPase 13A2
BiFC	Bimolecular fluorescent complementation assay
CNPase	2',3'-cyclic nucleotide 3'-phosphodiesterase
CNS	Central nervous system
DAPI	4',6-diamidino-2-phenylindole
DLB	Dementia with Lewy bodies
Drp1	Dynamin-related protein 1
EGF	Epidermal growth factor
ELISA	Enzyme-linked immunosorbent assay
EV	Extracellular vesicle
FFP	Free-floating protein
FGF2	Fibroblast growth factor 2
GFAP	Glial fibrillary acidic protein
GFP	Green fluorescent protein
HNE	4-hydroxy-2-nonenal
HPLC	High performance liquid chromatography
HRP	Horseradish peroxidase
IC	Intracellular
IgG	Immunoglobulin G
IP	Immunoprecipitation
LB	Lewy body
LRRK2	Leucine-rich repeat kinase 2
mAb	Monoclonal antibody
MFN1	Mitofusin 1
MSA	Multiple system atrophy
MVB	Multivesicular body
YFP	Yellow fluorescent protein
ONE	4-oxo-2-nonenal
PD	Parkinson's disease
PFF	Pre-formed fibril
PINK1	PTEN induced kinase 1
SEC	Size exclusion chromatography
SNARE	Soluble N-ethylmaleimide-sensitive factor attachment protein receptor

Introduction

Parkinson's disease: Epidemiology and clinical features

Parkinson's disease (PD) was first described in 1817 by James Parkinson in 'An essay on the shaking palsy' [1]. Six of his patients had various motor-related symptoms and were suspected to suffer from the same disorder. Today, PD is known to have a prevalence of 0.5-1 % [2] among persons at 65-69 years of age and is the second most common neurodegenerative disease after Alzheimer's disease (AD) [3]. With the increasing life expectancy, these diseases are becoming a major health problem. Although some inherited forms of PD have onset of symptoms at 30-35 years of age sporadic forms have an onset at 50 years or later [4]. The prevalence for the more common form of late onset PD has been predicted to double in the most populated countries within a 25 year period [5].

One of the neuropathological hallmarks of PD is the progressive loss of dopaminergic neurons in substantia nigra, pars compacta [6, 7]. Degeneration of the dopamine producing cells leads to failure in dopaminergic signalling, correlating with locomotor symptoms [8]. Typical symptoms are rigidity, resting tremor, hypokinesia and at later stages often cognitive impairment and dementia [9]. Less specific symptoms, which often are seen already at early disease stages, include depression [10], sleep disturbances [11], constipation [12] and olfactory dysfunction [13]. The development of symptoms may be explained by the progression of pathology, where the first changes typically appear in the brain stem with subsequent spreading to other regions, including neocortex [14].

Current treatments for Parkinson's disease

Disrupted dopamine homeostasis has been pointed out as an important factor in PD [15, 16] and the symptoms can be partly addressed to failure in dopaminergic neuronal signalling. Dopamine dysregulation has been associated with oxidative stress [16] and neurodegeneration, causing motor symptoms in animal models [15, 17].

There are currently no therapies against the underlying disease process in PD but the symptoms can, at early disease stages, be alleviated with levodopa (L-DOPA), a derivative of dopamine [18, 19] and other drugs that aim at

increasing or stabilizing disrupted dopamine levels in the brain. Dopamine is generated by the conversion of L-tyrosine by the enzyme tyrosine hydroxylase [20]. Possible causes for deficient signalling may be disturbed cellular dopamine homeostasis resulting from leakage from vesicles to the cytoplasm [15, 16] or failure in the synaptic signalling machinery [21]. By administration of L-DOPA cells convert the substance to dopamine and can thereby compensate for the reduction of dopamine. However, long term medication with L-DOPA brings risks for side effects such as dyskinesia [22] and sensitization [23], making dosage difficult [18]. These effects may be counteracted by using continuous drug delivery [24] of L-DOPA and dopamine stabilizing drugs [25, 26].

Lewy body pathology

Post mortem analyses of brains from PD patients display atrophy, cell death and inclusions of aggregated protein, predominantly in neurons (Figure 1) but also in glial cells. These aggregates, known as Lewy bodies and Lewy neurites, are also found in other neurodegenerative diseases, such as dementia with Lewy bodies (DLB) [27] and in the Lewy body variant of AD [28]. The Lewy bodies mainly consist of aggregated α -synuclein [27, 29], but have also been found to contain approximately 300 other proteins [30] involved in a broad spectrum of cellular processes, including several heat-shock proteins [30], neurofilaments [27], lysosomal proteins [31] and ubiquitin [32, 33] as well as lipids [34].

Alpha-synuclein aggregation resulting in Lewy body formation has been linked to toxicity and neurodegeneration [35]. The majority of pathological α -synuclein aggregates in PD and DLB are located to the pre-synapses and have been suggested to cause neurotransmitter deficiency and loss of dendritic spines [36]. The number of Lewy bodies in the brain is not proportional to the severity of the disease but their location correlates with the disease stage [14]. Moreover, cells containing Lewy bodies do not display any affected metabolism [37] or extensive cell death compared to surrounding cells [38]. The Lewy body is therefore believed to be an inert end stage deposit for the α -synuclein aggregation [39]. Another neurodegenerative disorder linked to α -synuclein aggregation is multiple system atrophy (MSA) [40]. In contrast to PD and DLB, MSA pathology is characterized by large α -synuclein aggregates found as cytoplasmic inclusions in oligodendrocytes [41, 42]. Usually, MSA has a sporadic background and the typical symptoms are autonomic failure, parkinsonism and cerebellar ataxia [43].

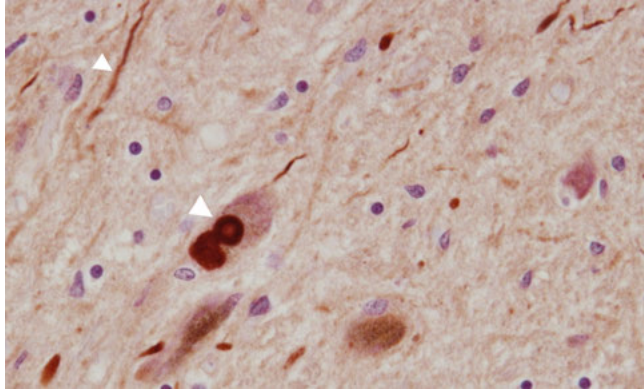


Figure 1. Lewy pathology detected with immunostaining in *post mortem* substantia nigra from a patient with Parkinson's disease. Alpha-synuclein pathology is detected with a monoclonal α -synuclein IgY antibody. Lewy bodies are seen as dark, spherical inclusions (large arrowhead), located to the soma of neuromelanin containing neurons. A Lewy neurite is marked with a small arrowhead. Nuclei are seen in blue (40x magnification).

Image: Leire Almandoz-Gil

Alpha-synuclein

Alpha-synuclein is a natively unfolded 140 amino acid long protein, which is expressed mainly in the central nervous system (CNS) [44] but also in peripheral cells [45, 46]. The primary structure of α -synuclein consists of three main regions. The N-terminal domain (aa 1-60) is forming an α -helix in its lipid bound form [47] (Figure 2), whereas the mid region (aa 61-95) is highly hydrophobic [48]. Both the N-terminal and mid regions are membrane binding and contain hydrophobic repeats [49], making the protein aggregation prone. The C-terminal domain (aa 96-140), however, is hydrophilic and negatively charged and is thought to be responsible for interactions with other proteins [50]. Moreover, α -synuclein has been suggested to form a physiological tetramer with α -helical content that resists aggregation [46].

Alpha-synuclein is partly located to the presynaptic space [51] where it has been suggested to act as a chaperone and promote the SNARE (soluble N-ethylmaleimide-sensitive factor attachment protein receptor) complex formation and regulate synaptic vesicle trafficking as well as neurotransmitter release and re-uptake [50, 52]. Interaction with mitochondrial membranes with an inhibiting effect of membrane fusion has also been reported [53]. In addition, α -synuclein has a nuclear localization [54] and has therefore been suggested to affect gene regulation [55]. Two other closely related proteins, β - and γ -synuclein, have shorter sequences and different aggregation propensities compared to α -synuclein. The β -synuclein has a shorter mid region

than α -synuclein and has a lower fibrillation rate [56]. The sequence of the mid region of γ -synuclein differs from that of α -synuclein and the C-terminal is shorter than in α -synuclein. Gamma-synuclein displays *in vitro* aggregation but to a lower degree than α -synuclein [49].

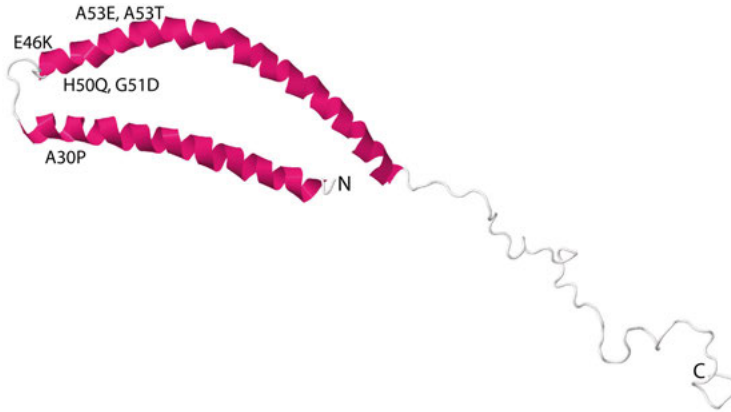


Figure 2. The membrane-bound structure of α -synuclein is α -helical in the N-terminal (1-60) and mid region (61-95). The disease-causing mutations are located in the N-terminal region. The C-terminal region (96-140) is unstructured, contains negatively charged residues and is responsible for protein interactions. (PDB structure 1XQ8).

Genetics of PD and other α -synucleinopathies

Dominantly inherited forms of PD [57] and DLB are caused by mutations in the α -synuclein gene (Figure 2) or in one of several other genes (Table 1) [58]. The familial mutations of α -synuclein result in single amino acid shifts [27, 57, 59-64]. Moreover, multiplications of the α -synuclein gene leading to increased levels of wild type α -synuclein have been found to cause early onset forms of PD [65-67]. The other genes leading to either dominantly or recessively inherited PD are involved in central cellular processes such as protein phosphorylation (LRRK2) [68], mitochondrial function (DJ-1, PINK1 and parkin) [53], lysosomal (ATP13A2) [69] and proteasomal degradation (UCHL-1) [70]. However, alterations in any of these genes are found in less than 10 % of the cases [71], making sporadic PD the most frequent disease form. Even though genetic forms of PD only represent a small fraction of the cases, the neuropathological picture is similar to the sporadic forms [65]. For the majority of the disease cases of PD and DLB, Lewy bodies can be found in various regions of the brain, indicating a central role of α -synuclein in the disease process [14, 72].

Table 1. The identified genes related to inherited forms of Parkinson’s disease. (AD: autosomal dominant, AR: autosomal recessive).

Symbol	Inheritance	Protein	Function
PARK1	AD	α -synuclein	Synaptic signaling
PARK2	AR	Parkin	Ubiquitin protein ligase
PARK5	AD	UCHL1	Ubiquitin thiolesterase
PARK6	AR	PINK1	PTEN induced kinase 1
PARK7	AR	DJ-1	Protein deglycase
PARK8	AD	LRRK2	Leucine-rich repeat kinase 2
PARK9	AR	ATP13A2	Lysosomal ATPase

Alpha-synuclein aggregation

Alpha-synuclein mutations, causing PD and DLB, have been found to accelerate its aggregation and formation of fibrils [35]. The aggregation cascade starts with misfolding of monomeric protein leading to the formation of smaller and larger oligomers before the appearance of fibrils, which are deposited as Lewy bodies and Lewy neurites (Figure 3). The underlying process behind misfolding and aggregation is not completely understood. However, an increased local concentration of α -synuclein might trigger the process [73] and truncation of the C-terminus may lead to an increased aggregation rate [74]. Moreover, association to lipid membranes, although being part of its physiological function, is also a factor that affects the aggregation of α -synuclein [75, 76].

