Studies of α-synuclein Oligomers-
with Relevance to Lewy Body Disorders

THERESE FAGERQVIST
The protein alpha-synuclein (α-synuclein) accumulates in the brain in disorders such as Parkinson’s disease (PD) and dementia with Lewy bodies (DLB). It is believed that the monomeric form of α-synuclein can adopt a partially folded structure and start to aggregate and form intermediately sized oligomers or protofibrils. The aggregation process can continue with the formation of insoluble fibrils, which are deposited as Lewy bodies. The oligomers/protofibrils have been shown to be toxic to neurons and are therefore believed to be involved in the pathogenesis of the actual diseases.

The overall aims of this thesis were to investigate the properties of α-synuclein oligomers and to generate and characterize antibodies against these species. In addition, the potential for immunotherapy of the α-synuclein oligomer-selective antibodies were evaluated in a transgenic mouse model with α-synuclein pathology.

Stable, β-sheet rich α-synuclein oligomers were induced by incubation with either one of the reactive aldehydes 4-hydroxy-2-nonenal (HNE) and 4-oxo-2-nonenal (ONE). The oligomers exhibited distinct morphological properties, although both types were toxic when added to a neuroblastoma cell line. The seeding effects of ONE-induced oligomers were studied in vitro and in vivo. The oligomers induced seeding of monomeric α-synuclein in a fibrillization assay but not in a cell model or when injected intracerebrally in transgenic mice. It seemed, however, as if the oligomers affected α-synuclein turnover in the cell model.

By immunizing mice with HNE-induced oligomers antibody producing hybridomas were generated. Three monoclonal antibodies were found to have strong selectivity for α-synuclein oligomers. These antibodies recognized Lewy body pathology in brains from patients with PD and DLB as well as inclusions in the brain from young α-synuclein transgenic mice, but did not bind to other amyloidogenic proteins. Finally, immunotherapy with one of the oligomer/protofibril selective antibodies resulted in lower levels of such α-synuclein species in the spinal cord of α-synuclein transgenic mice.

To conclude, this thesis has focused on characterizing properties of α-synuclein oligomers. In particular, antibodies selectively targeting such neurotoxic forms were generated and evaluated for passive immunization in a transgenic mouse model. Such immunotherapy may represent a future treatment strategy against Lewy body disorders.

Keywords: Alpha-synuclein, Parkinson’s disease, Lewy body dementia, Oligomer, Monoclonal antibody, Immunotherapy, Reactive aldehydes

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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.


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# Contents

## Introduction

- Parkinson’s disease ................................................................. 11
- Lewy bodies and Lewy neurites ............................................... 13
- Structure and genetics of α-synuclein ......................................... 14
- Beta- and gamma-synuclein .................................................... 17
- Alpha-synuclein aggregation .................................................... 18
- Proteinase K-resistant α-synuclein pathology ............................ 20
- Synaptic dysfunction related to α-synuclein .............................. 21
- Alpha-synuclein degradation .................................................... 21
- Alpha-synuclein and mitochondrial dysfunction ....................... 23
- Extracellular α-synuclein ........................................................... 23
- Oxidative stress and reactive aldehydes ................................... 24
- Animal models with α-synuclein pathology .............................. 26
- Transmission of α-synuclein pathology .................................... 27
- Seeding of prions, amyloid-beta and tau ................................. 29
- Alpha-synuclein immunotherapy .............................................. 30

## Aims

- Overall aim .............................................................................. 33
- Specific aims ........................................................................... 33

## Results and discussion

- Biochemical and seeding characteristics of aldehyde induced α-
  synuclein oligomers ................................................................. 34
- Properties of α-synuclein oligomer-selective antibodies and
  immunotherapy in an α-synuclein transgenic mouse model ....... 41

## Concluding remarks ................................................................. 49

## Method considerations .............................................................. 51

- Alpha-synuclein transgenic mice (paper II, III and IV) ............... 51
- Atomic force microscopy (paper I and II) ................................ 51
- Bimolecular fluorescence complementation assay (paper II) .... 52
- Circular dichroism (paper I) ...................................................... 53
- ELISA (paper I, III and IV) ....................................................... 53
- Fibrillization assay using Thioflavin-T (paper I and II) ............. 54
## Abbreviations

<table>
<thead>
<tr>
<th>Abbreviation</th>
<th>Description</th>
</tr>
</thead>
<tbody>
<tr>
<td>Aβ</td>
<td>Amyloid-beta</td>
</tr>
<tr>
<td>AFM</td>
<td>Atomic force microscopy</td>
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<td>APP</td>
<td>Amyloid precursor protein</td>
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<td>α-synuclein</td>
<td>Alpha-synuclein</td>
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<td>β-synuclein</td>
<td>Beta-synuclein</td>
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<tr>
<td>BBB</td>
<td>Blood-brain-barrier</td>
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<td>BSCB</td>
<td>Blood-spinal cord-barrier</td>
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<td>BSE</td>
<td>Bovine spongiform encephalopathy</td>
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<td>CD</td>
<td>Circular dichroism</td>
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<tr>
<td>CHIP</td>
<td>Carboxy terminus of Hsp70-interacting protein</td>
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<td>CJD</td>
<td>Creutzfeldt-Jacob disease</td>
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<tr>
<td>CNS</td>
<td>Central nervous system</td>
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<tr>
<td>CSF</td>
<td>Cerebrospinal fluid</td>
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<tr>
<td>DJ-1</td>
<td>Parkinson’s disease protein 7</td>
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<tr>
<td>DLB</td>
<td>Dementia with Lewy bodies</td>
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<td>ENS</td>
<td>Enteric nervous system</td>
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<tr>
<td>ER</td>
<td>Endoplasmic reticulum</td>
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<tr>
<td>γ-synuclein</td>
<td>Gamma-synuclein</td>
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<tr>
<td>GBA</td>
<td>Glucocerebrosidase</td>
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<tr>
<td>HNE</td>
<td>4-hydroxy-2-nonenal</td>
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<tr>
<td>LB</td>
<td>Lewy body</td>
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<tr>
<td>LBV-AD</td>
<td>Lewy body variant of Alzheimer’s disease</td>
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<td>LDH</td>
<td>Lactate dehydrogenase</td>
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<td>LN</td>
<td>Lewy neurite</td>
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<td>LRRK2</td>
<td>Leucine-rich repeat kinase 2</td>
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<tr>
<td>LTD</td>
<td>Long-term depression</td>
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<td>LTP</td>
<td>Long-term potentiation</td>
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<tr>
<td>MMP3</td>
<td>Matrix metalloproteinase 3</td>
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<tr>
<td>MPTP</td>
<td>1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine</td>
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<tr>
<td>MSA</td>
<td>Multiple system atrophy</td>
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<tr>
<td>NAC</td>
<td>Non-amyloid component</td>
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<tr>
<td>6-OHDA</td>
<td>6-hydroxydopamine</td>
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<td>ONE</td>
<td>4-oxo-2-nonenal</td>
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<tr>
<td>Abbreviation</td>
<td>Full Form</td>
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<tr>
<td>--------------</td>
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<tr>
<td>PD</td>
<td>Parkinson’s disease</td>
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<tr>
<td>PINK1</td>
<td>Serine/threonine-protein kinase 1</td>
</tr>
<tr>
<td>PK-PET-blot</td>
<td>Proteinase K paraffin-embedded-tissue blot</td>
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<td>PVDF</td>
<td>Polyvinylidene fluoride</td>
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<td>PRKN</td>
<td>Parkin</td>
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<td>ROS</td>
<td>Reactive oxygen species</td>
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<tr>
<td>SDS-PAGE</td>
<td>Sodium dodecyl sulfate polyacrylamide</td>
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<tr>
<td>SEC-HPLC</td>
<td>Size exclusion-high performance liquid chromatography</td>
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<tr>
<td>SNARE</td>
<td>Soluble NSF-attachment protein receptor</td>
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<td>SNCA</td>
<td>Alpha-synuclein</td>
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<tr>
<td>Th-T</td>
<td>Thioflavin-T</td>
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<tr>
<td>TSEs</td>
<td>Transmissible spongiform encephalopathies</td>
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<tr>
<td>UPDRS</td>
<td>Unified Parkinson’s disease Rating Scale</td>
</tr>
<tr>
<td>VAMP-2</td>
<td>Vesicle-associated membrane protein 2</td>
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Introduction

Neurodegenerative disorders, such as Parkinson’s disease (PD) and dementia with Lewy bodies (DLB), are becoming increasingly prevalent due to longer life expectancy, which gives rise to new medical and social challenges. Consequently, the estimated costs for the care of elderly people with neurodegenerative diseases are rapidly increasing. Since there are still no treatments that can cure these diseases, preventive strategies may be considered. Cognitive, physical and social activity together with a healthy diet has been proposed as preventive strategies although they have not proven successful in controlled trials [1].

Parkinson’s disease

Parkinson’s disease is the most common movement disorder affecting about 1-2% of the population older than 60 years or about 6 million people around the world [2]. The age of onset of PD is variable but often between 55-65 years. The disease was first described by James Parkinson in 1817 in “An assay on the shaking palsy” [3]. In the prodromal phase, before onset of motor symptoms, patients can experience constipation, depression and olfactory symptoms [4]. Later, the classical symptoms of PD, as rigidity, resting tremor and hypokinesia become evident. In addition, disturbed cognition is often seen and dementia may occur at the end stages of the disease course in 30-80% of affected individuals [5, 6]. Most cases of PD are idiopathic, but rare familial forms have been identified. As for environmental factors, exposure to various pesticides have been suggested [7]. The major risk factor is age which is also the best predictor for progression rate [8].

The disease severity is measured with the Unified Parkinson’s Disease Rating Scale (UPDRS). This scale contains four domains; I: mood and cognition, II: activities of daily living, III: motor symptom severity and IV: complications of treatment [9]. Positron emission tomography is sometimes employed for the diagnosis and monitoring of the disease progression of PD. \(^{18}\)F-FDOPA uptake in dopaminergic nerve terminals correlates with nigral dopamine cell numbers and striatal dopamine concentration. The binding activity of \(^{18}\)F-FDOPA is decreased in patients with PD [10].
Treatment with levodopa, a prodrug to dopamine, is used to increase dopamine concentrations in the brain. However, levodopa treatment is only symptomatic and is efficient only for a limited time. Also, the side effects of levodopa are often disturbing with motor fluctuations and dyskinesias [11]. Dopamin D2 agonists can also be used to increase the levels of dopamine in the brain [12].

Neuropathologically, PD is characterized by a loss of dopaminergic neurons in the substantia nigra, which consequently leads to a deficiency in striatal dopamine. It has been proposed that when typical PD symptoms begin at least 50% of the neurons in the substantia nigra are already lost [13]. The cell death could arise from mitochondrial- or endoplasmic reticulum (ER) dysfunction and oxidative stress, malfunction of the protein degradation system or aggregation/overexpression of the protein alpha-synuclein (α-synuclein) [14-16].

Other genes and their proteins that have been associated with PD or parkinsonism are PRKN (Parkin), PINK1 (Serine/threonine-protein kinase), DJ-1, LRRK2 (leucine-rich repeat kinase 2) and GBA (glucocerebrosidase) [17-21].

In addition, PD involves several other brain regions such as the brainstem and cerebral cortex, hence affecting also neurons with neurotransmitters such as noradrenaline, serotonin and acetylcholine [22]. Alpha-synuclein pathology in the brainstem is thought to be responsible for motor symptoms and cognitive impairment has been attributed to pathology in limbic and neocortical areas [23]. A staging system of α-synuclein pathology was suggested including early involvement of the brainstem, olfactory nucleus and peripheral and central autonomic neurons with progression via midbrain, forebrain, limbic cortex and finally to the neocortex [24].

Detection of α-synuclein in the enteric nervous system (ENS) in the gastro-intestinal tract as a biomarker for PD has recently been suggested. In one study α-synuclein pathology could be detected in submucosal plexus in the gastrointestinal tract of PD patients and it was observed that the pathology is more prominent in the upper part of the gastro-intestinal tract [25]. Interestingly, α-synuclein aggregates have been found in colon biopsies a few years before PD diagnosis [26]. These studies propose that peripheral abnormal protein aggregation occurs long before motor features become noticeable. In wild-type mice, intragastrically administered rotenone induced α-synuclein accumulation in the ENS, the dorsal motor nucleus of the vagus, spinal cord and substantia nigra, the same pathological staging as is found in PD patients [27]. All of these structures are primarily or secondarily connected to the ENS [28]. In the same mouse model the resection of some of the connecting nerves between the ENS and the central nervous system (CNS) delayed the appearance of motor symptoms and stopped the progression of α-synuclein pathology into the CNS [28]. Braak and colleagues reported that α-synuclein pathology, in the brain, starts in the
dorsal motor nucleus before the pathology occur in the *substantia nigra* [29]. Dorsal motor nucleus fibers innervate the gut and therefore they hypothesized that the PD pathology could originate from the gastro-intestinal nerve terminals [29]. Associated observations indicate that α-synuclein pathology in the myenteric plexus in the stomach are associated with postoperative delirium in patients undergoing gastrectomy, indicating a possible link to α-synuclein neurodegenerative disorders [30]. Also, α-synuclein deposits were found in sympathetic nerve fibers in skin biopsies from patients with pure autonomic failure, but not in healthy controls [31].

Dementia with Lewy bodies and multiple system atrophy

Dementia with Lewy bodies is the second most common cause of dementia after Alzheimer’s disease and 15-25 % of elderly demented patients have DLB pathology [32]. The patients suffer from cognitive failure, parkinsonism, postural instability, visual hallucinations and the symptoms are often fluctuating and related to α-synuclein pathology. In addition to the classical symptoms, the clinical criteria for DLB include REM sleep behaviour disorders, severe neuroleptic sensitivity and reduced striatal dopamine transporter activity. In most cases the disease occurs sporadically and genetic causes are rare. There are no pharmacological treatments aimed specifically at DLB associated symptoms, although dopamine therapy and cholinesterase inhibitors can be effective. The age of onset is between 65-80 years. Patients with DLB have a loss of a wide range of different neurons, including acetylcholine and dopamine producing cells [33].

According to the pathologic guidelines of DLB there are five types of the disease based on the localization of the Lewy body pathology; olfactory bulb only, brainstem predominant, limbic predominant, brainstem as well as limbic and finally neocortical [34]. In addition, concomitant Alzheimer’s disease related pathology is relatively often seen in DLB patients [24]. Remarkably, among elderly subjects with widespread Lewy body pathology, more than 50 % of the patients that did not have Alzheimer’s disease related pathology were cognitively unimpaired. Interestingly, 50-60 % of Alzheimer’s disease patients also have some DLB pathology [32].

In patients with the Lewy body variant of Alzheimer’s disease (LBV-AD) similar symptoms as DLB patients can be observed together with impaired short-term memory and other Alzheimer’s disease features. At post mortem examination α-synuclein pathology in both midbrain and cortical areas are observed [35].

In addition, multiple system atrophy is also associated with deposition of filamentous glial cytoplasmic inclusions of α-synuclein with neuronal loss in
some parts of the CNS, for example cerebellum and spinal cord [36, 37]. The disease is characterized by autonomic dysfunction, cerebellar ataxia or parkinsonism [38].

Lewy bodies and Lewy neurites

In 1912 the German neurologist Friedrich Lewy (1885—1950) first described protein inclusions, which later became known as Lewy bodies (LB:s) in brains from PD patients [39]. Filamentous intracellular protein inclusions in neuronal cell bodies, LB:s, and in neuronal processes, dystrophic Lewy neurites (LN:s), consist mainly of the protein α-synuclein and are the pathological hallmarks of DLB [33] and PD [29] (figure 1). Interestingly, the number of LB:s does not correlate well with either the duration or the severity of dementia in DLB patients [40]. Moreover, many elderly subjects have widespread LB pathology but reveal no neuropsychiatric symptoms [32]. In PD, it has been suggested that approximately 3-4% of neurons in substantia nigra contain LB:s, a number which is relatively constant regardless of disease stage [41].

In the toxin based MPTP (1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine) animal model the mice display motor symptoms and neurodegeneration in substantia nigra without obvious LB pathology [42]. Interestingly, although no LB pathology is present, α-synuclein was required for the MPTP-induced neurodegeneration, as shown by the resistance of α-synuclein knock-out mice to MPTP intoxication [43].

Lewy bodies occur in two types; brainstem and cortical. The classic brainstem LB:s are spherical intraneuronal inclusions between 8-30 µm in diameter with a densely packed core and radially arranged filaments in the periphery [44]. The cortical LB:s are poorly organized rounded structures which are composed of 7-27 nm wide filaments. Furthermore, “pale bodies” are rounded areas of granular, pale-staining eosinophilic material that are considered to be the precursor of LB:s [45]. The α-synuclein found in LB:s have been reported to be misfolded, phosphorylated and nitrosylated [45]. The LB:s and LN:s typically contain full-length and C-terminally truncated α-synuclein [46]. In addition to α-synuclein, about 300 other proteins have been found in LB:s [47]. Some of these proteins are ubiquitin, phosphorylated neurofilaments, chaperones and lipids [47-49].

The biological role of LB:s is not clear; are they detrimental, disturbing the normal cellular functions or do they represent an end stage of a mechanism to eliminate toxic proteins [41, 50]? There are a few reports suggesting that neurons containing LB:s are morphologically healthier than adjacent neurons [51]. In LN:s α-synuclein is accumulated in the axons and can damage the neuron by interfering with axonal transport. It has been suggested that LB:s are continuously formed during the disease and
disappear when the LB bearing neurons die, and that the lifespan of a LB is approximately six months [41].

Figure 1. Lewy body pathology (arrow) in a brain tissue section, mesencephalon, from a PD patient immunohistochemically stained with an α-synuclein antibody. Magnification 20x.

Structure and genetics of α-synuclein

Alpha-synuclein is mainly a synaptic protein expressed in neurons in the neocortex, hippocampus, substantia nigra, thalamus and cerebellum [52] but is also present in red blood cells [53]. It was first cloned from the neuromuscular junction of an electric eel [54] and later α-synuclein was identified as the non-amyloid component (NAC) purified from Alzheimer’s disease plaques. It was described as a peptide derived from a larger precursor, which now is known as α-synuclein [55]. Little is known about α-synuclein’s normal physiological function, but it has been suggested to be involved in the regulation of dopamine release [56] and later it has been discovered that α-synuclein is involved in SNARE (soluble N-ethylmaleimide-sensitive factor attachment protein receptor) complex assembly [57]. The gene encoding α-synuclein, SNCA, is located in the 4q21 region [58].

Alpha-synuclein is a natively unfolded protein of 140 amino acids. The protein consists of three regions; the N-terminal region, (residues 1-60) with four 11-amino acid imperfect repeats, the NAC region (residues 61-95),
which readily forms amyloid-like fibrils *in vitro* and are toxic to cells [59] and the C-terminal region (residues 96-140), which is rich in acidic residues and prolines [45] (*figure 2*).

The N-terminal part of α-synuclein interacts with cellular membranes (lipids) [60] and thereby adopts a highly helical conformation [61]. The structure of α-synuclein is sensitive to its surroundings and can easily be modulated by changes in its environment. The negatively charged and hydrophilic C-terminal of α-synuclein gives the protein its natively unstructured form [62].

The N-terminal region include the sites of the five familial mutations; A53T (Ala to Thr), A30P (Ala to Pro), E46K (Glu to Lys), H50Q (His to Gln) and G51D (Gly to Asp) (*figure 2*). The A53T mutation carriers have the earliest-onset parkinsonism (47 ± 11 years) often associated with cognitive decline and dementia [63, 64]. The A30P substitution is involved in later-onset parkinsonism (60 ± 11 years) with mild dementia [65, 66]. The A30P and A53T mutations have been shown to accelerate the formation of α-synuclein oligomers (soluble intermediates species) *in vitro* [67]. Furthermore, the E46K mutation leads to early-onset DLB [68]. The H50Q mutation has been described in one patient with parkinsonism showing a combination of motor problems and dementia [69]. This mutation is located between the E46K and A53T mutations and is likely to disrupt the same amphipathic α-helical structure, thereby suggesting that the disease mechanism may be similar for these mutations [69]. The G51D mutation carriers have early onset PD with a rapid progression [70, 71].

In addition, duplications and triplications of the *SNCA* gene have been described to cause PD or DLB [72, 73]. The phenotype in *SNCA* multiplications is dose-dependent: triplication carriers develop fulminant parkinsonism-dementia early, whereas duplication carriers manifest later-onset PD, DLB or multiple system atrophy [74]. Finally, two genome-wide association studies have demonstrated a significant association of *SNCA* with idiopathic PD [75, 76].
Figure 2. 3D structure of human α-synuclein bound to vesicles. The amino-terminal part of α-synuclein (shown in red) binds to cellular membranes and adopts a helical conformation. It also contains the sites of the five familial mutations A30P, E46K, H50Q, G51D and A53T. The middle part (shown in blue), the NAC region, consists of hydrophobic residues and is believed to promote aggregation. Finally, the carboxy-terminal part (shown in green) is negatively charged and hydrophilic. (Adopted from UniProtKB/SwissProt. Original structural data provided by [77]).

Beta- and gamma-synuclein

In addition to α-synuclein, there are two other proteins in the synuclein family. Human β- and γ-synuclein are 78 % and 60 % identical to α-synuclein. Beta-synuclein has the properties of a random coil, while α- and γ-synuclein are slightly more structured and compact. Both α- and γ-synuclein form fibrils while β-synuclein does not [78]. When β- or γ-synuclein are added to α-synuclein in vitro in a 1:1 ratio the aggregation is substantially delayed [78].

In animal models, β-synuclein has also been shown to inhibit α-synuclein aggregation [79]. Two mutations in the β-synuclein gene have been described in DLB patients [80]. These patients had widespread LB pathology and biochemically there was evidence for α-synuclein, but not β-synuclein, aggregation. Transgenic mice expressing one of these β-synuclein mutations developed progressive neurodegeneration, and cross-breeding with α-synuclein transgenic mice caused an enhanced phenotype [81]. In contrast to α-synuclein, the C-terminal of γ-synuclein can not bind to the proteins synaptobrevin-2/VAMP2 and can therefore not potentiate SNARE complex formation [82]. Patients with DLB were reported to express more α-synuclein and less β-synuclein compared to healthy controls [83]. This ratio could be critical for the pathological α-synuclein aggregation.
Alpha-synuclein aggregation

The aggregation of α-synuclein is believed to start with the monomer adopting a partially folded conformation, which leads to self-aggregation and formation of soluble intermediate species. Such species, commonly referred to as oligomers or protofibrils, may recruit more folded protein which results in the formation of insoluble fibrils [84, 85]. The term oligomer refers to smaller aggregates that can either be on their way of forming fibrils, i.e. on-pathway oligomers, or are locked in a conformation that does not permit further aggregation and formation of fibrils, i.e. off-pathway oligomers. Protofibrils are larger aggregates that are always on the way to forming fibrils [86, 87] (figure 3). In vitro, α-synuclein fibrillization is believed to be a nucleation-dependent polymerization process, which includes a lag phase where nuclei are built up, followed by a growth or elongation phase where aggregates grow rapidly [84]. Finally a steady state phase is reached with a thermodynamic equilibrium between the fibril and the monomer [88]. It is widely believed that native α-synuclein exists predominantly as a disordered monomer, but recently it was suggested that the native state of α-synuclein monomers is a helix rich tetramer [89, 90]. However, this was then counterclaimed by other studies showing that α-synuclein from human CNS, red blood cells and E. coli existed as a disordered monomer [91, 92]. The most consistent factor influencing α-synuclein aggregation is molecular crowding and α-synuclein concentration; the higher concentration the more likely α-synuclein is to form aggregates [93]. The aggregation of α-synuclein can also be influenced by post-translational modifications. For instance, phosphorylation at serine 129 has been shown to promote aggregation, [94, 95] but nitration or ubiquitination of α-synuclein has been associated with reduced aggregation [96, 97].
Figure 3. Schematic illustration of α-synuclein aggregation. The natively unfolded monomer can adopt a partially folded conformation and this induces aggregation which leads to the formation of off-pathway oligomers and/or on-pathway oligomers/protofibrils. The on-pathway species can continue to aggregate into insoluble fibrils.