Oligomeric α -synuclein species have a wide variety of morphologies and sizes [46, 77-79]. Those with a potential to aggregate further are defined as on-pathway oligomers, whereas species remaining thermodynamically stable in their conformation are defined as off-pathway oligomers [80]. On-pathway oligomers aggregate and form protofibrils that are believed to partially have the same structure as fibrils, i.e. a hydrophobic core where the mid regions of the subunits are arranged into anti-parallel β -sheets [81]. Such protofibrils can act as templates for larger fibrils to form [82, 83]. Further aggregation leads to deposition into the inert Lewy bodies and Lewy neurites, which are the highest structures of aggregated α -synuclein [39]. When studying the aggregation process of α -synuclein *in vitro*, heterogeneous fibrils are formed by different environmental factors. It is not known which of these fibril structures that correspond to the species present in the Lewy bodies.

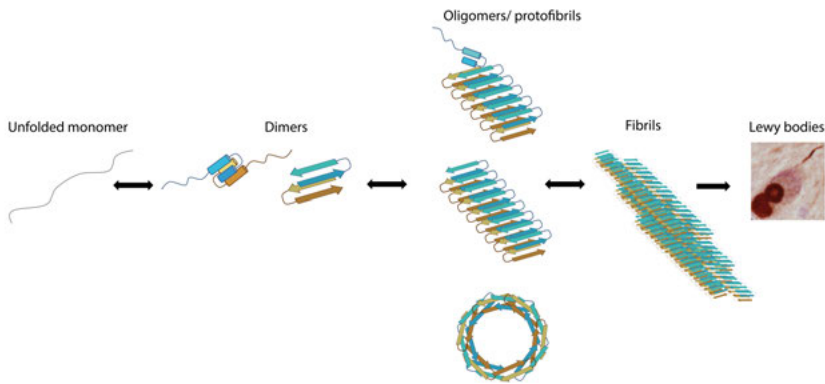


Figure 3. The aggregation cascade of α -synuclein starts with misfolding of monomers, associated with dimerization. Gradual elongation of oligomers leads to the formation of fibrils that accumulate into Lewy bodies. Different morphologies of oligomers/protofibrils can be toxic and can seed fibril formation to varying degree. The mid region of the subunits constitutes the core of the fibril structure.

Alpha-synuclein oligomers result in toxicity

Aggregation of native α -synuclein can be promoted by the presence of misfolded species of the same protein. Such seeding effects have been shown to occur from fibrils and pre-formed fibrils (PFF) [84-86]. The disease-causing mutation A30P led to increased protofibril formation but slower fibril formation compared to wild type (WT) α -synuclein, implying that the protofibrils are pathogenic [87]. These forms are soluble and may diffuse throughout the cell and recruit normal, functional forms of α -synuclein, thereby accelerating the aggregation. Some α -synuclein oligomers have membrane binding properties and may penetrate the cell membrane and spread to other cells, possibly also by exocytosis (reviewed by Hansen et al. 2012) [88]. Annular α -synuclein oligomers form pore-like structures [79] that could, when interacting with the plasma membrane, contribute to compromised membrane integrity.

Oligomeric α -synuclein species have been suggested to have effects on cells via direct or indirect mechanisms of action (Reviewed by Roberts et al 2015) [89] and are also believed to induce inflammation [90, 91]. Engineered artificial mutations in α -synuclein, stabilizing its oligomeric state, were found to cause degeneration of dopaminergic neurons when expressed *in vivo* via lentivirus [92], lending support to the idea that soluble α -synuclein aggregates are toxic. Oligomeric α -synuclein can also transfer between cells *ex vivo* [93], suggesting a high spreading potential for these species. Moreover, α -synuclein oligomers caused impaired axonal transport and led to disrupted neurite morphology in cultured dopaminergic neurons [94].

Generation of large α -synuclein oligomers

By co-incubating recombinant human monomeric α -synuclein with the reactive aldehyde 4-hydroxy-2-nonenal (HNE), large (2000 kDa) stable α -synuclein oligomers with a high degree of β -sheet structures can be generated [95]. Treatment with such oligomers led to impaired electrophysiological function in hippocampal preparations from rat brain [96]. However, the underlying mechanisms for oligomer-mediated cellular dysfunction have not been fully elucidated.

Oligomers of α -synuclein were found to be internalized in primary rat neurons [97] and in human cell lines, where they caused decreased cell viability [95], indicating that they exert their toxic effects via an intracellular influence. It could, however, not be excluded that the toxic effects are mediated via an extracellular route, i.e. via membrane disruption [92, 98, 99], interference with synaptic function [21] or via receptor mediated neurotoxicity (as seen for oligomeric amyloid β peptide (A β), the peptide that accumulates as plaques in the AD brain [100]). As the HNE-induced α -synuclein oligomers were detected also in the cell nuclei [95], one may speculate that they impair cellular function by affecting gene transcription. In fact, it could also be observed that α -synuclein oligomers, induced by the chemically similar reactive aldehyde 4-oxo-2-nonenal (ONE), that were added to cultured cells caused decreased levels both of α -synuclein and unrelated proteins [80]. Interestingly, despite having a similar size and secondary structure HNE- and ONE-induced oligomers differed greatly in morphology and compactness [95]. Moreover, neuronal cells treated with HNE had an increased secretion of α -synuclein, promoting cell-to-cell spreading of seeding-capable α -synuclein oligomers [101].

Extracellular propagation of α -synuclein pathology

Evidence suggests that α -synuclein can be transferred between cells, presumably via the extracellular space. Oligomeric α -synuclein has been detected in extracellular fluids, such as plasma and cerebrospinal fluid (CSF) [102, 103], and can also be secreted from cells in culture [104].

Interestingly, *post mortem* analyses 14-16 years after transplantation of embryonic stem cells into the brain of PD patients revealed Lewy body pathology also in the grafted cells, providing further evidence of cell-to-cell spreading of α -synuclein pathology *in vivo* [105]. When dopaminergic neurons were grafted into mice with a transgenic expression of human α -synuclein, a similar host-to-graft spreading of α -synuclein was seen [106].

According to the Braak hypothesis, the pathophysiology of PD involves hierarchical spreading of Lewy body pathology via the enteric nervous sys-

tem to the CNS, beginning in the olfactory bulb and/or in the gastrointestinal tract [14, 107, 108]. The severity of the disease often correlates spatially with the brain regions affected by Lewy pathology. Furthermore, the intercellular transmission of α -synuclein has been studied using co-cultures of neuronal cell lines. Cells expressing α -synuclein labelled with either green or red fluorescent protein were cultured together. After different incubation periods, increasing co-localization of the two fluorophores was seen in cells, suggesting a cell-to-cell spreading and seeding of aggregation. Using bimolecular fluorescent complementation, intracellular dimerization of α -synuclein from donor cells with α -synuclein from recipient cells, could be detected. Moreover, uptake of recombinant α -synuclein monomers, oligomers and fibrils was seen in cultured cells and, when injected, in cortical neurons of mice [106]. In a similar setting, extracellular *in vitro* generated α -synuclein oligomers could seed intracellular α -synuclein aggregation within primary neurons [78]. Moreover, cell-to-cell spreading of monomeric α -synuclein expressed with a fluorescent protein label was observed between neuronal cell lines [109]. A transfer of both monomeric and aggregated α -synuclein was also seen from neurons to glia [110]. In another study α -synuclein aggregates derived from Lewy bodies in PD patients were transferred from astrocytes to neurons in culture [111]. In yet another study, spreading and subsequent seeding of α -synuclein aggregates between co-cultured neurons was found to occur via tunnelling nanotubes [112].

Spreading of α -synuclein pathology via extracellular vesicles

Alpha-synuclein may be actively secreted from neuronal cells [104, 106] via Golgi-dependent exocytosis [113] or via non-conventional exocytosis [114]. Various cell types, including neurons and astrocytes, secrete exosomes [115]. These small vesicles of endosomal origin, are secreted via multivesicular bodies (MVB) [116], which consist of endosomes that have been redirected from the endosomal-lysosomal degradation pathway [117]. The release of exosomes involves fusion of the MVBs with the plasma membrane, reviewed by Coucci et al [118] and Kowal et al [119]. Exosomes are further characterized by the presence of various markers, such as flotillin-1 and alix [120]. Other extracellular vesicles (EVs) without endocytic origin are formed from direct budding of the plasma membrane and therefore contain cell membrane proteins and lipids [121]. All of the various EVs can be viewed as potential vehicles for the secretion and spreading of α -synuclein.

Exosomes have been implicated in different neurodegenerative disorders. For example, they were reported to contribute to the intercellular spreading of prion pathology [122, 123]. In addition, A β peptide aggregation was

found to be promoted through exosomal release in transgenic mice. In this study, exosomes were suggested as a therapeutic target, as inhibited exosomal release of the protein led to reduced amyloid plaque burden [124].

In a number of studies exosomal secretion of α -synuclein has been linked to impaired lysosomal degradation [93, 114, 125, 126]. Inhibition of lysosomal function combined with α -synuclein overexpression led to increased exosomal release of α -synuclein and uptake in recipient cells [126]. Neural cell lines expressing α -synuclein linked to fluorescent/luminescent reporter tags displayed high levels of oligomers associated to exosomes [93]. Also here, exosomal secretion of α -synuclein increased when degradation pathways were blocked. Another study showed that α -synuclein release via EVs was promoted by impaired degradation, mitochondrial function, induced oxidative stress or protein misfolding stress. In addition, the protein content in EVs displayed an increased degree of oxidative modifications, compared to cytosolic protein [114]. Exosomes from α -synuclein expressing cells promoted internalization of α -synuclein oligomers [93, 125] and led to increased toxicity in recipient cells [93, 120]. Exosomes have also been suggested as catalytic environments for α -synuclein aggregation as addition of exosomes accelerated *in vitro* aggregation of α -synuclein [127]. Furthermore, α -synuclein found in EVs displayed a higher aggregation propensity than α -synuclein in the cytosolic fraction [128].

The role of glial cells in α -synucleinopathies

In the healthy brain, vital factors such as synaptic signalling [129] and inflammatory processes are controlled by glial cells. Neurodegeneration in α -synucleinopathies is believed to be the result of toxic actions of misfolded protein [130], oxidative stress [91] and inflammation [110]. In the PD brain, glia counteract these pathological processes in a variety of ways. The role of professional phagocytes, such as microglia, in neurodegeneration has been widely studied [91, 131]. These cells are highly mobile even in their resting state [132]. Microglia take up and degrade extracellular α -synuclein aggregates via receptor-mediated endocytosis but activation leads to impaired degradation and α -synuclein accumulation [131]. Large α -synuclein oligomers caused pro-inflammatory upregulation in microglia, leading to neuronal loss [133].

Astrocytes also modulate inflammatory processes, thus regulating the inflammatory responses of other cells throughout the brain [134]. Moreover, astrocytic α -synuclein inclusions are found in patients with Lewy body pathology [110, 135]. In MSA, aggregated α -synuclein is found as glial cytoplasmic inclusions, mainly in oligodendrocytes [40]. However, mature oligodendrocytes do not express α -synuclein [41, 136] and are instead believed

to ingest aggregating α -synuclein from surrounding neurons [137]. In addition, oligodendrocyte precursor cells were found to display α -synuclein expression, which decreased upon maturation [138].

The role of astrocytes in α -synucleinopathies

Astrocytes are the most numerous type of glial cells in the brain and a major constituent of the blood-brain barrier [139, 140]. Their many functions include regulation of the blood flow in CNS blood vessels [141] and facilitation of the glymphatic flow [142]. They also protect the microenvironment by removal of debris and dead cells [143], support neurons by producing nutrients [144] and regulate neurotransmitters in the synapses [129, 145], enabling fast and accurate neuronal signalling. Moreover, they protect the neurons mechanically by protecting the synapses [139]. Astrocytes become activated by interaction with apoptotic cells [146], foreign antigens or toxic protein aggregates [91, 147] and go into reactive gliosis. In this state the morphology of the cell changes due to increased production of the structural protein *glial fibrillary acidic protein* (GFAP) and other intracellular filament proteins, such as nestin and vimentin [148].

Cytokine production of the astrocytes is increased as a part of inflammatory processes [149]. One study found that exposure to recombinant α -synuclein induced an inflammatory response and release of pro-inflammatory cytokines [134]. Such an activation of the astrocytes was induced by exposure to monomeric, fibrillar or C-terminally truncated α -synuclein [91]. The increase in cytokine expression and production of reactive oxygen species was found to be dependent of the surface protein toll-like receptor 4 [91]. In a study on *post mortem* MSA brains, the degree of astrocytic activation was found to be dependent of the distance to extracellular α -synuclein aggregates [150].