One line of evidence suggests that it is the oligomers rather than the fibrils of α-synuclein that are the disease causing form of α-synuclein [67, 98-100]. Alpha-synuclein oligomers can cause cell death [99] both intracellularly and in the extracellular space [101]. Among suggested theories for the mechanism of α-synuclein oligomer toxicity is that these protein forms could create pores in cellular membranes, resulting in ion influx [98] or to induce neuroinflammation [102]. Another mechanism whereby α-synuclein oligomers can cause toxicity is by disrupting the cellular protein homeostasis. In a bacterial system, α-synuclein oligomers inhibited chaperone function [103] and oligomers have also been shown to impair the chymotrypsin-like protease activity of the proteasome in vitro [104]. In transgenic α-synuclein mice it has been shown that oligomers can form and accumulate within the ER and thereby cause ER stress within the neuron [14]. Evidence for the accumulation of α-synuclein oligomers in the ER compartment from brainstem samples of PD patients creates a possible link between chronic ER stress and neurodegeneration [105].

Several different forms of oligomers have been described and they can e.g. adopt ring-forms and spheres [100, 106]. Oligomerization of α-synuclein, into off-pathway oligomers, can be promoted by several chemical modifiers such as the reactive aldehydes 4-hydroxy-2-nonenal (HNE) and 4-oxo-2-nonenal (ONE) [107], dopamine and its metabolites [108], baicalein [109], and epigallocatechin gallate [110]. In contrast, one study reported that HNE-induced oligomers can act as seeds and accelerate the fibrillation of monomeric α-synuclein and was therefore described as on-pathway oligomers [111]. The small molecule curcumin, found in the spice turmeric,
has been found to bind with high affinity to α-synuclein and promoted a conformation with a reduced propensity to aggregate [112].

Alpha-synuclein oligomers were found in brains of patients with triplications of the α-synuclein gene [113] and the A30P α-synuclein mutation has been suggested to accelerate oligomerization [67]. Moreover, elevated levels of soluble oligomers were detected in brain extracts from DLB patients compared to control brains [114]. Alpha-synuclein oligomers may be relevant to pathology also in transgenic mice. In mice expressing wild-type or A53T mutant α-synuclein, neurodegeneration could be observed also without fibrillar inclusions [115]. Therefore, halting the fibrillization process at early stages could possibly result in an increased oligomer formation, which could cause harm rather than cure.

The subcellular distribution of oligomers/protofibrils has been investigated in two different mouse models expressing A53T or A30P α-synuclein. The oligomers/protofibrils were present in the ER, nucleus and mitochondria fractions and the accumulation of oligomers/protofibrils in ER seemed to be related to the onset of behavioural disturbances in the mouse model [105, 116].

Proteinase K-resistant α-synuclein pathology

Given the severity of symptoms in patients with PD and DLB, the density of LBs is relatively low. It has therefore been suggested that they may not account entirely for the symptoms [51]. With the PK-PET-blot method (proteinase K paraffin-embedded-tissue blot), treatment with proteinase K is used to reveal more α-synuclein pathology compared to standard immunohistochemical methods [94]. Proteinase K is a stable serine protease with broad substrate specificity which digests soluble α-synuclein without affecting the pathological insoluble α-synuclein [117]. The use of proteinase K was first applied in the prion field for the detection of the scrapie prion [118, 119].

With PK-PET-blot a larger number of small α-synuclein aggregates can be detected in DLB brain [120]. Also for PD, a much more intense staining has been described in the substantia nigra and in the striatum with PK-PET-blot compared to standard immunohistochemical methods [94]. One can speculate that the proteinase K-resistant α-synuclein pathology may be a much better disease correlate and even represent the underlying cause of the symptoms in the actual disorders [120].

Alpha-synuclein forms proteinase K-resistant fibrils, whereas the non-fibrillogenic β-synuclein, lacking the NAC domain, does not. The NAC fragment of α-synuclein is the least accessible region for proteinase K digestion in α-synuclein aggregates [121] and the N-terminus appears to be more accessible then the C-terminal [94].
Synaptic dysfunction related to α-synuclein

The two main forms of synaptic plasticity in the brain are long-term potentiation (LTP) and long-term depression (LTD). The striatum plays an essential role in regulation of voluntary movement, behavioural control, cognitive function and reward mechanisms. In PD the dopaminergic neurons projecting to the nucleus striatum are degenerated and the synaptic plasticity is impaired, which has been proposed to lead to the impairment of adaptive motor control and procedural memory seen in PD patients [122, 123]. Moreover, proteinase K-resistant α-synuclein has been shown to accumulate in presynaptic terminals in human DLB cases [120]. The effect of α-synuclein oligomers on synaptic transmission and LTP, the molecular basis of learning and memory, was studied in rat hippocampal slices [124]. Slices treated with oligomers, but not those treated with monomers or fibrils, displayed increased basal synaptic transmission and impaired LTP [124].

Alpha-synuclein normally localizes to the presynaptic terminals but accumulates in cell bodies, neuronal processes and synapses in the diseased brain [125]. Synapses from α-synuclein overexpressing mice displayed a decrease in neurotransmitter exocytosis and endocytosis was in some synapses diminished or undetectable [126]. Their synaptic vesicles were enlarged and highly variable in size, whereas normal vesicles are usually small and relatively uniform [126]. There is little or no difference between wild-type and α-synuclein knock-out mice in terms of neurotransmitter release, implying that β-synuclein or γ-synuclein can replace α-synuclein [127].

Soluble NSF-attachment protein receptors (SNAREs) are usually thought to mediate intracellular membrane fusion. In some animal models lacking synaptic vesicular proteins, such as certain SNAREs, a reduced spontaneous synaptic response and deficits in neurotransmitter release have been observed [128]. In mice overexpressing α-synuclein the synapses lost some endogenous synaptic vesicular proteins likely caused by the sustained α-synuclein overexpression [126]. It has been suggested that α-synuclein directly binds to the SNARE-proteins synaptobrevin-2/vesicle-associated membrane protein 2 (VAMP-2) and promote SNARE-complex assembly [57]. Also, mice lacking all three synucleins (α-, β- and γ-synuclein) exhibited age-dependent neurological impairments and decreased SNARE-complex assembly [57].

Alpha-synuclein degradation

The ubiquitin-proteasome system and the autophagy-lysosome pathway are the most important ways for the cell to remove or repair misfolded or abnormal intracellular proteins [129]. In the ubiquitin-proteasome system mis-
folded proteins are polyubiquitinated at lysine residues which leads to their transfer to and degradation by the proteasome. Lysosomes are organelles within the cell that contain a variety of proteases whose activity maintains the turnover of membrane proteins and other macromolecules. Lysosomal malfunction has been associated with PD as well as other neurodegenerative disorders [129].

In a cell culture study, intracellular α-synuclein was proposed to be degraded via both the proteasomal and the lysosomal pathway, possibly directed by the C-terminus of Hsp70-interacting protein (CHIP) [130]. Alpha-synuclein monomers (secreted extracellularly) have been proposed to be degraded by extracellular proteases such as matrix metalloproteinase 3 (MMP3) [131]. Intracellular α-synuclein oligomers were suggested to be degraded by the 26S proteasome but were at the same time inhibiting proteasomal activity [132]. On the contrary, in another study with extracellularly added oligomeric and fibrillar forms of α-synuclein, both protein forms were taken up by endocytosis and were degraded in the lysosomal pathway [133]. The role of Cathepsin D, a lysosomal protease, in relation to α-synuclein has been investigated in a cell- and mouse model. Cathepsin D deficient mice displayed reduced levels of soluble endogenous α-synuclein, but elevated levels of insoluble aggregates. In contrast, overexpression of Cathepsin D in a dopaminergic cell line effectively degraded α-synuclein [134]. It has also been described that mutations in GBA1 gene, a genetic risk factor for PD and DLB, promotes α-synuclein accumulation in a cell culture model [135].

Cultured microglia incubated with α-synuclein oligomers caused the microglia to adopt an amoeboid phenotype characteristic of activated microglia [102]. Fibrillar α-synuclein caused only a slight activation of the microglia and β-synuclein had no effect on the microglia [102]. Alpha-synuclein oligomers injected into the brain of rats caused a clear microglial activation and a loss of dopaminergic neurons [102]. When the microglial activation was inhibited the neuronal cell death was reduced [102]. It has been suggested that a constant, long term activation of microglia is contributing to the neurodegeneration seen in various disorders such as Alzheimer’s disease, [136] which could also be relevant in PD and DLB pathogenesis.

Importantly, in brain tissue from sporadic PD cases the immunoreactivity of both lysosomal and proteasomal markers were reduced when compared to age matched controls [137]. In the aged matched controls, non-aggregated α-synuclein could be detected but there were no differences in the degradation markers between α-synuclein positive or negative neurons [137, 138]. These alterations in the degradation systems may account for the elevated levels of α-synuclein and, at least partly, for the neuronal loss seen in sporadic PD cases.
Alpha-synuclein and mitochondrial dysfunction

Events that can act synergistically to cause neuronal death are mitochondrial dysfunction, alterations in the ubiquitin-proteasomal system and oxidative stress. Progressive mitochondrial dysfunction leads to a loss of ATP, lower calcium buffering capacity and an increase in oxidative stress [139]. Mitochondria can also regulate apoptotic cell death. The N-terminal part of α-synuclein possibly contains mitochondria targeting like signals [139] and α-synuclein has been found inside the mitochondria in brains of humans and rodents [139-141]. In one study, elevated levels of α-synuclein were found inside mitochondria from PD patients compared to neurologically healthy controls [139]. Moreover, in post mortem PD brain a deficiency of the mitochondria complex I was discovered. A defect in mitochondria complex I leads to production of reactive oxygen species (ROS) [142]. In a conditional knock-out mouse model with disruption of mitochondrial functions there was evidence for progressive degeneration of dopaminergic neurons and impairment of motor functions [143]. An inhibitor of mitochondrial complex I, rotenone, causes PD like symptoms in rats [144].

Extracellular α-synuclein

There are currently no good biochemical biomarkers for PD and DLB available. Generally, a biomarker is of great importance to confirm a diagnosis, assess the progression rate of the disease and monitor drug effects in clinical trials. Several studies have reported lower levels of total α-synuclein in cerebrospinal fluid (CSF) from PD patients [145-147] and also lower levels of neurosin, a protease that degrades α-synuclein in vitro [148]. Alpha-synuclein oligomers were recently found to be elevated in CSF from PD patients compared to healthy controls and disease controls (patients with Alzheimer’s disease or progressive supranuclear palsy) [149-151]. In one of these studies, the levels of total α-synuclein were decreased in PD patients implying that the elevated levels of oligomers were not entirely caused by an overall increase in the expression of α-synuclein [150].

In yet other studies, PD patients were found to have elevated levels of α-synuclein oligomers in plasma [152] but decreased levels of total α-synuclein [153]. In LRRK2 mutation carriers the plasma levels of total α-synuclein were not significantly different compared to healthy controls [153]. The levels of α-synuclein in plasma were reported to show gender-related differences [154] and correlation analyses of clinical parameters with α-synuclein CSF levels have shown inconsistent results [145, 147].

In studies on DLB patients, the levels of total α-synuclein in CSF have generally been lower compared to the levels in patients with Alzheimer’s disease and other dementias or healthy controls [146, 148, 155, 156].
another study, different results were obtained, here the levels of α-synuclein in PD, DLB and control subjects were similar whereas Alzheimer’s disease patients had lower levels of α-synuclein in CSF compared to healthy controls [157]. Interestingly, in patients with a duplication of the SNCA gene and abundant α-synuclein pathology in the brain the levels of CSF α-synuclein were also reduced [155].

Additionally, it has been shown that the levels of α-synuclein in CSF does not fluctuate significantly over time [158] and drugs like L-dopa and dopamine agonists also do not affect the levels [156, 159]. The predominant source for α-synuclein in the CSF is considered to be the CNS [160].