It has also been found that cultured astrocytes can take up [91] and degrade extracellular α -synuclein aggregates [131]. Neuron-derived α -synuclein monomers and aggregates were internalized in cultured astrocytes via dynamin-mediated endocytosis, leading to inflammatory responses [110]. In that study, it was also reported that astrocytic α -synuclein inclusions were present in brains from α -synuclein transgenic mice and in *post mortem* tissues from DLB cases. Astrocytes in culture have also been reported to efficiently internalize α -synuclein aggregates derived from PD brain. When astrocytes in their turn released such protein species, they caused higher neuronal death than when the original aggregates were given directly to neurons in the absence of astrocytes [111]. Together, these findings suggest that astrocytes may be centrally involved in α -synuclein pathology and neuronal death in the diseased brain.

Immunotherapy as a treatment for α -synucleinopathies

Immunotherapy has emerged as a promising approach for neurodegenerative disorders. For AD, several clinical trials, based on both active [151] and passive [152] immunization against A β are ongoing or have been finished. As for PD, a phase 1 study with active immunization, using short peptides mimicking parts of the α -synuclein sequence has been finished [153] and the same approach is currently at a phase 2 stage. Moreover, in a phase 1b study with intravenous passive immunization against α -synuclein it was found that a single dose resulted in a 96 % reduction of free α -synuclein in serum. Moreover, the antibody had a proven safety and tolerability profile [154].

Immunotherapy studies based on active immunization targeting α -synuclein in animal models have been reported to promote clearance of α -synuclein aggregates [155] and reduce neurodegeneration [156]. However, to avoid side effects such as autoimmunity and to achieve a better control of antibody titers, passive immunization has become the more common approach (reviewed in Lannfelt 2014) [157].

Peripheral passive immunization of transgenic α -synuclein mice with antibodies targeting α -synuclein reduced the amount of aggregated forms of α -synuclein in the brain [155]. Furthermore, treated mice showed milder motoric [158, 159] and cognitive symptoms compared to untreated mice [97, 160-163]. An antibody raised against the C-terminal domain of α -synuclein was used for immunization in mice with transgenic expression of human α -synuclein. Treated mice displayed ameliorated motor skills and milder behaviour deficits. The synaptic pathology in CNS was also reduced and treatment effects were addressed to antibody-mediated clearance of intracellular or membrane bound α -synuclein via autophagy [163].

In studies of antibody treatment in cell models for α -synuclein pathology, extracellular forms of α -synuclein have been targeted [97, 159]. Antibodies with C-terminal epitopes were reported to induce Fc γ receptor mediated uptake of extracellular α -synuclein aggregates and clearance via lysosomes, leading to reduced cellular α -synuclein accumulation [97]. Moreover, α -synuclein antibodies prevented exogenously added α -synuclein PFFs from internalization and seeding of intraneuronal aggregation [159]. Here, cell-to-cell propagation of α -synuclein aggregates between primary neurons was blocked by the presence of antibodies. In another study, antibodies targeting a C-terminal truncation site of α -synuclein were reported to reduce cell-to-cell spreading in cell lines and blocked extracellular truncation of α -synuclein [160].

Oligomeric α -synuclein as immunotherapy target

Our laboratory has contributed to the development of antibodies that are highly selective against oligomeric/protofibrillar α -synuclein. These antibodies were raised in mice against HNE-induced α -synuclein oligomers and recognize both Lewy bodies in human PD brain as well as early α -synuclein pathology in a transgenic mouse model [80]. When using these antibodies on a cell model for α -synuclein aggregation, it was found that the oligomer-selective antibodies reduced the oligomerization of α -synuclein [164]. Moreover, the antibodies have been assessed for immunotherapy on mice. Fourteen months old α -synuclein transgenic mice were given intraperitoneal injections with one of the antibodies weekly. Treated mice were found to have lower levels of α -synuclein oligomers/protofibrils in the CNS and showed decreased lethal motor symptoms as compared to PBS treated animals [158]. Another treatment strategy, where single chain antibodies towards oligomeric α -synuclein, linked to a cell-penetrating peptide were administered by intracerebral injection, have also reduced α -synuclein accumulation and ameliorated symptoms in transgenic mice [165].

Functions of Fc γ receptors in the CNS

Fc γ receptors are transmembrane receptor proteins expressed by different immune cells in mammals. These molecules bind to IgG antibodies and play central roles in the immune response and are involved in the inflammatory response in neurodegeneration [166]. Expression of Fc γ receptors has been reported in CNS cells [97, 167].

The Fc γ receptors bind to the Fc fragment of either monovalent IgG antibodies or IgG in complex with its antigen. The high affinity receptor Fc γ RI is expressed by macrophages as microglia [168] as well as in other cell types and its sequence contains two Fc recognizing regions [169]. Activating receptors have an *immune receptor tyrosine-based activation motif* (ITAM) on the cytosolic side of the cell membrane. Binding of activating receptors as Fc γ RI to IgG antibody (subclass 1, 3 or 4) leads to high-affinity binding and transmembrane signalling which may lead to an inflammatory signalling cascade including production of cytokines and up-regulation of phagocytosis [166]. In contrast, inhibitory Fc γ receptors as Fc γ RII b contain an intracellular ITIM *immune receptor tyrosine-based inhibitory motif*. Therefore IgG binding to this receptor type results in down-regulation of inflammatory signalling. Cross-linking of activating and inhibitory receptors through IgG complex binding also leads to down-regulation of cellular activation [169].

Immune cells often express a variation of these different receptor types to regulate the degree of cellular activation and phagocytosis [170]. The high affinity receptor as well as low-affinity receptors may bind to immune com-

plexes and mediate phagocytosis and degradation of the antigen. When designing therapeutic strategies directed to the CNS, these mechanisms are crucial to understand in order to optimize antibody mediated clearance of toxic protein aggregates and to avoid cell activation/inflammatory processes.

Mitochondrial integrity in α -synucleinopathies

Some of the high-risk genes linked to PD and other α -synucleinopathies are directly associated to mitochondrial homeostasis and function. The PARK2 gene product ubiquitin E3 ligase (parkin) and the PARK6 gene product PINK1 (PTEN induced kinase 1) are central in the process of targeting mitochondria for mitophagy [171, 172]. Mutations in these genes cause impaired mitochondrial turnover and compromised cellular energetics. The PARK7 gene product DJ-1 is crucial for mitochondrial integrity [173] and protects mitochondria from fragmentation during oxidative stress [174]. Mechanisms in the dynamics of mitochondrial fusion and fission have also been implicated in PD and other α -synucleinopathies. Mitochondrial fusion involves the association of mitochondrial membranes by mitofusins and other proteins, leading to formation of new mitochondria and construction of mitochondrial networks [175]. This process was inhibited upon α -synuclein expression in *Caenorhabditis elegans*, leading to increased mitochondrial fragmentation [53]. Mitochondrial fission is the process where mitochondria are dividing under the influence of dynamin-related protein 1 (Drp1) and other proteins. This process leads to the generation of new functional mitochondria [176] and also to isolation of dysfunctional mitochondria prior to their degradation via mitophagy [177]. In a mouse model exposure to the toxin MPTP led to hyperactivation of Drp1, resulting in mitochondrial fragmentation, which caused neurotoxicity and parkinsonian motor symptoms [178].

Other neurotoxins have also been found to cause parkinsonian symptoms and neurodegeneration. Exposure to the toxin 1-methyl-4-phenyl-1,2,5,6 tetrahydropyridine (MPTP) caused motor symptoms identical to idiopathic PD and selective neuronal death in substantia nigra [179]. The effect is caused by the MPTP metabolite 1-methyl-4-phenylpyridinium (MPP⁺) which enters dopaminergic neurons via dopamine receptors [180] and inhibits mitochondrial complex 1, causing reduced ATP levels [181] and increased production of reactive oxygen species [182]. Alpha-synuclein knockout mice were found to be resistant to MPTP induced dopaminergic degeneration [183]. Moreover, rats treated with the toxin rotenone developed parkinsonian symptoms correlating with cytoplasmic α -synuclein inclusions and dopaminergic neurodegeneration, which was addressed to rotenone-induced inhibition of mitochondrial complex I [184]. Altogether, these findings suggest an involvement of α -synuclein in mitochondrial dysfunction in PD.

Aims

The overall aim of this thesis was to study the cellular and molecular events that are central for the disease-associated aggregation and spreading of α -synuclein. In addition, we sought to assess the effects of α -synuclein oligomer-selective antibodies in relevant cell models of α -synuclein disorders.

Specific aims

- I** To study cellular uptake and associated mechanisms of α -synuclein antibodies.
- II** To investigate the uptake and processing of α -synuclein oligomers in co-cultures of astrocytes, neurons and oligodendrocytes.
- III** To study effects of α -synuclein oligomer-selective antibodies on the accumulation of α -synuclein oligomers in neuronal-glia co-cultures.
- IV** To assess how different forms of α -synuclein are processed via exosomes and other extracellular vesicles in cultured cells.

Results and discussion

In an earlier study made by our group, transgenic A30P α -synuclein mice were treated with α -synuclein oligomer/protofibril-selective antibodies, resulting in ameliorated motor symptoms and reduced protofibril levels in the spinal cord [158]. The correlation of these treatment effects implies that the oligomers and protofibrils play a central role in the disease process, at least in this mouse model. Possible mechanisms behind the observed antibody effect include: steric hindrance of further aggregation, increased phagocytosis of antibody: α -synuclein complexes or IgG-induced signaling leading to increased processing of α -synuclein.

To further investigate possible mechanisms of the *in vivo* treatment effects, antibodies were applied to a cell model for α -synuclein oligomerization. The α -synuclein dimerization/oligomerization BiFC assay was expressed in human neuroglioma cells. Here, the addition of α -synuclein oligomer-selective antibodies led to lowered intracellular levels of fluorescent α -synuclein oligomers [164]. This reduction was addressed to antibody-mediated clearance via promoted cellular degradation.

Characterization of the cellular uptake of α -synuclein oligomer-selective antibodies in human cell lines

In **Paper I**, we elucidated if extracellularly administered α -synuclein oligomer-selective antibodies may get internalized in the H4 neuroglioma cell line and, if so, whether such an uptake could be promoted by the presence of α -synuclein. Firstly, the uptake of α -synuclein antibodies was assessed in non-transfected cells. Both α -synuclein antibodies selective for oligomers or regular linear epitopes were assessed, as well as a non-specific isotype control. The highest uptake was seen with the α -synuclein oligomer-selective antibody mAb47, which reached a maximum of intracellular accumulation after 4 h of incubation. The other antibodies, including non-specific isotype control IgG, also had a detectable uptake at this time point. Next, the same set of antibodies was applied to cells overexpressing the α -synuclein:Venus BiFC oligomerization assay. This construct is almost identical to the one used by Näsström et al. 2011 [164], except that α -synuclein is fused to the N- and C-terminal halves of the yellow fluorescent protein Venus (instead of GFP) and

that the cells are stably transfected as they are kept in selection media containing geneticin (G418). The antibody uptake in the BiFC cells reached a maximum at 4 h and mAb47 displayed the highest increase, as compared to the uptake in non-transfected cells (**Paper I**, Figure 1 B, C). Importantly, the non-specific IgG control did not display any increased uptake in BiFC expressing cells. The uptake pattern of the α -synuclein oligomer-selective antibodies was seen as small perinuclear punctae. The punctae were found to be inside cells and were partially co-localizing with BiFC signal, as seen with confocal microscopy (**Paper I**, Figure 2). The increased antibody uptake upon α -synuclein BiFC expression implied that the presence of α -synuclein enhanced internalization and accumulation of antibody. To sort out if extracellular α -synuclein levels were critical for this effect, antibody uptake was studied in non-transfected cells given 4 h old conditioned media from BiFC expressing cells. At 4 h the α -synuclein levels in BiFC media were approximately 50nM, ten times higher than in media from non-transfected cells. After 30 min of incubation the antibody uptake was higher in cells given α -synuclein BiFC enriched conditioned media, as compared to cells given regular growth media (Figure 4 & **Paper I**, Figure 4). The increased uptake under these conditions suggests that extracellular α -synuclein is critical for the mAb47 uptake. The fact that this setup led to a much faster accumulation than adding mAb47 directly to BiFC stable cells, also implies that the extracellular levels build up over time. It is therefore likely that cellular mAb47 uptake is dependent of extracellular α -synuclein levels. A mechanistic explanation could be that antibodies form immune complexes with extracellular oligomers, which are phagocytosed by the cells [97]. By principle, cellular uptake of a treatment antibody depends on its affinity for its antigen in the extracellular space as well as its propensity to interact with cell membranes and surface proteins [185].