The decrease of α-synuclein in CSF in patients could be due to a dysfunction in the metabolism or clearance of the protein in the brain. Another possible explanation could be that the α-synuclein aggregation is enhanced in patients, yielding lower total levels of α-synuclein which would be consistent with the elevated levels of oligomeric species found in PD patients.

Because the LB pathology is intracellular, α-synuclein has in the past been considered an exclusively intracellular protein. However, it has been shown that α-synuclein, both monomeric and aggregated forms, can be secreted from cells via ER/Golgi-independent exocytosis [161-163]. This exocytosis has been suggested to be dependent on intracellular calcium levels and increase under certain stress conditions, such as heat shock and serum deprivation [161, 163]. When treating cells overexpressing α-synuclein with HNE, a reactive aldehyde, α-synuclein translocated from the cytosol to vesicles and increased amounts of α-synuclein were released from the cells [111]. In a co-culture system the effect of HNE on cell-to-cell transfer of α-synuclein were investigated. The transferred α-synuclein increased almost two-fold when the cells were subjected to HNE [111].

**Oxidative stress and reactive aldehydes**

The brain is sensitive to ROS and this vulnerability could be explained by it’s high use of oxygen, low antioxidant defences and high content of polyunsaturated lipids [164]. Alpha-synuclein has been proven to have affinity for unsaturated- and polyunsaturated fatty acids, [165] which are sensitive to oxidative stress. The production of ROS has been suggested to be involved in a number of neurodegenerative diseases, such as PD [166]. Dopaminergic neurons are thought to be particularly prone to oxidative stress because of dopamine’s ability to participate in oxidative reactions [167]. Reactive oxygen species induce peroxidation of lipids (polyunsaturated fatty acids), which generates reactive aldehydes [168].

Interestingly, lipid peroxidation has been reported to be enhanced in the substantia nigra of PD patients [168, 169] which may influence the
The reactive aldehydes HNE and ONE (figure 4) are products of lipid peroxidation [168, 170] and HNE has been implied in the pathogenesis of PD [166]. These aldehydes may also be involved in other neurodegenerative diseases, in some regions of Alzheimer’s disease brain HNE levels were found to be increased [171]. HNE is present in normal brain tissue at low micromolar concentrations, but in pathological conditions it can increase up to 5 mM [172].

Figure 4. Chemical structures of the reactive aldehydes 4-oxo-2-nonenal (ONE) and 4-hydroxy-2-nonenal (HNE).

In vitro, co-incubation of α-synuclein and HNE or ONE was shown to lead to formation of stable oligomers [106, 107, 172, 173]. It has been described that HNE and ONE form adducts to cysteine, histidine and lysine [107]. Alpha-synuclein has one histidine and 15 lysine residues [173] and HNE modifies histidine 50, lysine 60 and lysine 96 [111]. Conflicting results on the extent of β-sheet structure in the oligomers formed by HNE co-incubation has been reported [107, 111, 173].

It has been shown that both HNE and ONE in them self induces apoptosis [174] and furthermore, HNE is neurotoxic by causing impairment of glutamate transport and mitochondrial function [175] whereas ONE has been implied to be even more toxic than HNE in vitro [176]. Both HNE- and ONE-induced oligomers reduced the cell viability compared to monomeric α-synuclein when added to neuroblastoma cells [107]. In PD brain HNE is colocalized with LB:s [177]. Both ONE and HNE can act locally or enter the general circulation [178]. Interestingly, α-synuclein aggregation in the membrane fraction, where HNE concentrations would be relatively high, was reduced by treatment with antioxidants, highlighting the importance of oxidative modifications [179].

In general, an increase in oxidative stress can lead to lipid peroxidation which generates HNE and other reactive aldehydes that can modify α-synuclein. This process can result in increased oligomerization and secretion which in turn may facilitate seeding in recipient cells. By preventing lipid peroxidation the abnormal modification and aggregation of α-synuclein could perhaps be regulated [111]. Furthermore, using aldehydes to create α-synuclein oligomers generates stable conformations that can be useful in many experimental settings.
Animal models with α-synuclein pathology

The first animal model of PD was generated in 1968 by Dr. Ungerstedt who demonstrated that injection of 6-hydroxydopamine (6-OHDA) into the striatum or substantia nigra generated a depletion of dopamine in these areas [180]. For PD there are two different kinds of animal models; toxin-based (MPTP, 6-OHDA and rotenone) and genetic models (α-synuclein, LRRK2, PINK1, DJ-1 and the “MitoPark” mouse with mitochondrial dysfunction) [181]. Currently there is no animal model that can fully recapitulate all symptoms and the pathology in PD or DLB. A good animal model of PD should be age dependent and show progressive pathology with neuronal loss in the substantia nigra along with the presence of oligomers/protofibrils, LB:s and LN:s. Also, the model should display motor dysfunction and cognitive impairment. The most common mouse models are based on α-synuclein overexpression.

Studying familial PD in mouse models can lead to insights into sporadic PD since LB:s and LN:s are present in both forms of PD. The phenotype of the α-synuclein overexpression is largely determined by which promoters that has been used, for example the Thy-1-, Prion-, PDGFβ- and tyrosine hydroxylase-promoter. None of the existing transgenic models have a progressive loss of dopamine neurons. One mouse model has a loss of nigrostriatal dopamine neurons, but it occurs during embryogenesis [182].

All promoters drive the expression of α-synuclein in neurons with very little expression in glia [183]. Alpha-synuclein viral models drive the expression in both neurons and glia and such models have been described to cause neurodegeneration of dopaminergic neurons [184, 185].

The (Thy-1)-h[A30P] α-synuclein transgenic mice have a robust expression of the mutated human α-synuclein throughout the brain, which is roughly twofold compared to the endogenous α-synuclein expression [186]. This mouse model develops age-dependent impairment in fear conditioning behaviour that coincides with the appearance of phosphorylated α-synuclein (Ser-129) pathology [187]. It has been suggested that phosphorylation of Ser-129 of α-synuclein promotes fibrillization and ubiquitinylation [95]. When the mice are 17 months old they have impaired locomotor capabilities compared to wild-type mice [188]. In the brainstem and spinal cord the α-synuclein pathology is also stained with Thioflavin-S, which represent a more dense structure of α-synuclein pathology [187]. Staining that resembles dystrophic neurites similar to those found in human DLB brain is also present. Moreover, from twelve months of age the mice develop proteinase K-resistant pathology, predominantly in the brainstem, midbrain and spinal cord. The proteinase K-resistant α-synuclein is also phosphorylated at Ser-129. The same brain areas also show evidence of astrogliosis. The substantia nigra, however, is relatively spared from pathology, which may be due to the
fact that the Thy-1 promoter used in this model does not express the transgene at high levels in that area [94].

Biochemically, the mice develop measurable levels of \( \alpha \)-synuclein oligomers/protofibrils already at three months of age and this increase as the mice age. At 15 months of age some mice have SDS resistant aggregates in both brain- and spinal cord fractions [116].

No neuronal cell loss has been observed for this model. The dopamine content in homozygous (Thy-1)-h [A30P] \( \alpha \)-synuclein transgenic mice was not significantly different from wild-type mice [94]. The mice die prematurely at an average age of 540±96 days [188]. This mouse model has been utilized in the work related to this thesis.

Transmission of \( \alpha \)-synuclein pathology

A number of patients suffering from PD have received brain transplants of embryonic mesencephalic neurons. The clinical outcome has been relatively modest, although the grafts have survived for up to 16 years and were found to contain neuromelanin, a marker of nigral neurons [189-191]. A double-blind, sham-controlled study did not find any clinical benefit of the transplantation but in a subpopulation of patients with milder disease severity there was a significant improvement [192]. Two studies on post mortem brains demonstrated that \( \alpha \)-synuclein pathology was present in the grafts and it was suggested that the pathology had spread from the host to the grafted neurons [189, 190]. Both LB:s and LN:s were present in the grafts and the LB:s were similar to those found in the host tissue with ubiquitin, phosphorylated proteins and Thioflavin-S positivity. The transplanted patients that survived 11-16 years after surgery had developed pathology in the grafts but patients that lived only four or nine years did not [189, 190]. This could mean that it takes at least a decade for healthy neurons to develop LB:s and LN:s. However, all transplanted patients did not demonstrate \( \alpha \)-synuclein pathology in the grafts [191]. Moreover, the pathology seemed to be progressive: one patient received transplants 16 and four years (different hemispheres) before death, and the older transplant was found to contain more LB:s. These findings might be important for understanding the progression of pathology in the brains of PD patients. Many mechanisms, including inflammation, oxidative stress, loss of growth factors and protein transfer between cells have been proposed to generate LB:s in the grafted neurons [193]. The LB:s could either develop as a consequence of protein transmission from the host or arise primarily in the transplant.

The transmission of \( \alpha \)-synuclein pathology in a transgenic mouse model was recently explored. Young (2-5 months) asymptomatic A53T \( \alpha \)-synuclein overexpressing mice were intracerebrally injected in the neocortex and striatum with either brain homogenate from older, symptomatic mice, or
with preformed α-synuclein fibrils [194]. This single inoculation resulted in limited formation of LB/LN pathology close to the injection sites 30 days after injection. After 90 days post injection, abundant α-synuclein pathology was detected throughout the CNS, indicating a time dependent increase and spread of the pathology [194]. Inoculation with brain homogenate or fibrils induced identical consequences and although the injection was unilateral the α-synuclein deposits were distributed bilaterally. Regions that were directly or indirectly connected to the sites of injection (frontal cortex and thalamus) developed the most prominent α-synuclein pathology, which suggests that the pathology propagates most readily between associated neurons. Also substantia nigra, which provides innervation to the striatum, displayed accumulation of hyperphosphorylated α-synuclein [194]. Interestingly, the pathology had also spread to areas that do not share direct innervation with the injection sites, e.g. the brainstem, indicating that transsynaptic spreading also might be involved in the propagation of LB pathology. The occurrence of pathology was associated with a dramatic reduction in survival and the appearance of motoric symptoms [194].

In a related study, the effects of intracerebrally injected α-synuclein fibrils in wild-type mice were investigated [195]. When fibrils assembled from recombinant mouse α-synuclein were injected into the striatum, LB- and LN-like pathology appeared in several interconnected brain regions, including the substantia nigra. The α-synuclein pathology co-localized with markers for ubiquitin and Thioflavin-S, indicating that these inclusions resemble authentic LB:s in human PD. In contrast, no α-synuclein pathology could be observed when wild-type mice were injected with either monomeric α-synuclein or PBS [195]. Alpha-synuclein pathology in the substantia nigra was accompanied by dopamine neuronal cell loss. Norepinephrine and serotonergic neuronal populations remained unaffected, suggesting that they are not equally susceptible to the spread of α-synuclein pathology. The injected mice displayed deteriorated motor coordination and balance [195].

In cell culture models α-synuclein can spread from overexpressing cells to acceptor cells [196]. After prolonged incubation, inclusion bodies, similar to human LB:s, were formed in the acceptor cells. It was suggested that the transmission of α-synuclein occurs via the endocytic pathway. When neuronal stem cells were injected into α-synuclein transgenic mice the cells were quickly infiltrated with α-synuclein from surrounding cells [196]. It has also been proposed that α-synuclein oligomers can induce transmembrane α-synuclein seeding in vitro, in a time- and dose dependent manner [197]. In cultured primary neurons from wild-type mice it was described that exogenously added α-synuclein fibrils can enter the neuron and promote recruitment of endogenous α-synuclein leading to the formation of insoluble PD-like pathology [198]. First, LN-like pathology developed in the axons and then propagated to form LB-like aggregates in perikarya. The
accumulation of α-synuclein leads to impairments in neuronal excitability and connectivity and later neuronal cell death [198].

Based on these observations it has been discussed whether α-synuclein behaves like a prion and that PD in fact might be a prion disorder. The reason for this is that both α-synuclein and the prion protein (PrP\(^C\)) normally adopts a α-helical rich conformation under physiological conditions, and both are capable of refolding into a β-sheet rich structures which facilitates the aggregation into fibrils or rods, respectively, that can eventually form LB:s or amyloid plaques. In addition, both types of protein aggregates have been shown to promote the misfolding of additional proteins [199-201].