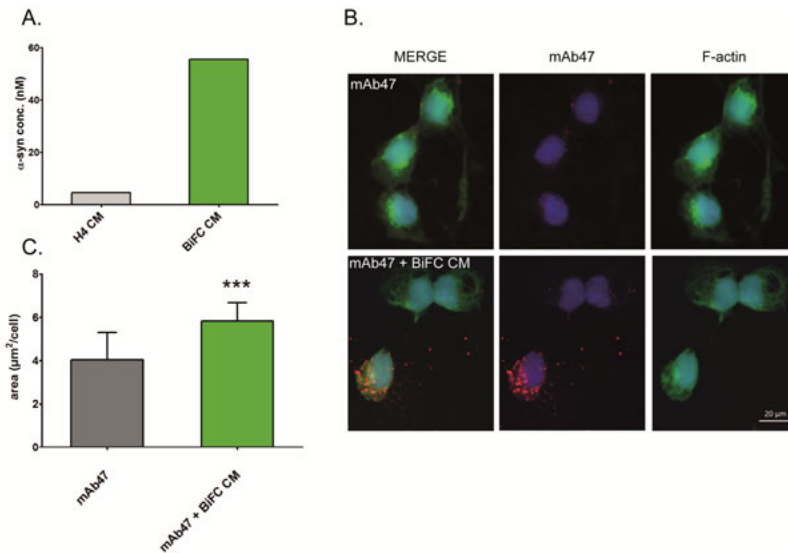


Figure 4. Increased uptake of mAb47 due to extracellular presence of α -synuclein. **A)** H4 cells expressing the α -synuclein BiFC assay displayed a ten-fold increase in extracellular α -synuclein levels compared to non-expressing cells, as measured by ELISA. **B)** H4 cells were incubated with mAb47 in combination with either conditioned media from BiFC expressing cells or with fresh growth media. After 30 min, cells incubated with BiFC conditioned media displayed a higher uptake of mAb47 (red). Blue = DAPI, scale bars = 20 μm . **C)** The uptake of mAb47 was significantly increased, as measured with quantitative fluorescence microscopy ($p < 0.0001$).

Moreover, the involvement of immune receptors in the antibody uptake was assessed. H4 cells with a stable expression of BiFC were incubated at 4 $^{\circ}\text{C}$ in regular media and then blocked with polyclonal antibodies against the human receptors Fc γ RI, Fc γ RIIB/C or Fc γ RIIIA/B for 10 min prior to addition of mAb47. After 2 h, the cells blocked for Fc γ RI and Fc γ RIIB/C displayed a marked decrease of mAb47 uptake, as compared to the non-blocked control (**Paper I**, Figure 5). This finding suggests that the immune receptors were mediating antibody uptake when α -synuclein was present in cell media [97]. Thus, these observations suggest that the treatment effects seen in transgenic mice could be addressed not only to steric hindrance of aggregation in the extracellular space but also to antibody interactions with immune cells and neurons and internalization of mAb47:oligomer complexes.

Activation of glia via immune receptors could lead to pro-inflammatory responses, reviewed by Fuller et al [169] and Nimmerjahn et al [186]. However, the mAb47 treated transgenic α -synuclein mice did not display any signs of astrocytic or microglial reactions to the antibody treatment [158]. The reduced α -synuclein BiFC fluorescence seen in H4 cells after mAb49G treatment [164] might be due to increased cellular processing of oligomers as a result of phagocytosis of immune complexes. Since α -synuclein is associated to the plasma membrane, the antibody could find the α -synuclein oli-

gomer epitope in the cell membrane. Alternatively, the antibody recognizes secreted α -synuclein species in the extracellular space. Alpha-synuclein oligomers can also get secreted and are then capable of spreading to other cells via exosomes [93], possibly exposing epitopes in the extracellular space.

Effects of α -synuclein oligomer uptake and accumulation in cultured astrocytes

In **Paper II** we investigated uptake, degradation and toxic effects of oligomeric α -synuclein in a co-culture system of primary neurons, astrocytes and oligodendrocytes. In neurodegenerative disorders such as AD and PD, a central part of the pathology is related to CNS inflammation and progressive spreading of neuronal death. Glial cells respond to α -synuclein pathology by going into an activated state where cytokines and reactive oxygen species are released and cells increase in size [91]. Astrocytes may have a particularly important role, as they have been found to engulf dead neurons to protect surrounding cells from contact-induced apoptosis [143]. They have also been reported to ingest α -synuclein [91, 110]. However, it is not known whether this uptake prevents or accelerates the propagation of pathology.

We studied cellular accumulation of α -synuclein oligomers in a co-culture of 20 % neurons, 75 % astrocytes and 5 % oligodendrocytes. Here, we found that astrocytes and oligodendrocytes rapidly accumulated oligomers, whereas the neurons did not display any α -synuclein accumulation. Large α -synuclein inclusions were found in astrocytes and oligodendrocytes after 24 h of oligomer exposure (Figure 5 & **Paper II**, Figure 1). When co-cultures were exposed to α -synuclein fibrils, the uptake pattern was different and suggested that the fibrils attached to the cell membrane of astrocytes rather than being ingested (**Paper II**, Figure 3). Moreover, time-lapse microscopy revealed a rapid accumulation of α -synuclein oligomers in the astrocytes. To study the degradation of ingested α -synuclein, cells were first exposed to α -synuclein oligomers for 24 h, followed by washing and incubation in media without α -synuclein for up to 12 days.

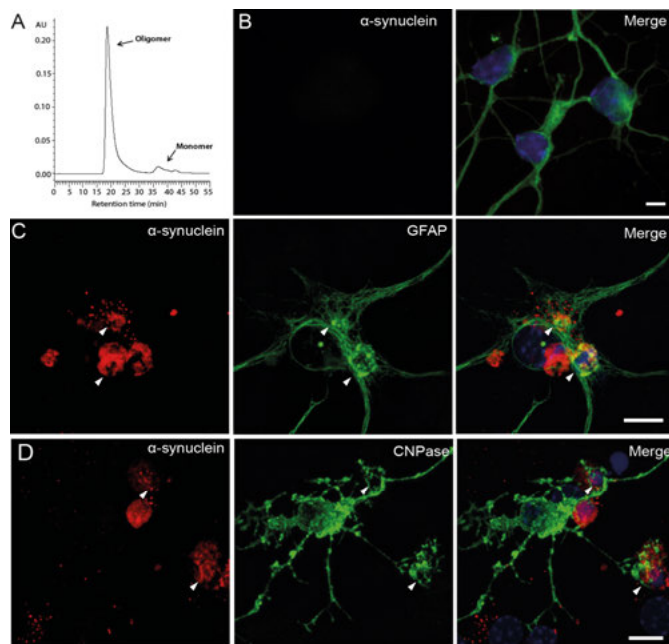


Figure 5. Accumulation of α -synuclein oligomers in astrocytes and oligodendrocytes. **A)** Analysis of HNE-induced α -synuclein oligomers by SEC-HPLC displayed conversion of monomers into 2000 kDa oligomers. Co-cultures were exposed to $0.5\mu\text{M}$ α -synuclein oligomers for 24 h. **B)** Immunofluorescence towards β -III tubulin (green) displayed no accumulation of α -synuclein in neurons. **C)** A high uptake of α -synuclein oligomers (red) was seen in astrocytes (GFAP, green) and in **D)** oligodendrocytes (CNPase, green). Blue = DAPI, scale bars = $10\mu\text{m}$.

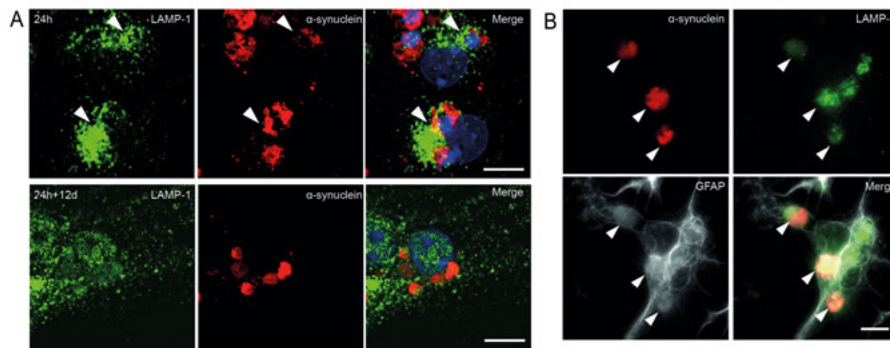


Figure 6. Ingested α -synuclein is degraded by lysosomes in astrocytes. Co-cultures were exposed to $0.5\mu\text{M}$ α -synuclein oligomers for 24 h. **A)** After 24 h, α -synuclein deposits (red) localized to lysosomal compartments, as seen with LAMP-1 staining (green). After 12 days, α -synuclein deposits were still present but displayed lower localization to LAMP-1 positive compartments, suggesting a re-direction from the lysosomal degradation pathway. **B)** After 24 h, the large α -synuclein deposits localized to LAMP-1 positive compartments within astrocytes (GFAP, white). Blue = DAPI, scale bars = $10\mu\text{m}$.

The size distribution of ingested aggregates was shifted over time so that α -synuclein inclusions became smaller and more numerous over a 12 day period (**Paper II**, Figure 5), but the inclusions remained throughout the experiment. This finding suggests that, even though astrocytes started to process and degrade the engulfed α -synuclein oligomers, the degradation was not completed as deposits remained at later time points. Biochemical analysis of lysates by Western blot and ELISA displayed decreasing levels of internalized α -synuclein over the first 6 days (**Paper II**, Figure 6). This apparent decrease could be a result of truncation or changed conformation of the accumulated α -synuclein, possibly interfering with antibody detection of epitopes. The ingested α -synuclein localized to the endosomal/lysosomal pathway at early time points as seen with LAMP-1 staining. At later time points the remaining deposits did not localize to LAMP-1 positive compartments, suggesting that the material had been re-directed from the lysosomal pathway (Figure 6 & **Paper II**, Figure 7). To assess for toxic effects mediated by the α -synuclein deposits, mitochondria were analyzed with a set of techniques. Transmission electron microscopy (TEM) analysis revealed disrupted mitochondrial morphology after α -synuclein oligomer exposure. A lost structure of outer mitochondrial membranes and inner cristae suggested a mitochondrial stress response (**Paper II**, Figure 8 B, C). Moreover, ATP levels had decreased in oligomer exposed cultures, further indicating mitochondrial dysfunction (**Paper II**, Figure 8 D). In line with this observation, analysis with QPCR revealed an increase of mitochondrial DNA after oligomer exposure, suggesting that astrocytes compensated for the loss of functional mitochondria (**Paper II**, Figure 8 E). Transfection with Cell light Mitochondria-GFP plasmid and fluorescence microscopy revealed fragmented mitochondrial networks and mitochondrial swelling in the oligomer exposed astrocytes (**Paper II**, Figure 9 A, B). An increase of the mitochondrial marker Mitofusin 1 and increased translocation of Drp1 indicated that the mitochondrial machinery was stressed by the α -synuclein inclusions (**Paper II**, Figure 9 C-F). This finding suggested an impaired mitophagy as a result of an overburdened lysosomal system.

Conclusively, this study sheds light on the possible role of astrocytes in the PD brain. The astrocytes may protect surrounding neurons by ingesting toxic α -synuclein oligomers. However, large intracellular α -synuclein deposits impair the lysosomal degradation and lead to detrimental processes in the astrocytes. In the long term these mechanisms may instead drive the progression of pathology further.

Antibody-mediated effects on astrocytic α -synuclein accumulation and mitochondrial stress in neuronal-glia co-cultures

In **Paper II** high uptake and accumulation of α -synuclein oligomers was seen in the astrocytes, leading to impaired astrocytic function. To further investigate whether this accumulation and the resulting cellular effects could be affected, α -synuclein oligomer-selective antibodies were applied to the co-cultures used in **Paper II**. Treatment with the same antibodies in transgenic A30P mice resulted in reduced levels of toxic oligomers/protofibrils and ameliorated motor symptoms [158]. However, the mechanisms behind this oligomer clearance remained unknown. In **Paper III**, we studied how the astrocytes in co-cultures processed pre-formed antibody: α -synuclein oligomer complexes. We could show that antibody: α -synuclein oligomer complex formation in the extracellular space strongly reduced the α -synuclein accumulation in astrocytes. Importantly, by preventing accumulation antibodies also rescued astrocytes from mitochondrial stress effects.

The co-cultures were exposed to pre-formed complexes of α -synuclein oligomers and three different α -synuclein oligomer-selective antibodies. This setup corresponds to a treatment situation where α -synuclein pathology is propagating in the PD brain by cell-to-cell transfer and oligomeric epitopes are accessible in the extracellular space. Before cell exposure, oligomers and antibodies were pre-incubated at a 1:1 ratio. All assessed α -synuclein oligomer-selective antibodies led to reduced α -synuclein accumulation (**Paper III**, Figure 1) and mAb47 had the strongest reducing effect. Interestingly, this antibody had proven efficacy in the A30P transgenic mice [158]. In addition, mAb47 displayed the highest intracellular presence during α -synuclein expression in **Paper I**. We therefore focused on this antibody in **Paper III**. In a set of control experiments, mAb47 was added to cells either in combination with, before or after oligomers. The pre-formed mAb47: oligomer complex resulted in lower accumulation compared to the other settings (Figure 7 & **Paper III**, Figure 2), suggesting that the extracellular complex formation was crucial for the effect to occur. An isotype control (IgG1) had a significantly weaker effect on α -synuclein accumulation (**Paper III**, Figure 3), implying that the effect seen with mAb47 was dependent of specific antigen binding and not a result of general IgG-mediated cell activation [166]. Furthermore, mAb47 co-localized with the ingested α -synuclein (**Paper III**, Figure 4) and the complex was found in LAMP-1 positive compartments.

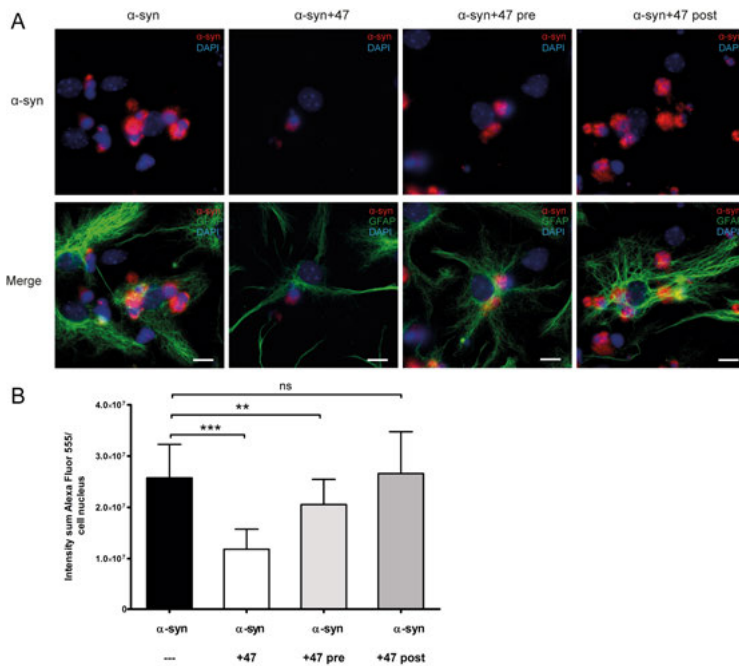


Figure 7. Extracellular binding of mAb47 and α -synuclein oligomers is critical for intracellular effects on α -synuclein accumulation. **A)** Co-cultures exposed to α -synuclein oligomers for 24 h (α -syn) displayed high α -synuclein accumulation (red) in astrocytes (GFAP, green). The mAb47 antibody was added to cells either at the same time as oligomers (α -syn+47), 24 h before (α -syn+47 pre) or after (α -syn+47 post) oligomers. Cultures were carefully washed between incubations. The strongest reducing effect on intracellular α -synuclein accumulation was seen when mAb47 was added at the same time as oligomers (α -syn+47). When mAb47 had been added before oligomers, there was a moderate reduction (α -syn+47 pre) whereas addition of antibody after the oligomers (α -syn+47 post) did not reduce accumulation. Blue = DAPI, scale bars = 10 μ m. **B)** Quantitative fluorescence microscopy confirmed that reduction of α -synuclein accumulation was only achieved when mAb47 was added to cells before (α -syn+47 pre) ($p < 0.01$) or at the same time (α -syn+47) ($p < 0.001$) as oligomers.

Thus, the mAb47:oligomer complex partially located to the same degradation pathway as the α -synuclein oligomer alone (**Paper III**, Figure 5). When monitoring mitochondria with Cell light Mitochondria-GFP after oligomer exposure, mitochondrial fragmentation and swelling was observed. In cells exposed to mAb47:oligomer complexes, the mitochondrial fragmentation was reduced to the levels of untreated controls (Figure 8 & **Paper III**, Figure 6), implying that alleviation from sustained α -synuclein deposits was enough to prevent mitochondrial impairment. To investigate the mechanism behind the reduced accumulation, cells were treated with inhibitors of the endosomal-lysosomal pathway.

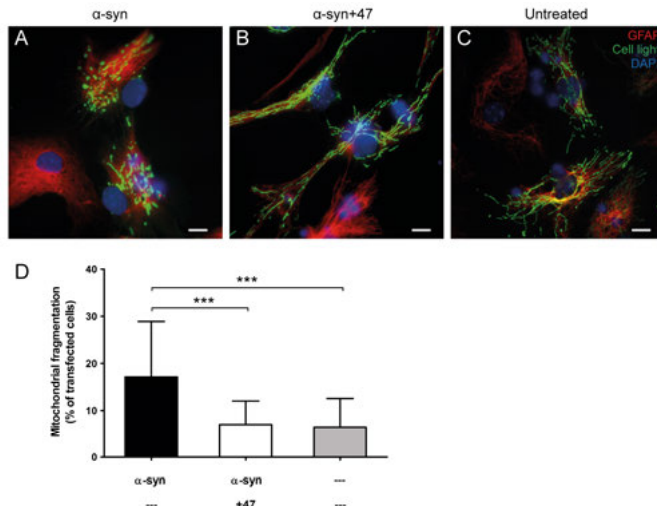


Figure 8. Treatment with mAb47 prevents mitochondrial stress effects in astrocytes. Co-cultures were exposed to α -synuclein oligomers for 24 h. Transfection with Cell light mitochondria-GFP allowed visualization of mitochondria (green). **A)** Oligomer exposure led to disruption of mitochondrial networks in astrocytes (GFAP, red). **B)** When oligomers had been pre-incubated with mAb47, cells displayed an elongated mitochondrial network with lower degree of fragmentation, similar to **C)** untreated control cells. **D)** Cells with disrupted mitochondrial networks were counted and normalized against the number of transfected cells. Exposure to oligomers led to a significant increase of mitochondrial disruption (***) $p < 0.001$). When mAb47 was present, the level of mitochondrial disruption was lowered and similar to control levels. Blue = DAPI, scale bars = 10 μ m.

However, none of the inhibitors affected the mAb47-mediated reduction (**Paper III**, Figure 7), indicating that the pre-formed complex was degraded by another route than the endosomal-lysosomal pathways. In time-lapse recordings, the pre-formed mAb47:oligomer complex showed a low accumulation in astrocytes, compared to oligomers alone (**Paper III**, Figure 8), suggesting a slowed down accumulation or increased degradation of the α -synuclein oligomers in the presence of mAb47. When conditioned media were analyzed with α -synuclein ELISA, extracellular α -synuclein levels were significantly lower when antibody was present (**Paper III**, Figure 9). The levels of α -synuclein bound to mAb47 were also measured by immunoprecipitation of mAb47 from conditioned media. This fraction of α -synuclein was also heavily reduced after 24 h (**Paper III**, Figure 9), supporting the explanation that the overall α -synuclein clearance was promoted by mAb47. On the other hand, the extracellular levels of mAb47 did not decrease as much as oligomers (**Paper III**, Figure 9). It could be hypothesized that antibodies, ingested as complexes, could be re-cycled to the extracellular space and were not degraded to the same extent as the α -synuclein oligomers. The overall α -synuclein clearance could have been due to different effects of the

complex formation, including promoted phagocytosis, shifted degradation pathways or a combination of the above mentioned effects (Figure 9).

Altogether, our findings suggest that astrocytes and their neuroprotective functions should be taken into account when designing therapeutic strategies for α -synucleinopathies.

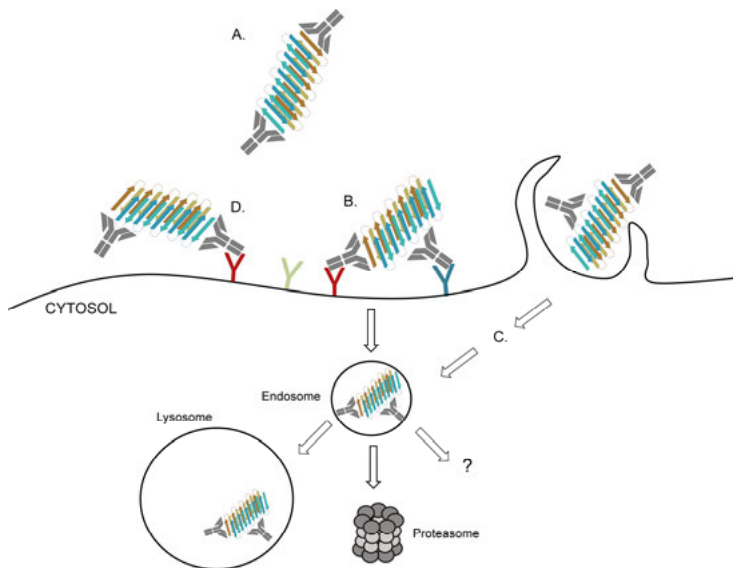


Figure 9. Cellular processing pathways of antibody: α -synuclein oligomer complexes. Possible modes of action for antibody-mediated clearance of α -synuclein aggregates may be **A)** extracellular binding of aggregates and blocking of further aggregation, **B)** promoted receptor-mediated uptake of antibody: α -synuclein complexes, **C)** shifted intracellular trafficking/degradation pathways or **D)** binding to cell surface receptors inducing transmembrane signalling and increased autophagy.

Secretion of α -synuclein via extracellular vesicles

Spreading of α -synuclein pathology has been suggested to be mediated by extracellular forms of α -synuclein. Oligomeric α -synuclein was found in EVs and these vesicle-associated species had a more efficient uptake in recipient cells than free-floating extracellular α -synuclein and caused toxicity [93], suggesting that the EVs promote cell-to-cell spreading of pathology. The EV secretion of α -synuclein in the form of exosomes is promoted by impairment of lysosomal degradation [93, 114, 126].

In **Paper IV**, we evaluated how different fluorescent tags or mutations affected the cellular distribution of overexpressed α -synuclein to EVs. To investigate how the cells distributed the different forms of α -synuclein, lysates,

conditioned media or enriched EVs were isolated (Figure 13 & **Paper IV**, Figure 1) and analyzed with α -synuclein ELISA. Conditioned media were centrifuged at 100 000x g in order to get a pellet of various extracellular vesicles. These structures were positive for flotillin 1 and had a size range of 100-500 nm (**Paper IV**, Figure 2). When α -synuclein was expressed in fusion with the N-terminal peptide of fluorescent Venus protein, the ratio of α -synuclein in EVs compared to its corresponding free-floating fraction, was significantly increased (Figure 10 & **Paper IV**, Figure 3). Denaturing the EVs resulted in a small increase in ELISA signal, indicating that most of the α -synuclein was located on the outside of EVs.