Seeding of prions, amyloid-beta and tau

In this context, seeding implicates that various protein species can accelerate the rate of aggregation and/or increase the amount of aggregated protein.

Seeding or transmission of pathology by proteins was first observed in the field of prions. Examples of prion diseases, also called transmissible spongiform encephalopathies (TSEs), are kuru and Creutzfeldt-Jakob disease (CJD) in humans, spongiform encephalopathy (BSE) in cattle and scrapie in sheep. These unusual disorders may arise either spontaneously, via inheritance (prion protein mutations), or by infection. The spreading of disease by infection has drawn interest, especially because of the outbreak of BSE (‘mad cow’ disease) in the United Kingdom and indications that BSE has likely been transmitted to humans, resulting in a new variant of CJD (vCJD) [202]. The brains of diseased individuals display characteristic spongiform degeneration, astrogliosis and accumulation of misfolded protein [202]. These diseases are associated with a conformational conversion of the cellular prion protein, PrP\(^C\), into a β-sheet rich form, PrP\(^Sc\), which alone can act as an infectious agent. PrP\(^Sc\) is believed to propagate by binding to PrP\(^C\) and acting as a template to force its refolding into the harmful PrP\(^Sc\) form [203]. Interestingly, inoculations of diseased brain material into individuals of the same species will reproduce the disease [203]. Moreover, it has been demonstrated that PrP-deficient mice are resistant to inoculation by the PrP\(^Sc\) form, which established a link between the prion protein and TSE pathogenesis [204].

Amyloid-beta (Aβ) is the central pathological protein in Alzheimer’s disease and in vivo seeding of Aβ pathology was seen when brain homogenate from an Alzheimer’s disease patient and transgenic amyloid precursor protein (APP) mice were injected into young transgenic APP mice [205, 206]. The induced pathology appeared after two to five months and was similar to what is normally observed in older transgenic APP mice. Some of the induced Aβ pathology was surrounded by activated microglia and astrocytes. If the homogenates were immunodepleted of Aβ or
denatured, the seeding was completely prevented. However, injection of only synthetic soluble, oligomeric or fibrillar Aβ did not produce any detectable seeding [205]. Recently it was shown that intraperitoneal inoculation with transgenic APP mice brain extract into transgenic APP mice induced Aβ pathology in the brains of the mice [207].

In the case of the microtubule-associated protein tau, which is also involved in Alzheimer’s disease, brain homogenate from a transgenic tau mouse that develops filamentous tau aggregates was injected into the brain of transgenic wild-type tau-expressing mice that normally do not have tau inclusions [208]. The injection induced tau to aggregate and the pathology spread from the injection site to other brain regions. The induced pathology was present intracellularly 6, 12 and 15 months after injection. There were no signs of astrogliosis or inflammation in the injected mice. Homogenates that contained predominantly insoluble tau species induced more tau pathology compared to homogenates with mostly soluble tau. No neuronal loss was observed in the injected mice suggesting that transmission and toxicity are not caused by the same tau species [208].

**Alpha-synuclein immunotherapy**

There are two forms of immunization; active and passive. Active immunization (vaccination) stimulates the immune system to produce antibodies against the injected protein species. Passive immunization, on the other hand, is based on the direct administration of antibodies generated against a specific protein form. Active immunization has proven to be effective in many mouse models of neurodegenerative diseases, by reducing Aβ [209], tau [210], PrP [211] and huntingtin [212].

In Alzheimer’s disease, both passive treatment with antibodies directed against Aβ [213-215] and active [216] vaccination has proven successful in transgenic APP mice. The first immunotherapy study on humans, with active Aβ vaccination (AN1792), was prematurely stopped when 6 % of treated patients developed meningoencephalitis. However, *post mortem* examination on almost a dozen patients have revealed decreased plaque burden in selected brain areas and some patients had slower rates of decline of cognitive functions [217, 218]. Ongoing studies, mainly based on passive immunization, will show whether Aβ immunotherapy will be successful in halting the disease.

Amyloid-beta is a secreted protein and the pathology is mainly extracellular and could therefore be readily recognized by antibodies. Alpha-synuclein is an intracellular protein, but it has recently been described that different forms of α-synuclein accumulate in the cell membrane and can be excreted [196, 219, 220]. This has provided a rationale for immunotherapy directed against α-synuclein pathology.
One active immunization study has been conducted in α-synuclein transgenic mice [221]. The mice were vaccinated with recombinant human α-synuclein and showed a reduction in α-synuclein accumulation in cell bodies and synapses accompanied by a reduced neurodegeneration [221]. The antibodies produced by the mice with higher affinity were directed against the C-terminal of α-synuclein. In this study the immunization did not trigger an inflammatory response. This aspect is important since inflammatory reactions, such as vasculitis and autoimmune responses, is a frequently reported side effect in active immunization studies [222]. A different approach has been investigated in rats receiving vaccination with recombinant α-synuclein before stereotactic delivery of an adeno-associated viral vector overexpressing α-synuclein [223]. This resulted in a reduced number of α-synuclein aggregates and activated microglia. It was suggested that the vaccination resulted in a induction of regulatory T-cells [223]. Recently a phase I clinical trial of a PD vaccine was initiated evaluating a peptide-carrier conjugate consisting of a short peptide designed not to induce a T-cell response [224] (ClinicalTrials.gov, NCT01568099). This conjugate has been shown to reduce the levels of α-synuclein pathology in two different mouse models of α-synucleinopathies.

After the promising results obtained with active immunization, passive immunization studies were initiated to design a protocol that potentially could reduce or avoid the side effects seen with active immunization but still retain the beneficial effects on α-synuclein clearance. The first passive immunization study in transgenic mice, expressing human α-synuclein under the control of the promoter PDGFβ, resulted in ameliorated motor and learning deficits using an α-synuclein antibody (IgG1) directed against the C-terminal of the protein. The antibody treatment also lowered the levels of calpain-cleaved α-synuclein aggregates in the neocortex and hippocampus and it did not perturb the microvasculature [225]. Furthermore, the antibody was fluorescently labelled and it was proven that it could traffic into the CNS and bind to α-synuclein [225]. In the second in vivo study the focus was to elucidate the possible mechanisms by which immunization prevents α-synuclein related deficits [226]. Here, an IgG_{2a} C-terminal targeting antibody was used in an α-synuclein transgenic mouse model that at the start of treatment already displayed α-synuclein pathology. After four weeks of treatment the mice performed similarly to non-transgenic mice in a functional motor coordination test and displayed a considerable reduction (30-80 %) of α-synuclein pathology both in astroglia and in neurons. Importantly, the treatment prevented neuronal loss and reduced synaptic deficits normally seen in this mouse model [226]. The antibody assisted clearance of α-synuclein was suggested to occur mainly in microglia mediated by the Fc\(\gamma\) receptor by facilitating the delivery to lysosomes for degradation [226]. The Fc\(\gamma\) receptors are located on the cell surface and bind to the Fc domain of IgG antibodies which triggers a range of immune
responses, including phagocytosis, release of inflammatory mediators and clearance of immune complexes [227].

So called intrabodies, or intracellular antibodies, are antibodies expressed inside cells by transfection with plasmids or infection with viruses [228]. Three scFv (single-chain variable fragment) antibodies binding α-synuclein have been described to inhibit the aggregation of α-synuclein and reducing toxicity in cells [229-231].

Immunotherapy might be most successful if the treatment is initiated before or in the earlier stages of the disease thereby preventing or slowing the progression of the pathology [232]. Preventive immunization could be protective by limiting the build-up of α-synuclein aggregates that might act as seeds for more protein deposition. The studies on passive immunization suggest two different scenarios for antibody-mediated clearance of α-synuclein. First, it is possible that the antibodies could recognize abnormal intracellular α-synuclein accumulating in the plasma membrane within glial or neuronal cells and thereby stimulate degradation via autophagy [179, 225]. The other alternative would be to use antibodies targeting extracellular α-synuclein after it has been released upon cell death or while propagating between cells [226].

Naturally occurring autoantibodies belong to the innate immune system which means that they are generated without specific immunization and exposure to foreign antigens. Such antibodies usually have a low affinity but a broad specificity against both foreign- and self-structures [233]. Different forms of α-synuclein are released into the CSF and plasma, both in PD and DLB patients as well as in healthy control individuals, [150, 152] which may lead to an induction of autoantibodies. In one study investigating the prevalence of α-synuclein autoantibodies it was discovered that the presence of such antibodies in plasma was more common in familial- (90 %) and sporadic PD (51 %) compared to control sera (31 %) [234]. Two more recent studies have reported conflicting results; as measured by ELISA, both higher and lower levels of anti-α-synuclein autoantibodies in plasma from PD patients as compared to healthy controls or Alzheimer’s disease patients were reported [235, 236].
Aims

Overall aim
The general aim of this thesis was to study the formation and properties of α-synuclein oligomers, induced by reactive aldehydes, and the seeding capabilities of such oligomers both in vitro and in vivo. In addition, the objective was to generate and characterize antibodies selective for α-synuclein oligomers/protofibrils and investigate such antibodies for immunotherapy in a transgenic mouse model.

Specific aims
I To study the biochemical, morphological and functional properties of HNE- and ONE-induced α-synuclein oligomers.

II To investigate the aggregation properties of the ONE-induced oligomers in a fibrillization assay, in a cell model and in transgenic mice.

III To develop and characterize α-synuclein oligomer-selective antibodies.

IV To explore the potential of the oligomer-selective antibodies to reduce levels of oligomers/protofibrils in transgenic mice overexpressing α-synuclein.
Results and discussion

Biochemical and seeding characteristics of aldehyde induced α-synuclein oligomers

HNE-modified proteins have been found in brainstem and cortical LB:s in both PD and DLB cases [177, 237]. The highest intracellular HNE concentrations can be found close to the cellular membrane where also the initial stages of α-synuclein aggregation has been suggested to take place [168]. However, the pathological significance of ONE- and HNE-induced α-synuclein oligomers is not clear and they have not yet been described in diseased brain tissue. Oligomers can either be on their way of forming fibrils, i.e. on-pathway oligomers, or be locked in a conformation that does not allow fibrils to be formed, i.e. off-pathway oligomers.

In paper I, HNE or ONE was co-incubated with α-synuclein which generated two different types of oligomers. Upon size exclusion-high performance liquid chromatography (SEC-HPLC) analysis, both species eluted with a molecular weight of ~2000 kDa. In comparison, monomeric α-synuclein eluted as a single peak corresponding to a molecular weight of ~50 kDa (figure 5) (paper I, fig. 1). The discrepancy between the molecular weight of α-synuclein calculated by the sequence determined mass (14460 kDa) and by SEC-HPLC can be explained by α-synucleins unfolded tertiary structure. Almost no monomeric protein could be detected in neither of the oligomeric samples, indicative of a complete conversion from monomers to oligomers (paper I, fig. 1).
Figure 5. SEC-HPLC showing the retention time of ONE-induced oligomers (A), HNE-induced oligomers (B) and monomeric α-synuclein (C). Both types of oligomers eluted at approximately 20 minutes corresponding to a molecular weight of ~2000 kDa. The monomers eluted with a corresponding weight of ~50 kDa.

As determined by far-UV CD spectra both oligomer types exhibited a negative minimum absorption wavelength near 217 nm corresponding to a high degree of β-sheet structure (paper I, fig. 2). The oligomers share some common properties but when analyzing the ONE- and HNE-induced oligomers with atomic force microscopy (AFM) a clear difference in morphology could be observed. The ONE-induced oligomers had a round and amorphous appearance, 4-8 nm in height and a diameter of 40-80 nm (paper I, fig. 3 and paper II, fig. 1). In contrast, the HNE-induced oligomers displayed a more elongated, protofibrillar structure with a size ranging from 2-4 nm in height and 100-200 nm in length, as determined by line profile image analysis (paper I, fig. 3). HNE and ONE have similar chemical structures except that ONE has a carbonyl group instead of a hydroxyl group at the C4 position. This chemical difference makes ONE a more potent cross linker, which could explain the different morphologies of the resulting oligomers [176]. As comparison, native monomeric α-synuclein appeared as homogenous round structures with a diameter of 20-30 nm and a height of 1-2 nm. Aggregated α-synuclein were also analyzed, displaying amyloid-like structures, 30 nm in width and 200-1000 nm in length (paper I, fig. 3).

The stability of the generated oligomers was evaluated using SDS- and urea denaturing conditions and treatment with proteinase K. When analyzed with SDS-PAGE ONE-induced oligomers could be observed as a high molecular weight smear exceeding 350 kDa (paper I, fig. 5A). HNE-induced oligomers dissociated under denaturing conditions and appeared as a wide range of both high and low molecular weight species (paper I, fig. 5B). HNE-induced oligomers were also more sensitive to increasing concentrations of urea (paper I, fig. 5E). This urea sensitivity is in line with the fact that ONE is a better chemical cross linker than HNE, yielding more covalent bonds. In contrast, the HNE-induced oligomers were more resistant to proteinase K-treatment compared to ONE-induced oligomers (paper I, fig.
5A, B and D). These results provided some insight into the structural features of the oligomers, which indicated that a part of the HNE-induced oligomer is more compact and less accessible to proteinase K digestion.

In paper I and II the aggregation and seeding properties of HNE- and ONE-induced oligomers were investigated with a fibrillization assay using Thioflavin-T (Th-T). Firstly, when incubating the oligomers separately they did not continue to form fibrils in comparison to the monomeric \( \alpha \)-synuclein, which generated a large increase in Th-T signal after ten days of incubation (paper I, fig. 4). The fact that the oligomers did not form fibrils indicates that both oligomer types are off-pathway species. In another study the HNE-oligomers were described as on-pathway oligomers since they were described to induce seeding [111]. In a separate fibrillization assay the ability of ONE-induced oligomers to induce seeding was studied. The addition of 1 % ONE-induced oligomers to monomeric \( \alpha \)-synuclein resulted in an earlier start of the aggregation process compared to addition of 1 % fibrils or no seed, but the final Th-T signal after 96 h was lower (figure 6) (paper II, fig. 2A). This indicates that ONE-induced oligomers could promote the aggregation of \( \alpha \)-synuclein, at least \textit{in vitro}. The resulting fibrils were then analyzed with AFM and the fibrils formed with addition of ONE-induced oligomers were found to be smaller and thinner compared to the other fibrils (paper II, fig. 2B), which explains the lower Th-T signal. Normally, off-pathway oligomers do not induce seeding or form fibrils. Our ONE-oligomers do not continue to form fibrils but surprisingly they showed a seeding effect.
Figure 6. Fibrillization assay using Th-T. The addition of 1 % ONE- induced oligomers (2) to α-synuclein monomers gave rise to an earlier start of aggregation (at 24 h) but showed a lower final Th-T signal compared to when either no seeds (1) or 1 % fibrillar seeds where added (3).

The cellular uptake of the oligomers was studied in a neuroblastoma cell line (SH-SY5Y). The two oligomer types and monomeric α-synuclein were labeled with a fluorescent tag that enabled detection inside the cell. ONE-induced oligomers were taken up after four hours and were mainly located close to the nucleus (paper I, fig. 8C). In contrast, the HNE-induced oligomers were taken up already after one hour of incubation and the signal increased over time. The HNE-induced oligomers had a slightly different distribution in the cell, located throughout the cell soma and close to the nuclei (paper I, fig. 8D, E and F). One could speculate that the different localizations could induce different effects on the cell and that the oligomers might be processed differently in the cell.

To study the effect of the oligomers in cells a MTT assay (2-(4,5- dimethylthiazol-2-yl)) was used, measuring cell viability. In the MTT assay the neuroblastoma cells was treated with 0.05-50 nM of ONE-, HNE-induced oligomers and monomers for 24 h. At 50 nM both types of oligomers generated a significant reduction in cell viability compared to monomers (paper I, fig. 7). How the oligomers exhibit their toxic effect remains to be elucidated, but some discussed mechanisms involves disruption of the cellular membrane causing an influx of Ca$^{2+}$ [98] or via neuroinflammation [102].
To further characterize the properties of the ONE-induced oligomers a BiFC (bimolecular-fluorescence complementation) assay was used to follow the effect on the formation of α-synuclein dimers/oligomers in a neuroblastoma cell line in the presence of ONE-induced oligomers. In this assay, the Venus molecule (modified YFP molecule) is split in half and linked to full-length α-synuclein. When two or more α-synuclein molecules associate with each other the Venus halves reconstitute and starts to fluoresce [99] (method considerations p. 52). When ONE-induced oligomers were added to the cells for four hours and the dimerization/oligomerization of α-synuclein was measured after 16, 24 and 40 h a reduction in Venus signal could be observed at all time points compared to addition of monomeric α-synuclein or PBS (figure 7) (paper II, fig. 3A-M).

Figure 7. BiFC assay measuring the dimerization/oligomerization of α-synuclein with Venus fluorescence in a cell model. When ONE-induced oligomers (A-C) were added to the cells there was a reduction in Venus signal compared to cells treated with α-synuclein monomers (E-G) or PBS (I-K) at all time points up to 40 h. The number of cell nuclei was counted using DAPI-staining and was similar between the groups. D, H, L shows magnification of cells from respective treatment group (40 h). Scale bars 50 µm.
The decreased fluorescence was not due to reduced cell growth since the total number of cells was not different between the groups (paper II, fig. 4A). One possible explanation for the observed decrease in fluorescence could be the formation of hybrid α-synuclein oligomers, consisting of both exogenously added α-synuclein and α-synuclein-hemi-Venus lacking Venus complementation.

To elucidate the reason for the reduction of Venus fluorescence, cell lysates from cells treated in the same way as in the BiFC study was analyzed with western blot. The α-synuclein-hemi-Venus monomers were detected both in the supernatant and to a lesser degree in the pellet fraction after centrifugation at 15 000 x g (paper II, fig. 5A, B). The levels of α-synuclein-hemi-Venus monomers were reduced in the supernatant in cells treated with oligomers at 16, 24 and 40 h (paper II, fig. 5A). The exogenously added ONE-induced oligomers were detected as a high molecular weight smear in the pellet fraction (paper II, fig. 5B). Since the ONE-induced oligomers usually do not become pelleted at 15 000 x g we speculated that the oligomers might have associated with certain cellular structures. On the other hand the exogenously added monomers were probably degraded after the uptake since they could not be detected in the supernatant nor in the pellet (paper II, fig. 5A, B). The high molecular weight α-synuclein smear observed with western blot in the pellet fraction contained no α-synuclein-hemi-Venus protein (paper II, fig. 5C), indicating that this material solely represented the exogenously added ONE-induced α-synuclein oligomers. Thus, the reduced Venus signal in cells treated with oligomers was most likely not due to the formation of hybrid-oligomers. The total protein levels in cell lysate measured with the BCA assay showed no differences between the groups indicating that the reduced levels in Venus fluorescence seen in oligomer treated cells the BiFC assay was not due to a general decrease in protein production (paper II, fig. 6).

To elucidate if the reason for the protein reduction was due to general cell toxicity a cell membrane integrity assay that measures the release of LDH (lactate dehydrogenase) was used. This study had a different set up compared to the MTT assay, as the cytotoxicity of only ONE-induced oligomers was compared to monomers but at a higher concentration and with a shorter incubation time (4 h). The cells (H4 neuroglioma) were treated with 0.5 mM of protein and the toxicity was measured after 16, 24 and 40 h. The toxicity in cells treated with oligomers or monomers was approximately 10-15 % at all time points and were not significantly different compared to cells treated with PBS (paper II, fig. 4B). Thus, the overexpression of the α-synuclein-hemi-Venus constructs seems to be responsible for the cytotoxicity, whereas addition of ONE-induced oligomers did not generate any additional toxicity. These findings are well in line with a previous report using the same constructs where the same level of toxicity was observed [99]. The reduction of α-synuclein-hemi-Venus levels might be explained by
a decreased expression or an increased degradation upon addition of oligomeric α-synuclein.

To further explore the effects of the ONE-induced off-pathway oligomers 13 months old homozygous male and female (Thy-1)-h[Δ30P] α-synuclein transgenic mice were intracortically injected with either 40 ng or 400 ng of oligomer in wild-type mouse brain homogenate or wild-type mouse brain homogenate alone (7 mice/group). After four months the mice were sacrificed and the brains were evaluated using the PK-PET-blot method (method considerations p. 57). No clear seeding effect could be observed in the mice injected with either concentration of ONE-induced oligomers (figure 8) (paper II, fig. 7).

![Figure 8](image)

**Figure 8.** Mouse brain sections stained for α-synuclein using PK-PET-blot after intracerebral injection of ONE-induced oligomers or only wild-type mouse brain homogenate. Three groups of animals were injected with either wild-type mouse brain homogenate alone (controls, A), or together with 40 ng oligomers (low dose, B) or 400 ng oligomers (high dose, C). In the control group 1 of 7 animals displayed proteinase K-resistant α-synuclein staining at the injection site, in the low dose group 3 of 7 mice displayed α-synuclein staining. In the high dose group none of the mice had α-synuclein staining at the injection site. Scale bar 10 µm.

The lack of effect could perhaps be explained by the fact that the ONE-induced oligomers are off-pathway species and although they gave rise to a seeding effect in the Th-T fibrillization assay they were not able to generate a clear seeding effect in vivo. In some recent studies it has been shown that fibrils assembled from mouse α-synuclein injected in nontransgenic wild-type mouse brain do induce transmission of α-synuclein pathology in interconnected brain regions [195]. When young transgenic mice were intracerebrally injected with either fibrils or brain homogenate from older transgenic mice the recipient mice displayed accelerated formation of α-synuclein pathology and an earlier onset of symptoms [194]. This might indicate that more fibrillar structures rather than oligomers are needed to induce pathology in vivo. Also, such findings indicate that “normal” mouse α-synuclein in wild-type mouse brain can be recruited and participate in the formation of α-synuclein pathology. Mouse α-synuclein has 95 % sequence...
homology to human α-synuclein and faster aggregation kinetics in vitro [238]. In another study α-synuclein protofibrils were injected into the substantia nigra of rats; the potential seeding effects were not investigated but a loss of neurons and activation of microglia could be observed at the site of injection [102]. Moreover, an earlier study showed that α-synuclein oligomers with toxic properties did not induce aggregation whereas non-toxic oligomers had seeding properties [98]. Similarly, the results from paper II indicates that ONE-induced oligomers, which we have previously shown to be toxic to neuroblastoma cells, after prolonged incubation [107] did not cause seeding in the cell model or in mice. Further studies are needed to understand which oligomeric species cause seeding and which cause neurotoxicity.

Properties of α-synuclein oligomer-selective antibodies and immunotherapy in an α-synuclein transgenic mouse model

The aim was to find antibodies that could selectively target α-synuclein oligomers/protofibrils in vitro and in vivo. The previously described HNE-induced α-synuclein oligomers (paper I) was used to immunize mice to generate antibody producing hybridomas. An advantage of using HNE as a cross linker is that the resulting oligomers are stable and not easily degraded. Another advantage is the almost complete conversion of monomers to oligomers when α-synuclein is incubated with a molar excess of HNE [107]. Previous protocols have only been able to convert 15 % of α-synuclein monomers into oligomers/protofibrils [239]. The generated antibodies, called mAb38F, mAb38E2, mAb15 and mAb47, were screened with inhibition-ELISA [240] (method considerations p. 53) to select antibodies with high selectivity for α-synuclein oligomers. The advantage with this type of ELISA is that the antibody and antigen interact in solution. If, on the other hand, the antigen is bound to a surface there is a risk that the true conformation of the aggregate will be lost.

The mAb38F, mAb38E2 and mAb47 antibodies showed between 150-300 times stronger selectivity for oligomers compared to monomers (figure 9) (paper III, fig. 1A, B and paper IV, fig. 1). The selectivity could be explained by the fact that some epitopes may be more exposed in the oligomer compared to the monomer. As a comparison, a commercially available antibody, Syn-1, which showed selectivity for α-synuclein monomers was included (paper III, fig. 1D). Yet another of the generated antibodies, mAb15, showed equal binding to both species (figure 9) (paper III, fig. 1C). In addition, to further ensure that the antibodies did not have
any affinity to HNE modifications per se, HNE-modified BSA was co-incubated with the antibodies and no binding was detected (data not shown).