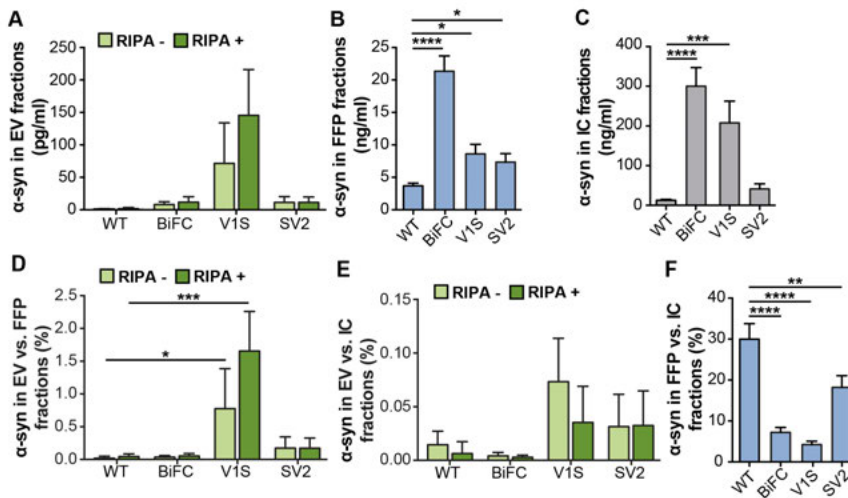


Figure 10. Alpha-synuclein expressed with a non-physiological protein tag is directed towards EV secretion. Centrifugation pellets (EVs) and supernatants (free-floating protein, FFP), originating from conditioned media and lysates (intracellular fraction, IC) were analysed with α -synuclein ELISA. **A)** The α -synuclein levels were in the range of 1-200 pg/ml in EVs. RIPA+ fractions were equal to or higher than RIPA- fractions. **B)** The WT α -synuclein transfection led to significantly lower α -synuclein levels in FFP compared to the other transfections. **C)** BiFC and V1S transfections led to significantly higher α -synuclein levels than WT in IC fractions. **D)** When analysing the EV to FFP ratio, both RIPA+ and RIPA- fractions from the V1S transfection were significantly higher than the respective fractions from WT, suggesting a direction towards EV secretion caused by single transfection of the N-terminal hemi-Venus tag. **E)** EV to IC ratios. **F)** FFP to IC ratios. Bars represent mean \pm SD of 3 independent experiments. Statistical significance was calculated by 1-way ANOVA with Dunnett's posthoc test compared to WT (*p<0.05, **p<0.01, ***p<0.001).

When another neurodegenerative protein, tau (the major constituent of the neurofibrillary tangles in the AD brain) fused to GFP was expressed in the

SH-SY5Y cells, the fluorescent tag did not affect the distribution of tau to EVs (**Paper IV**, Figure 4). Thus, the observed re-distribution seemed to be specific for α -synuclein. Next, cell-to-cell spreading of α -synuclein:BiFC in either the EV of free-floating fraction was assessed. The EV-associated form of α -synuclein:BiFC had a markedly higher uptake in recipient cells (**Paper IV**, Figure 5 A), suggesting EVs as effective mediators in cell-to-cell spreading of pathological α -synuclein. Furthermore, cells displayed toxicity when given EV-associated α -synuclein:BiFC (**Paper IV**, Figure 5 B). This toxicity was significantly higher than what could be seen with EV-associated WT protein, possibly due to higher oligomeric content. On the other hand, the corresponding FFP fractions resulted in equal toxicities. Introducing the disease-causing mutation A53T to α -synuclein increased the EV to lysate ratio, as compared to WT expression (**Paper IV**, Figure 6). The other disease-causing α -synuclein mutations did not cause a shift in the EV to lysate ratio. However, the lipophilic mutations H50Q [61] and G51D [187] displayed increased intraluminal localization.

In summary, we detected subtle changes in the cellular α -synuclein distribution to EVs when introducing either a non-physiological protein tag or a disease-causing mutation to α -synuclein. In the PD brain, such fine-tuned changes caused either by altered membrane binding or increased aggregation of α -synuclein, may in the long term contribute to the intercellular spreading of α -synuclein pathology.

Further studies

In **Paper III** the α -synuclein oligomer-selective antibodies were found to reduce the high astrocytic accumulation of α -synuclein in cultures. However, it was not clear by which mechanism the overall clearance was promoted by the antibodies. Low levels of α -synuclein oligomers were detected in cells when antibody was present and these weak accumulations localized to the endosomal/lysosomal pathway. Even though only a small fraction of the α -synuclein oligomers were degraded via lysosomes, the possibility remains that the majority of ingested oligomers may have been processed via another pathway. Chemical inhibition of endosomal maturation or fusion of endosomes with lysosomes did not alter the antibody-mediated reduction of accumulation. However, it is likely that oligomers taken up in the form of complexes were directed to proteasomal degradation, as they were seen to co-localize with ubiquitin (**Paper II**), or processed via an as of yet not described trafficking pathway (Figure 9). To investigate these mechanisms further studies are needed. It would also be of importance to elucidate by which mechanisms and in which structures the astrocytes ingest and store α -synuclein oligomers. Moreover, the IgG1 mAb47 displayed high bioactivity

in **Paper I** and **Paper III**. To understand the biomolecular basis for these properties, further characterization of the antigen binding properties in the extracellular milieu and also receptor/ membrane binding properties need to be carried out. Such data would be important in order to develop robust treatment strategies employing this type of conformation-selective monoclonal antibodies. In **Paper IV**, the EV secretion of α -synuclein was found to be promoted by introducing protein tags or disease causing mutations. Studies should be carried out in order to better understand the mechanisms causing this cellular re-distribution. Such information would be important in the mapping of extracellular spreading of α -synuclein pathology.

Concluding remarks

The work in this thesis has shed light on the cellular processes associated with α -synuclein aggregation, extracellular propagation and the mechanisms behind antibody treatment on cell models for α -synuclein disorders. In **Paper I**, we could show that mAb47 had an increased intracellular presence in human neural cell lines, due to expression of the α -synuclein BiFC oligomerization assay. The IgG internalization was significantly promoted by extracellular presence of the antigen, suggesting extracellular, oligomeric forms of α -synuclein as plausible therapeutic targets. Moreover, the observation that immune receptors could mediate uptake of mAb47 suggests that this interaction may also happen in the human CNS and activate immune cells, and needs to be taken into account when designing future treatments. In **Paper II**, we could demonstrate that astrocytes are highly involved in α -synuclein pathology as they rapidly engulfed and stored the toxic α -synuclein oligomers. This action leads to impaired degradation and mitochondrial stress in the astrocytes. In the PD brain, this process might in the long term perturb vital astrocytic functions such as glymphatic clearance and neuroprotection. In **Paper III**, we could show that mAb47 and two similar mAbs reduced the astrocytic accumulation of α -synuclein oligomers described in **Paper II**. This effect was addressed to extracellular antigen binding and increased overall clearance of α -synuclein in the cultures. In turn, astrocytes were rescued from mitochondrial stress. In the PD brain, this type of passive immunization targeting α -synuclein pathology might shift the mode of clearance and preserve astrocytic functionality. In line with the findings in **Paper I**, it was evident that extracellular antibody: antigen binding was crucial for the intracellular antibody-mediated effects. In **Paper IV**, we found that expression of α -synuclein in fusion with a non-physiological protein tag led to a shift in cellular distribution towards increased secretion via EVs. A similar effect was seen upon expression of α -synuclein with the disease-causing mutation A53T. These effects could be due to an altered physiological function or changed aggregation properties of α -synuclein.

Moreover, EV-associated α -synuclein:BiFC oligomers displayed a more efficient cell-to-cell transfer and were associated with toxic effects. These findings highlight the molecular properties of α -synuclein in relation to EV-mediated spreading of α -synuclein pathology.

Altogether, the work in this thesis provides new insight into the role of extracellular forms of α -synuclein in the spreading of pathology. Assessment of α -synuclein oligomer-selective antibodies in the cell models suggests extracellular α -synuclein oligomers as plausible therapeutic targets in development of future treatment strategies against PD and other α -synucleinopathies. By interfering with the extracellular spreading of toxic α -synuclein species, progression of neurodegeneration may be halted.

Methods

Detailed protocols of the experimental methods are described in the respective papers.

Human cell lines derived from the central nervous system (Paper I, IV)

The H4 cell line originates from human neuroglioma and is a heterogeneous population of cells with neural characteristics [188]. In **Paper I**, the H4 cell line was used in the experiments for antibody uptake and for expression of plasmids encoding the two α -synuclein-BiFC fusion peptides. The plasmids contain the antibiotic resistance gene against geneticin (G418) and after transfection cells were cultured in selection media with G418 added to maintain plasmids. In this system, developed by Tiago Outeiro, α -synuclein-BiFC is constantly expressed (Figure 11). In an earlier study from our laboratory, H4 cells expressing α -synuclein BiFC displayed reduced oligomerization after treatment with α -synuclein oligomer-selective antibodies [164]. In **Paper IV**, the human SH-SY5Y neuroblastoma cell line was used to study α -synuclein secretion and uptake via extracellular vesicles during expression of various forms of α -synuclein. The SH-SY5Y cell line was transiently transfected with plasmids encoding α -synuclein allowing expression for up to 48h. An earlier study from our laboratory revealed that exogenous α -synuclein oligomers were internalized and caused toxicity in SH-SY5Y cell lines [95]. Alpha-synuclein expression in SH-SY5Y cells has also been found to reduce viability by impairment on a transcriptional level, leading to apoptosis [189].

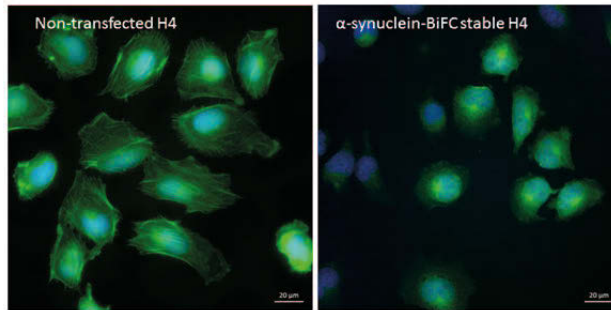


Figure 11. Human neuroglioma H4 cells. Non-transfected cells are visualized by staining of the actin cytoskeleton with FITC-conjugated phalloidin (left). In the BiFC stable H4, green fluorescence is seen throughout the soma as a result of dimerization/oligomerization of the fusion proteins (right) (63x magnification).

Neural cell culture (Paper II, III)

In **Paper II** and **III**, mixed neural cell cultures from embryonal mouse cortex were used to study the effects of α -synuclein oligomers on neurons and glial cells. Cortices from C57B16 E14 mouse embryos were dissected and dissociated in HBSS. This cell model had been earlier characterized [143, 190-192]. The cells were expanded as neurospheres in the presence of the mitogens *Fibroblast growth factor 2* (FGF2) and mouse *Epidermal growth factor* (EGF). The neural stem cells were passaged by dissociating the neurospheres after 3-5 days and re-suspending the cells in fresh media. Neurospheres of passage 1-3 were used for experiments. In order to differentiate the cortical stem cells, neurospheres were dissociated and seeded on pre-coated cover slips in cell culture plates. After one day the growth factors were removed and cells were differentiated for one week. The stem cells differentiate into a population of 20 % neurons, 75 % astrocytes and 5 % oligodendrocytes (Figure 12) [146]. Differentiated cells were exposed to HNE-induced α -synuclein oligomers for 24 h to assess cellular accumulation of oligomers at various conditions (**Paper II**) and in the presence of α -synuclein oligomer-selective antibodies (**Paper III**).

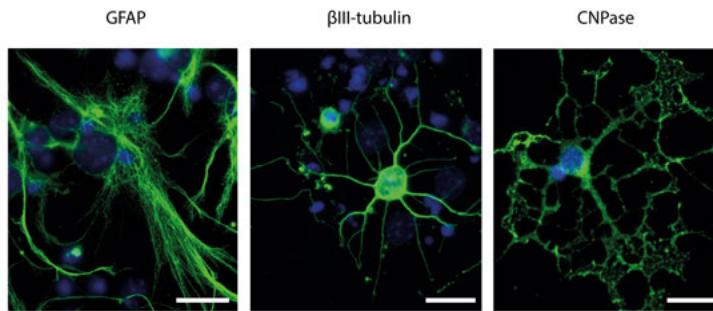


Figure 12. Mixed neural cell cultures from embryonal cortical stem cells. Differentiation of the neural stem cells leads to development into a co-culture of astrocytes (GFAP), neurons (β III-tubulin), and oligodendrocytes (CNPase).

Bimolecular fluorescence complementation assay (BiFC) (Paper I, IV)

In **Paper I** and **IV**, α -synuclein fused to either the N- or C-terminal half of the reporter molecule *enhanced yellow fluorescent protein* (EYFP) *Venus* was expressed in human cell lines [193]. Heterodimerization of the two fusion proteins will generate a fluorescence signal from the re-constituted fluorophore (Figure 13 A) that is detectable by fluorescence microscopy [194]. Overexpression of the assay gives rise to fluorescent dimers and oligomers. However, it also leads to generation of non-fluorescent dimers and oligomers. Importantly, the fusion protein may affect the molecular properties of α -synuclein depending on the fusion site. The N-terminal Venus peptide, fused N-terminally is 157 amino acids and may for example affect the membrane binding properties of the N-terminal of α -synuclein [47, 51]. The Venus protein is a modified version of GFP that in the BiFC setting has an earlier developed and stronger fluorescence signal. The reconstitution of the fluorophore is optimized by mutations, also causing an excitation and emission red-shift towards the yellow spectrum, as compared to wild type GFP [195]. In an earlier study from our group, the GFP version of BiFC was used to measure anti-oligomer effects by α -synuclein oligomer-selective antibodies [164]. In **Paper I**, cellular uptake of α -synuclein oligomer-selective antibodies was studied. This setup required a fluorescence assay with a more robust expression and fluorescence signal. Therefore the Venus BiFC assay was used in this study.