![Figure 9](image)

**Figure 9.** Binding characteristics of the oligomer-selective antibody mAb38F and the non-selective antibody mAb15 using inhibition ELISA. The mAb38F displayed a marked selectivity for oligomers with an IC$_{50}$ value of 3nM, compared to IC$_{50}$ of 1µM for monomers. The mAb38E2 and mAb47 had similar binding characteristics as mAb38F (data not shown). The mAb15 showed similar binding to both species.

To determine if the generated antibodies were specific for α-synuclein the binding to different aggregated forms of other amyloidogenic proteins such as tau and Aβ, both involved in Alzheimer’s disease, and IAPP (Islet amyloid polypeptide), which is involved in type-2 diabetes, was investigated. None of the newly generated oligomer-selective antibodies bound to any of the other aggregates (paper III, fig. 4). Hence, they did not recognize a general amyloid epitope. Neither did they show any binding to monomers of the homologous proteins β- and γ-synuclein (paper III, fig. 4).

A previously described oligomer-selective antibody [241] were found to bind a wide range of oligomeric/protofibrillar forms of different proteins, e.g. Aβ and α-synuclein. Another polyclonal antibody, recognizing both oligomers and fibrils of α-synuclein, has been used to assess the levels of α-synuclein oligomers in human brain [114]. Therefore, we also investigated if our oligomer-selective antibodies bound to Aβ protofibrils using the inhibition ELISA, but no such binding could be observed (paper III, fig. 3), showing that the antibodies are truly α-synuclein specific.

To study whether our antibodies also recognized oligomers/protofibrils formed in the brains from α-synuclein transgenic mice we set up a sandwich-ELISA, which employs the same oligomer-selective antibody both for capture and detection. With this ELISA the chance of detecting monomers is low since two or more of the same epitope needs to be present in order for the antibodies to bind. Since the mouse model overexpresses mutant A30P α-synuclein it was important to establish that the antibodies also bound to oligomers made from A30P α-synuclein (paper III, fig. 2). When measuring the levels of oligomers/protofibrils in brain and spinal cord of young and aged (Thy-1)-h[A30P] α-synuclein transgenic mice [186] (method
considerations p. 51) there was a clear elevation in older mice (15 months) in the TBS- and Triton fraction compared to young mice (3 months) (paper III, fig. 8A-D). This elevation indicates that the oligomers/protofibrils increase as the disease progresses and that these species could be a relevant target both for diagnostics and for therapy. Some mice in the aged group had also started to develop SDS-resistant aggregates (paper III, fig. 8B, D). It can be discussed if the oligomeric/protofibrillar levels seen at three months of age in this mouse model have any impact on the cognition and behaviour. Since no such disturbances can be observed in young mice [188] these levels are likely to be tolerated by the neurons. But at 15 months of age the mice have developed behavioural disturbances and at this age the levels of oligomers/protofibrils are much higher (paper III, fig. 8B, D). There is a large variability in the oligomer/protofibril levels between different mice in the same age group, making it more difficult to draw general conclusions. It is also interesting to observe that although the mice suffer from behavioural disturbances no neuronal cell death has been observed in this model [94], highlighting the discussion whether the same species cause cognitive/motoric symptoms and the neuronal cell loss. Another important point is that, in this mouse model, the levels of oligomers/protofibrils are related to the presence of motor disturbances, indicating a central role of such species in the disease (figure 10) (paper IV, fig. 2). The total levels of α-synuclein were also measured in the formic acid fraction, representing fibrillar α-synuclein, using a total α-synuclein ELISA. Also here there was a clear difference between the age groups with overall higher levels in older mice, although with large variability (paper III, fig. 9). These findings indicate that, as the disease progresses, some of the oligomers/protofibrils might evolve into more insoluble aggregates.

![Figure 10](image-url)

*Figure 10. Levels of oligomers/protofibrils in the TBS fraction of the spinal cord of 14-17 months old (Thy-1)-h[A30P] α-synuclein transgenic mice as measured by the oligomer-selective ELISA. Mice displaying motor symptoms have significantly higher levels of oligomers/protofibrils. (T-test, p< 0.0005).*
To explore the intracellular distribution of α-synuclein oligomers/protofibrils, they were measured in subcellular fractions from the ER, mitochondriae and nuclei in brain samples from α-synuclein transgenic mice using the oligomer-selective ELISA. Oligomers/protofibrils were present in all fractions, and the levels were similar between the ages in both the mitochondriae and nuclei. Remarkably, the mean levels in the ER in 12 months old mice were higher compared to 6 and 18 months old mice, and significantly higher compared to 18 months old mice (paper III, fig. 10A). The increase in oligomers/protofibrils at this particular age is interesting because it coincides with the onset of cognitive impairments [188] and the appearance of proteinase K-resistant pathology seen in this mouse model [94]. Similar results have been reported from a study using a different transgenic mouse model [105]. It is tempting to speculate that the increase in oligomers/protofibrils at this time point is involved or initiates the cognitive impairments. The formation and accumulation of α-synuclein oligomers/protofibrils in the ER might be favored by the lack of the anti-aggregational β-synuclein and sequestration of ER chaperons by increasing amounts of α-synuclein [14]. This accumulation can lead to ER dysfunction and chronic ER stress [242, 243] which in turn have been shown to cause neurodegeneration [14].

The oligomer-selective antibodies mAb38F and mAb38E2, the commercially available Syn-1 antibody together with an antibody recognizing phosphorylated α-synuclein (PS129 α-synuclein) was evaluated on brainstem sections from α-synuclein transgenic mice at different ages (4, 12 and 18 months). In general, the staining was more pronounced for all antibodies as the mice aged (figure 11) (paper III, fig. 7). Interestingly, the oligomer-selective antibody recognized pathology earlier, already at four months (figure 11A), compared to the other antibodies (figure 11D and G). Furthermore, when comparing the oligomer-selective antibodies and the phosphorylation-dependent antibody in 18 months old mice there were a clear difference in the staining pattern. The oligomer-selective antibody gave rise to a rounded cytosolic staining, typically localized in the vicinity of the cell nuclei (figure 11C). In contrast, the phosphorylation-dependent antibody gave rise to a neuritic-like staining (figure 11I) (paper III, fig. 7). It appeared as if the respective antibodies did not stain exactly the same structures, which implies that structures stained by the oligomer-selective antibodies is not phosphorylated, at least not in transgenic mouse brain.
Figure 11. As shown by immunohistochemistry of brainstem from (Thy-1)-h[A30P] α-synuclein transgenic mice, the oligomer-selective antibody mAb38F (A-C) recognizes α-synuclein pathology already at four months of age (A) in comparison to the monomer selective antibody Syn-1 (D-F) that does not bind to this early pathology (D). In older mice the staining was more pronounced with all antibodies (C, F, I). The oligomer-selective antibody gave rise to a rounded cytosolic staining at 18 months of age (C) whereas the phosphorylation dependent antibody (PS129) (G-I) gave rise to a more neuritic-like staining at 18 months (I). Staining with mAb38E2 was similar to mAb38F (data not shown). Magnification 40 x.

To validate the specificity of the newly generated antibodies on human tissue, PD and DLB brain sections were stained and compared to non-neurological control cases. A distinct staining of LB:s and neurite like structures were seen in both substantia nigra and cortex of diseased brains but no staining could be observed in the control cases with the oligomer-selective antibodies (figure 12) (paper III, fig. 5). Thus, the antibodies recognize physiologically relevant aggregates and not only oligomers formed in vitro. The fact that the oligomer-selective antibodies recognized LB:s could mean that oligomers are included in the LB or that the α-synuclein fibrils that make up a large part of the inclusion have exposed epitopes or structures in common with the oligomer. When comparing the staining pattern between the oligomer-selective antibodies and the non-oligomer-selective antibodies there were no obvious differences (paper III, fig. 5). This might be explained by the fact that the human tissues included...
here represent the end stage of the disease. Possibly, the oligomer-selective antibodies might be able to recognize earlier pathology than traditional antibodies on tissues from cases at less advanced disease stages. Consecutive sections were stained with an oligomer-selective antibody (mAb38F) and a commercially available α-synuclein specific antibody. Both antibodies bound to the same LB, clearly showing that the oligomer-selective antibody binds α-synuclein deposits (paper III, fig. 6).

Figure 12. The oligomer-selective antibody mAb38E2 recognize LB and neurite-like structures in both PD (A) and DLB brain (B, C), as shown by immunohistochemistry. No staining could be observed in the control section (D). Similar stainings were observed for mAb38F (data not shown). Magnification 20 x.

Several genetic and biochemical observations strongly suggest that α-synuclein oligomers/protofibrils are the main toxic forms and may be central in the pathogenesis of PD and DLB [67, 98, 100, 113]. Therefore, passive immunization targeting such species should be an attractive therapeutic option. In paper IV the effect of the oligomer-selective antibody mAb47, which exhibit similar properties to those described in paper III, was evaluated in 14 months old homozygous (Thy-1)-h[A30P] α-synuclein transgenic mice. The mice were treated with intraperitoneal injections of antibody (10 mg/kg) once every week for 14 weeks and were compared to mice injected with PBS. The reason for starting treatment at 14 months was related to observations in paper III that the levels of oligomers/protofibrils were high in this age span. During the study the mice were monitored and the antibody treatment seemed to be well tolerated. Oligomer/protofibril levels were measured in the TBS- and Triton fractions (method considerations p. 58) from spinal cord and brain homogenates using the oligomer-selective ELISA (method considerations p. 53). A significant reduction in the levels of oligomers/protofibrils in the spinal cord of antibody treated mice could be observed in both fractions when compared to placebo-treated mice (figure 13) (paper IV, fig. 3). No statistically significant reduction was seen in the brain (paper IV, table 1).
Figure 13. Levels of oligomers/protofibrils in spinal cord of (Thy-1)-h[A30P] α-synuclein transgenic mice measured with the oligomer-selective ELISA. In mice treated with the oligomer-selective antibody mAb47 the levels of oligomers/protofibrils were reduced both in the TBS- (A) and Triton fraction (B) compared to mice treated with PBS. (T-test, p= 0.0137 and p=0.0373).

The reason for the differences in effect between spinal cord and brain could possibly be explained by the fact that there was a higher antibody concentration in the spinal cord (paper IV, table 2). Approximately 0.15-0.24 % of the antibody had passed the blood-brain barrier (BBB) and the blood-spinal-cord-barrier (BSCB), respectively (paper IV, table 2). This difference may be due to the BSCB being more permeable than the BBB (reviewed in [244]). Alpha-synuclein pathology in spinal cord is common in PD patients [245-248] and is likely to be of clinical significance (reviewed in [249]). Interestingly, no effect of antibody treatment on levels of fibrillar or monomeric α-synuclein could be observed (paper IV, table 1). The lack of effect on other α-synuclein species could be beneficial since the monomeric form of α-synuclein is believed to have important cellular functions and a clearance might have a negative impact on the brain. Moreover, fibrils are believed to be more inert and less toxic [250] compared to oligomers/protofibrils and may therefore also not be a suitable primary target for immunotherapy.

The brains were also evaluated using Thioflavin-S and with immunohistochemistry for phosphorylated α-synuclein. When analyzing changes in α-synuclein pathology there was no reduction in antibody-treated mice (paper IV, fig. 4). The lack of effect on Thioflavin-S and phosphorylated α-synuclein pathology could perhaps be explained by the fact that the antibody is mainly directed against the oligomeric/protofibrillar form of α-synuclein. Thioflavin-S binds to more mature aggregates and fibrillar forms and as described in paper III, not all oligomeric α-synuclein seem to be phosphorylated. These findings might indicate that the oligomer-
selective antibodies do not clear fibrillar and phosphorylated forms of α-synuclein.