Chemically induced α -synuclein oligomers (Paper II, III)

In **Paper II** and **III**, α -synuclein oligomers were generated by incubating recombinant human monomeric α -synuclein with the reactive aldehyde 4-hydroxy-2-nonenal (HNE) at a molar ratio 1:30 for up to 72 h at 37 °C [95]. Conversion of α -synuclein monomers into large oligomers was confirmed by size exclusion chromatography (Figure 5 A). Oligomers were fluorescently labelled with Cy3 dye and used for exposure to cell cultures.

Immunofluorescence on cell cultures (Paper I-IV)

In **Paper I**, H4 cells were treated with α -synuclein oligomer-selective antibodies. Immune fluorescence was applied on permeabilized and blocked samples to monitor the uptake of α -synuclein antibodies. To monitor the cell contours in non-transfected cells, the fibrillary actin cytoskeleton was labelled with FITC conjugated phalloidin. In BiFC transfected cells, the green fluorescent signal emitted from the Venus fluorophore was used to monitor the cell contours. In addition, the linear epitope α -synuclein antibody C20 (Santa Cruz Biotechnology) was used to label α -synuclein in the cells independently of conformation. Nuclei were stained with DAPI in all cells. To detect the expression of Fc γ immune receptors in H4 cells, polyclonal goat antibodies were used. In **Paper II**, the different cell types in the cortical co-culture were labelled with specific markers. Neurons were labelled with a mouse-anti- β III-tubulin antibody, astrocytes with a rabbit-anti-GFAP antibody and oligodendrocytes with mouse-anti-2'3'-cyclic nucleotide 3'-phosphodiesterase (CNPase) antibody. In **Paper II** and **III**, the HNE-induced α -synuclein oligomers added to cultures were fluorescently labelled with Cy3-dye before experiments. To assess degradation of ingested α -synuclein oligomers, cells were stained with antibodies against the lysosomal marker Lysosomal-associated membrane protein 1 (LAMP-1) (**Paper II** and **III**) and ubiquitin (**Paper II**). To elucidate whether the glial α -synuclein accumulations had fibrillary content, cells were stained with Thioflavin S (Thio S) (**Paper II**). In **Paper II** and **III** Mitochondria were visualized by transfection of Cell light Mitochondria-GFP plasmid, leading to mitochondrial expression of GFP. In **Paper II** immunofluorescence with an antibody against the mitochondrial marker Mitofusin 1 was used to study mitochondria levels in cells treated with HNE-induced α -synuclein oligomer. Moreover, immunolabelling of the mitochondrial fission protein dynamin related protein 1 (Drp1) was carried out for assessment of mitochondrial stress effects. In **Paper IV**, the uptake of α -synuclein:BiFC in recipient cells was monitored by immunofluorescence with a polyclonal antibody against GFP.

Immunofluorescence on mouse brain tissue (Paper II)

In paper II hemispheres from 19 mo old transgenic (Thy-1)-h [A30P] were immunostained to investigate the involvement of astrocytes in α -synuclein pathology. The mouse model has a neuronal expression of human A30P α -synuclein under the Thy-1 promoter. Hemispheres were sectioned in 20 μ m sagittal sections. Antigen retrieval in heated citrate buffer was used to increase the possible binding sites for detection antibodies. The tissues were permeabilized with Triton-X, allowing detection of intracellular epitopes. To allow detection of α -synuclein with mouse IgG, blocking of unspecific interaction was performed with M.O.M. Immunodetection kit (Vector laboratories). The primary detection antibodies mAb1338 (R&D systems) and C20 (Santa Cruz), directed towards human α -synuclein were used in combination with anti-GFAP or anti- β III tubulin antibodies. Mounting of sections was made with mounting medium containing DAPI, visualizing cell nuclei.

Immunofluorescence on human brain tissue (Paper II)

In Paper II, human brain tissue from PD or DLB patients was examined to investigate the role of astrocytes in α -synuclein pathology *in vivo*. Paraffin embedded sections from substantia nigra were used for immunohistochemistry. The paraffin was removed in xylene and hydrated in a series of decreasing concentrations of ethanol. After permeabilization and blocking of unspecific interaction, sections were incubated with primary antibodies against α -synuclein and GFAP. Autofluorescence in the tissue caused by lipofuscin was removed by incubation with Sudan black. Sections were incubated with fluorescently labelled secondary antibodies before mounting with DAPI containing mounting media.

Fluorescence microscopy (Paper I-IV)

In **Paper I** fluorescent α -synuclein oligomers formed in the BiFC assay were detected with wide field fluorescence microscopy. The aggregation of the α -synuclein-BiFC peptides was monitored through the green signal generated upon dimerization/oligomerization. To monitor the internalization of antibody or α -synuclein oligomers in a three-dimensional way, confocal microscopy was used (**Paper I, II and III**). By stacking a series of confocal images, a three-dimensional image is generated. This image gives spatial information about subcellular location of fluorescently labelled molecules. In **Paper II and III**, cells were monitored with time lapse microscopy. This method allows visualization of movements of the cells, interactions and uptake of fluorescently labelled proteins in a time-resolved manner on a scale of minutes

and up to several days. Fluorescent images may then be collected and overlaid with light microscopy images. Quantitative fluorescence microscopy was carried out to determine the extent of cellular antibody uptake (**Paper I**) or accumulation of HNE-induced α -synuclein oligomers (**Paper II** and **III**). As a measure of mitochondrial levels, immunostainings of the mitochondrial marker Mitofusin 1 were quantified (**Paper II**). Fluorescence signal was measured with Zen 2012 software using count, area or intensity as parameters. These values were normalized against the number of cells per image. Morphologies of Cell light Mitochondria-GFP positive mitochondria were divided into categories of either elongated or fragmented networks. The groups were normalized against the total number of Cell light expressing cells per image, as seen by GFP fluorescence. In **Paper IV** immunostainings of α -synuclein:BiFC uptake were monitored by wide field fluorescence microscopy.

Isolation of extracellular vesicles (Paper IV)

In **Paper IV**, cell media from α -synuclein expressing SH-SY5Y neuroblastoma cell lines was centrifuged to isolate extracellular vesicles (Figure 13). After 48h of transfection with plasmids encoding α -synuclein, conditioned media was collected and filtered through a 0.45 μm filter. In addition cell lysates were collected. Cell media were then centrifuged with a fixed angle rotor in an ultracentrifuge at 100 000x g to pellet extracellular vesicles. The supernatant was saved to analyse extracellular, free-floating forms of α -synuclein. The vesicle containing pellets were washed in PBS, transferred to clean tubes and pelleted again prior to re-suspension in either RIPA lysis buffer (Abcam) or PBS with 0.1 % bovine serum albumin. The RIPA lysis buffer causes vesicles to break up, therefore allowing analysis of the α -synuclein content both inside and on the outside of the vesicles. The PBS fraction on the other hand, reflects the native vesicles and allows analysis of molecules exposed on the surface of the vesicles. Sandwich ELISA was used to measure α -synuclein levels in all fractions.

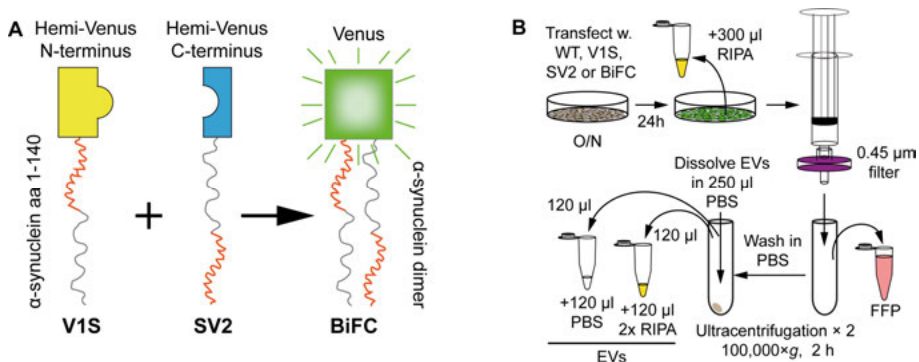


Figure 13. Sample preparation to study α -synuclein secretion from human neuroblastoma cell lines. **A)** SH-SY5Y cell lines were transfected with plasmids encoding full length α -synuclein or α -synuclein in a bimolecular fluorescent complementation (BiFC) assay. The gene for α -synuclein was either fused to N-terminal or C-terminal hemi-Venus protein and double transfection allowed fluorescent dimerization. **B)** Cells were transfected for 24 h, washed and incubated in EV depleted media for 24 h. Conditioned media was collected and filtered prior to centrifugation. Lysates were collected and stored at -20°C . Conditioned media was centrifuged and the supernatant was saved as free-floating protein (FFP). The pellet was washed, moved to a new tube and centrifuged again. The resulting EV pellet was dissolved and split in two, either with PBS or with RIPA lysis buffer.

Enzyme-linked immunosorbent assay (ELISA) (Paper I-IV)

In **Paper I-IV** ELISA was used to detect and quantify various forms of α -synuclein in protein samples from cell cultures. A sandwich ELISA was used to detect the total α -synuclein content (Figure 14 A). The mAb1338 (R&D systems) or Syn-1 (BD Biosciences) was used as capture antibody. The protein samples were added to wells, leading to antigen binding to the capture antibody. For the primary detection step the polyclonal rabbit-anti- $\alpha/\beta/\gamma$ -synuclein FL-140 antibody was used. For secondary detection a monoclonal goat-anti-rabbit antibody conjugated to *horseradish peroxidase* (HRP), was added and interaction was detected by addition of HRP substrate. The absorbance of the reaction product was measured at 450 nm. In **Paper II** and **Paper III**, α -synuclein oligomer ELISA was also used to detect oligomeric forms of α -synuclein. The generated oligomer-selective antibody 38F11 was used as capture and primary detection antibody (Figure 14 B). The primary detection variant was coupled to biotin and secondary detection was carried out by adding streptavidin linked to HRP. This setup excludes the detection of monomers as only species that have more than one accessible epitope for 38F11 will be recognized [196].

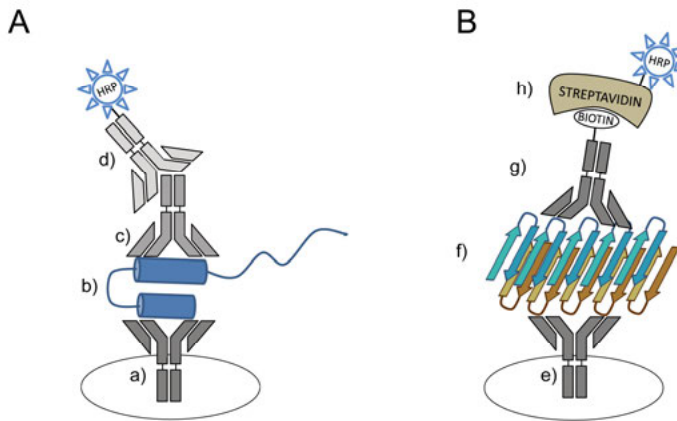


Figure 14. Alpha-synuclein Sandwich ELISA. **A)** Total levels of α -synuclein are detected with Sandwich ELISA using linear epitope antibodies. The **a)** capture antibody mAb1338 binds to **b)** α -synuclein irrespective of conformation and **c)** the polyclonal rabbit-anti-synuclein FL-140 works as primary detection antibody. Secondary detection is carried out with **d)** a HRP-tagged anti-rabbit antibody. **B)** The α -synuclein oligomer ELISA is based on oligomer-selective **e)** mAb38F11 as a capture antibody, recognizing **f)** oligomeric α -synuclein. Biotinylated **g)** mAb38F11 works as primary detection and **h)** streptavidin-linked HRP is added for secondary detection.

Western blot analysis (Paper I, II, IV)

In **Paper I**, Western blot was used to measure extracellular levels of α -synuclein. In **Paper II**, Western blot analysis was used to study cellular levels of lysosomal marker LAMP-1 as well as internalized α -synuclein oligomers in neural co-cultures exposed to HNE-induced α -synuclein oligomers. In **Paper IV**, Western blot was used to measure enrichment of markers for extracellular vesicles. Samples were first denatured by heat and loaded onto a SDS-polyacrylamide gel (SDS-PAGE). Denatured proteins bound to SDS become negatively charged, allowing separation with respect to molecular weight. Applying an electrical voltage allows the proteins to migrate over the gel. Proteins with low molecular weight migrate faster through the grid of PAGE matrix, whereas high molecular weight proteins migrate slowly. After the migration, the gel was laid over a transfer membrane in a transfer device. Electrical voltage was applied in order to have the protein content transferred from the gel to the membrane. Primary detection antibodies were then used to label proteins in the membrane. Horseradish peroxidase-linked secondary antibodies were used and the protein bands were detected with a chemiluminescence system. The detection gives a quantitative measure of the amount of protein loaded on the gel. The method provides information about molecular weight by using a protein molecular weight standard. The HNE-induced α -synuclein oligomers were not completely denatured in SDS

and are therefore seen as different oligomeric or monomeric bands or a smear on the membrane (**Paper II**).

Immunoprecipitation (Paper II, III)

To measure the normally low levels of Drp1, immunoprecipitation of the lysates was carried out prior to Western blotting (**Paper II**). This method concentrates the antigen of interest prior to the SDS-PAGE step. First, the lysate was incubated with antibody directed towards Drp1, allowing antibody:Drp1 complex formation. Protein G-coupled magnetic beads were then linked to the antibodies by protein G binding to the Fc region of IgG:s. The beads were immobilized to the wall of the tube by placing the tube next to a magnet. The remaining sample could then be removed, leaving Drp1 bound to antibody and the magnetic beads. After washing, complexes were dissociated by heat denaturation as mentioned above and the sample containing free Drp1 was loaded on SDS-PAGE. In **Paper III**, immunoprecipitation was used to pull down complexes of treatment antibodies and α -synuclein oligomers from conditioned media, prior to Western blotting (Figure 15). In this experiment, conditioned media were thawed and directly incubated with Protein G-coupled magnetic beads, allowing pulldown of antibody: α -synuclein complexes. Heat denaturation allowed elution and Western blot analysis of both treatment antibodies and α -synuclein.

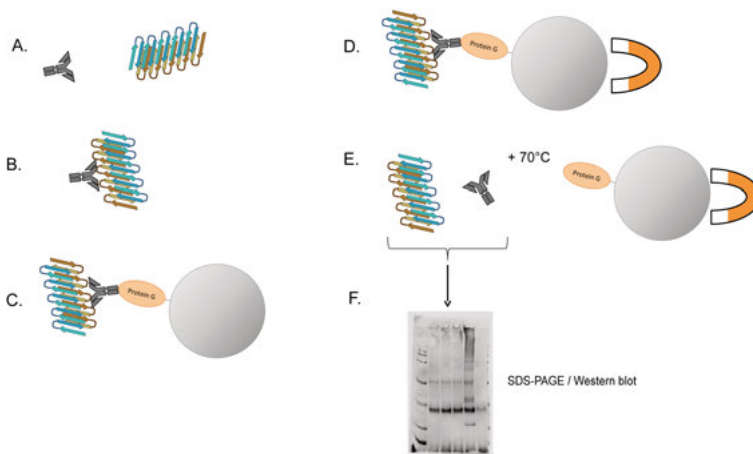


Figure 15. Immunoprecipitation of antibody: α -synuclein complexes. **A)** Prior to addition to cells, α -synuclein oligomer selective antibodies were allowed to form **B)** complexes with α -synuclein oligomers in regular growth media. **C)** Addition of protein G-coupled magnetic beads allowed binding of protein G to the antibodies. **D)** By placing samples next to a magnet, antibody: α -synuclein complexes bound to beads were immobilized and washed. **E)** Heat denaturation dissociated the protein complex and α -synuclein and antibodies were eluted in sample buffer. **F)** The concentrated samples were analysed with Western blot using α -synuclein antibodies.

Transmission electron microscopy (TEM) (Paper II, IV)

In **Paper II** neural co-cultures treated with HNE-induced α -synuclein oligomers were analysed with transmission electron microscopy to study the mitochondrial morphology. The method provides high resolution images of subcellular structures at nanometer scale [197]. Samples fixed in glutaraldehyde were incubated with osmium tetroxide in cacodylate buffer before dehydration in a series of increasing ethanol concentrations. Plastic was used to rinse the surface and three layers of plastic were applied before polymerization at 60 °C for 48 h. Images were taken with 50 000x magnification. In **Paper IV**, extracellular vesicles from human neuroblastoma cells were studied with TEM. Cell media were collected from cells after 24 h of incubation and immediately fixed in 2.5 % glutaraldehyde for 3 days. As a control, petri dishes with no cells containing regular growth media were incubated for 24 h. Solutions were then filtered through 0.45 μ m syringe filters and ultracentrifuged according to the isolation protocol. Both centrifugation pellets and supernatants were collected and stained with uranyl acetate prior to analysis.

QPCR quantification of mitochondrial DNA (Paper II)

In **Paper II**, mitochondrial DNA was isolated from cells exposed to HNE-induced α -synuclein oligomers. A quantitative polymerase chain reaction (QPCR) based assay was used to analyse the relative number of DNA lesions in order to assess mitochondrial damage [198, 199]. Cell lysates were collected and mitochondrial DNA was isolated by centrifugation at 10 000x g for 20 min. The pellet was frozen at -20 °C and analysed with QPCR. The fraction of DNA lesions per kilobase was calculated for both HNE-oligomer treated and non-treated cells.

ATP luminescence assay (Paper II)

In **Paper II**, total ATP levels were measured in co-cultures treated with HNE-induced α -synuclein oligomers for 24 h. A luminescent ATP detection assay based on luciferase was used [200]. As a negative control, the mitochondrial inhibitor Antimycin A [201] was incubated with cells for 2 h prior to measurement. Cells were lysed by addition of detergent prior to addition of a mixture of the enzyme luciferase and its substrate. The ATP dependent substrate conversion by luciferase and generated luminescence was measured with a plate reader. The relative ATP levels were then calculated and compared to untreated control cells.

Populärvetenskaplig sammanfattning

Parkinsons sjukdom är den näst vanligaste neurodegenerativa sjukdomen efter Alzheimers sjukdom. I Sverige lever idag ca 20 000 personer med denna diagnos. De klassiska symptomen vid Parkinsons sjukdom är stelhet, skakningar och rörelsearmod, men vid senare stadier utvecklas ofta demens. Sjukdomen utbryter vanligtvis efter 50 års ålder, men ärftliga former kan debutera tidigare än så. De motoriska symptomen beror på brist av signalsubstansen dopamin i hjärnan som i sin tur orsakas av att de dopaminproducerande nervcellerna skadas och slutligen dör. Sjukdomen är kronisk och fortfarande finns ingen behandling som bromsar eller stoppar förloppet. Genom att tillsätta L-DOPA går det dock att mildra symptomen. Genetiskt nedärvda former av Parkinsons sjukdom förekommer hos upp till 10 % av patienterna medan man i övriga fall inte känner till vad som orsakat sjukdomen. Parkinsonshjärnan kännetecknas nästan alltid av inlagringar av proteinet alfa-synuklein i vissa av hjärnans celler. Dessa s.k. Lewykroppar uppträder i de drabbade områdena och tidigare trodde man att dessa orsakade själva celldöden men på senare tid har forskning visat att små förstadier till Lewykropparna, s.k. alfa-synuklein oligomerer, har en mer skadlig inverkan. Alfa-synuklein bildas framförallt i nervceller och förefaller ha en roll vid nervsignalerings. Under aggregeringen antar proteinet en felveckad struktur och friska, funktionella former som kommer i kontakt med oligomerer kan i sin tur ombildas till skadliga, felveckade, former. Som en strategi för behandling av Parkinsons sjukdom skulle man därför behöva förhindra förekomsten av oligomerer och samtidigt bevara de funktionella formerna av alfa-synuklein. Behandling av musmodeller för sjukdomen med antikroppar som är selektiva för alfa-synuklein oligomerer har påvisat minskade symptom och lägre nivåer av sådana skadliga proteinformer. För att undersöka mekanismerna bakom behandlingseffekterna har antikroppsbehandling även studerats på cellmodeller.

I denna avhandling har effekterna av alfa-synuklein oligomerer och antikroppsbehandling studerats på cellnivå. I det första arbetet användes en särskild form av alfa-synuklein, som möjliggör detektion av oligomerer i levande celler med fluorescensmikroskopi. Vi kunde påvisa att det cellulära upptaget av tillsatta behandlingsantikroppar ökades i närvaro av alfa-synuklein oligomerer. Detta upptag ökade speciellt när alfa-synuklein fanns tillgängligt i cellernas tillväxtmedium. Den antikropp som tidigare gett positiva behandlingseffekter i musmodellen visade sig här ha det högsta upptaget

i celler jämfört med andra antikroppar. Vidare gick det att påvisa att vissa immunreceptorer på cellerna bidrog till upptaget av antikroppar, vilket skulle kunna leda till nedbrytning av antikroppsbundna oligomerer. I det andra arbetet studerades ansamling av alfa-synuklein oligomerer i cellkulturer bestående av nervceller och gliaceller (hjärnans stödjeceller). Den största inlagringen sågs i astrocyter, en typ av gliacell som dessutom är den vanligaste celltypen i den mänskliga hjärnan. Dessa celler skyddar nervceller och reglerar de inflammatoriska processer som har visat sig vara inblandade vid Parkinsons sjukdom. Astrocyterna började bryta ner oligomererna men en överbelastning av nedbrytningsmaskineriet ledde till en försämrad funktion hos cellernas mitokondrier och därmed till minskade energinivåer. I det tredje arbetet applicerades de oligomeraselektiva antikropparna på samma cellsystem och en markant minskning av både alfa-synuklein inlagringar och mitokondriestress i astrocyterna uppnåddes i närvaro av antikroppar. Dessutom hade antikropparna en utrensande effekt av alfa-synuklein även i cellmediet, vilket tyder på att astrocyterna lättare kunde bryta ner de skadliga oligomererna med hjälp av antikropparna. Resultaten tyder på att astrocyter har en viktig roll vid alfa-synuklein patologi och att inlagringarna orsakar cellstress som i längden kan leda till nervcellsdöd. Således kan ansamling av alfa-synuklein oligomerer i astrocyter vara en relevant process att rikta framtida behandlingsstrategier emot.

En rad studier visar att spridningen av skadligt alfa-synuklein i hjärnan sker från cell till cell och att alfa-synuklein utsöndras från nervceller och glia. Extracellulära vesikler är en slags vektor för biomolekyler som RNA, fettsyror och protein. I tidigare studier har man påvisat att sjuklig spridning av alfa-synuklein medierats via sådana strukturer. I det fjärde delarbetet undersökte vi på odlade celler hur olika varianter av alfa-synuklein processas och fördelas till vesikler. Genom centrifugering av cellmediet vid höga varvtal anrikades vesikler och nivåerna av alfa-synuklein i dem jämfördes med nivåerna av alfa-synuklein som förekom fritt utanför cellerna. När vi lät cellerna tillverka alfa-synuklein som ett hybridprotein tillsammans med en del av ett annat protein ökades fördelningen till vesikler jämfört med när cellerna tillverkade fysiologiskt alfa-synuklein. En liknande omfördelning sågs när vi lät cellerna tillverka alfa-synuklein med den sjukdomsorsakande mutationen A53T. Dessa data indikerar att en rubbad fysiologisk funktion hos alfa-synuklein eller en förändrad benägenhet att aggregera ökar utsöndringen via extracellulära vesikler och att en sådan ökning kan bidra till utvecklingen av Parkinsons sjukdom.

Sammanfattningsvis har vi kunnat påvisa att upptag och spridning av alfa-synuklein till både nervceller och astrocyter kan ha en viktig roll i sjukdomsförloppet vid Parkinsons sjukdom och andra sjukdomar med alfa-synuklein patologi. Våra resultat tyder också på att immunterapi mot alfa-synuklein kan påverka olika patologiska processer i båda dessa celltyper.

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A handwritten signature in black ink, appearing to be 'Sven Allgulander', written in a cursive style.

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