To follow the inflammatory response the tissue sections were also stained with the astrocytic marker GFAP and the microglial marker Iba-1. The reason for looking at inflammatory markers was previously reported side-effects from active and passive immunotherapy trials targeting Aβ in Alzheimer’s disease patients consisting of meningoencephalitis and vascular edema [251, 252]. However, no increase in the inflammatory markers could be observed in the antibody-treated mice compared to placebo-treated mice (paper IV, fig. 5), which suggest that the treatment did not cause increased inflammation in the brain. The antibody used in this study was an IgG1, a subclass with anti-inflammatory properties mediated through binding to the inhibitory FcγRIIB [253]. FcγRIIB is conserved in mice and humans and is the most broadly expressed FcγR [254].
Concluding remarks

In the now famous “Assay of the shaking palsy” by James Parkinson in 1817 the symptoms of what we now call Parkinson’s disease (PD) was first described [3]. Almost 100 years later the German neurologist Friedrich Lewy described abnormal protein deposits in the brains of PD patients, that later would be named after him [39]. In 1993 further clues in the PD puzzle were discovered when the protein α-synuclein was described as the non-amyloid component purified from plaques in brains from patients with Alzheimer’s disease. It was described as a peptide derived from a larger precursor, which now is known as human α-synuclein [55]. In 1997 Maria Spillantini and colleagues described the biochemical nature of the Lewy body concluding that the main component was α-synuclein [48]. Since then the field of α-synuclein research has expanded significantly and lately much attention has been focused on the mechanisms underlying the α-synuclein pathology found in PD and other α-synucleinopathies, such as dementia with Lewy bodies (DLB) and multiple system atrophy.

In recent years several lines of evidence suggest an important role of α-synuclein oligomers in the neurodegeneration and toxicity seen in these disorders. To further study the biochemical and biological properties of such oligomers we generated and characterized stable oligomers induced by the reactive aldehydes HNE and ONE, described in paper I. Incubation of α-synuclein with either HNE or ONE generated an almost complete conversion of monomers to oligomers that were approximately 2000 kDa in size. The oligomers were stable, β-sheet rich species that were readily internalized and had toxic properties when applied to cells. The HNE-induced oligomers were also shown to inhibit LTP in rat hippocampal slices [124].

The propagation and seeding effects of different α-synuclein aggregates has been the topic of several studies providing a better understanding of how the pathology may spread both within the CNS and from peripheral sites to the CNS. The results from paper II describe the effects of the ONE-induced oligomers in both in vitro and in vivo settings. In a fibrillization assay the oligomers were found to seed the formation of fibrils but in a cell system, where dimerization/oligomerization of α-synuclein can be visualized fluorescently, no seeding effects could be observed. Instead, the oligomers seemed to influence the cellular α-synuclein turnover. When the ONE-induced oligomers were injected intracortically in α-synuclein transgenic mice no clear seeding effects could be observed four months after the
injection. Other studies injecting fibrillar α-synuclein in mouse brain have shown a clear transmission of α-synuclein pathology throughout large parts of the CNS [194, 195].

As the oligomeric forms of α-synuclein seem to be central in the pathogenesis of several neurodegenerative disorders, tools to study such aggregates would be valuable. **Paper III** describes the generation and characterization of monoclonal antibodies binding selectively to oligomeric forms of α-synuclein. These antibodies also bind Lewy body pathology in tissue sections from human PD and DLB brain, indicating that they recognize physiologically relevant structures. Used for immunohistochemistry, the antibodies detect pathology earlier than a commercially available monomer selective α-synuclein antibody in brain sections from transgenic α-synuclein mice. An ELISA designed to measure mainly oligomeric α-synuclein was set up, using the same oligomer-selective antibody both for capture and detection. With this assay the levels of oligomers/protofibrils in transgenic mice brain were measured and increasing amounts of oligomers/protofibrils were detected as the mice aged. In a related report, it was shown that an oligomer-selective antibody could reduce the levels of α-synuclein dimers/oligomers in a cell model [255]. In an unpublished study, it was observed that one of these oligomer-selective antibodies could traffic into the CNS after intraperitoneal injection, using in vivo imaging.

As α-synuclein oligomers have been described as particularly neurotoxic immunotherapy directed selectively against such species is an attractive approach. This selectivity might also be important to avoid side effects from binding and potentially clearing the monomeric, physiologically active form of the protein. **In paper IV** one oligomer-selective antibody, similar to those described in paper III, were used to immunize aged α-synuclein overexpressing mice. After treatment for 14 weeks with intraperitoneal injections of antibody the levels of oligomers/protofibrils in spinal cord, (but not in the brain) of these mice were reduced, as measured by ELISA. It was observed that the treatment antibody could pass into the CNS, although with significantly higher levels in spinal cord fractions compared to the brain fractions. The antibody treatment did not increase the neuroinflammation. The immunologic response is an important aspect since both active and passive immunization targeting Aβ in Alzheimer’s disease patients have resulted in inflammatory side effects [251, 252]. Furthermore, evidence for a correlation between the levels of oligomers/protofibrils and motor dysfunction in this mouse model was also reported in paper IV. The next step would then be to investigate whether the antibody treatment also reduces the motor- and cognitive deficits seen in this mouse model. In a longer perspective our findings may contribute to the development of a novel treatment strategy against PD and related disorders.
Method considerations

Alpha-synuclein transgenic mice (paper II, III and IV)
The homozygous (Thy-1)-h[A30P] α-synuclein line 31H mouse model [94, 187, 188, 256] on a C57/bl6 background express mutated human α-synuclein approximately two fold compared to endogenous levels [186]. A30P α-synuclein has been shown to have higher propensity for oligomer formation compared to wild-type protein [67]. Since one aim was to study oligomers this mouse model was the preferred choice. From previous studies it is known that the mice develop proteinase K-resistant pathology from twelve months of age and later Thioflavin-S positive inclusions predominantly in the brainstem, spinal cord and midbrain [94]. The mice exhibit hyperphosphorylated α-synuclein and there is also evidence for astrogliosis [94]. Behaviorally, the mice suffer from cognitive impairment at twelve months of age, whereas motor deficits are usually seen from 17 months of age [188]. There have been no reports on neuronal cell death arising from the α-synuclein pathology and substantia nigra is relatively spared from pathology [188]. This mouse model have been central in this thesis and in paper II the mice were employed to look at the possible seeding effects from α-synuclein oligomers that were intracerebrally injected into the cortex of aged mice. In paper III the progression of α-synuclein pathology was investigated using oligomer-selective antibodies. The level of oligomers in brain and spinal cord homogenate was measured at different ages using a sandwich-ELISA. Brain tissue sections from the mice at different ages were also used for immunohistochemical staining. Finally, in paper IV the mice were treated with an oligomer-selective antibody intraperitoneally for 3.5 months to evaluate if immunotherapy could lower the levels of oligomers/protofibrils in these mice.

Atomic force microscopy (paper I and II)
Atomic force microscopy (AFM) is a technique utilized to visualise surface structures at high resolution. The principal of AFM is to scan a tip over a surface while sensing the interactions between the tip and sample, the force interacting between the tip and sample is measured with the deflection of a cantilever which is detected with a laser beam [257]. This high-resolution
scanning microscopy technique provided information of the morphology of the ONE- and HNE-induced oligomers in paper I and II and the resulting fibrils seeded with oligomers and fibrils in paper II.

Bimolecular fluorescence complementation assay (paper II)

The Bimolecular fluorescence complementation (BiFC) assay allows the study of α-synuclein dimerization/oligomerization in living cells by simultaneously expressing two non-fluorescent Venus (modified YFP) halves fused to either the C-terminal or N-terminal of full-length α-synuclein [99]. When at least two α-synuclein molecules come together the Venus molecule is reconstituted and starts to fluoresce (figure 14). It has been shown that the complementation is driven by α-synuclein and not by the Venus protein [99]. Dimers/oligomers formed in the BiFC assay appear as a high molecular weight smear on native-PAGE, but on SDS-PAGE the aggregates are dissociated to monomeric species [99]. When expressed in cells it has been shown that the dimerized/oligomerized α-synuclein is localized to the soma and nucleus. This overexpression and formation of aggregates also leads to some degree of cytotoxicity [99]. In paper II the effect on α-synuclein dimerization/oligomerization after addition of oligomers was evaluated using the BiFC assay.

Figure 14. Schematic illustration of the BiFC assay. Two hemi-Venus molecules each linked to either the N-terminal (VN) or C-terminal (VC) of full-length α-synuclein. When two or more α-synuclein molecules aggregate the Venus molecule is reconstituted and becomes fluorescent.
Circular dichroism (paper I)

Measurements with circular dichroism (CD) give structural information, such as secondary structure, of proteins. In this case, the secondary structure of both ONE- and HNE-induced α-synuclein oligomers were examined in paper I. The secondary structure is measured in the far-UV spectral region (190-250 nm) and the chromophore is the peptide bond. The conversion of peptides into fibrils can be measured with CD. The shape of the spectrum curve and the positive- and negative maxima give information of the protein conformation. Alpha-helical structures cause a minimum at 208 and 222 nm and β-sheet structures generates a minimum at 217-220 nm [258].

ELISA (paper I, III and IV)

Enzyme linked immunosorbent assay (ELISA) is a widely used assay to detect and/or quantify proteins [259]. There are different types of ELISAs; in some the antibody binds the antigen in solution and in others either the antibody or the antigen is fixed on a surface before detection. The interaction can be revealed by an anti-Ig antibody coupled to an enzyme, for example horseradish peroxidase (HRP) or alkaline phosphatase (ALP).

In paper I, an indirect ELISA set up was used to map the surface epitopes of the two different types of oligomers that were generated. In paper III, an indirect ELISA was used to exclude the possibility that the newly generated antibodies bound to HNE modified proteins in general. In an indirect ELISA the antigen is coated onto a surface and is detected with one primary antibody binding to the antigen and thereafter an anti-IgG secondary antibody coupled to an enzyme. This set up is easy and can be used for quantification.

In paper III an inhibition-ELISA [240] (figure 15) was used to establish the selectivity of the newly generated antibodies. The advantage of the inhibition ELISA is the fact that the antibodies bind the antigen in solution and thus more physiological relevant epitopes will be assessable for the antibody to bind. The conformation of the monomers/aggregates may be changed or lost if they are coated onto a surface. Using the inhibition-ELISA, oligomer-selective antibodies were chosen and used to set up a sandwich-ELISA which is selective for oligomeric species. The oligomer-selective sandwich-ELISA have the same antibody both for capture and detection which means that at least two or more of the same epitope needs to be present to generate a signal. The oligomer-selective ELISA was used in paper III and IV to measure the levels of oligomers/protofibrils in brain homogenates from transgenic α-synuclein mice.
In the inhibition-ELISA the antibodies are first pre-incubated in solution with either α-synuclein oligomers or monomers (step 1). In step 2 the antibodies that have not bound in the first step binds to the monomer coated surface of the ELISA well. The amount of antibody bound in step 2 is then a measure of how well the antibody bound to the antigen in step 1. Consequently, high binding in step 1 generates a low signal in step 2.

**Fibrillization assay using Thioflavin-T (paper I and II)**

To study the aggregational properties of the ONE- and HNE-induced oligomers in paper I and the possible seeding effects of the ONE-induced oligomers in paper II a fibrillization assay using Th-T was utilized. Thioflavin-T is widely used to detect fibrils both in vitro and in vivo. Thioflavin-T is a benzothiazole salt that binds to β-sheets of proteins and is therefore used to measure the aggregation and formation of fibrils. When Th-T binds β-sheets it generates a large enhancement in fluorescence and it changes the emission spectra from 445 to 482 nm [260].

**Immunohistochemistry (paper III and IV)**

Different proteins can be detected with specific antibodies on tissue sections using immunohistochemistry. The quality of the staining depends on the specificity of the antibodies used, the fixation of the tissue and what method for antigen retrieval that is chosen. Additionally, the condition of the tissue before fixation and embedding is crucial. The advantage of
immunohistochemistry over for example ELISA is that it provides information about the localization of the proteins, either within cell compartments or brain regions. Also, it provides valuable information on the appearance of the pathology of interest. On the other hand, immunohistochemistry is perhaps not the easiest method for quantitative measurements. But by using blinded analyses it is at least possible to make quantitative estimates. In paper III, both mice and human brain sections were stained with the oligomer-selective antibodies and compared to other α-synuclein antibodies. Immunohistochemical staining for both α-synuclein pathology and inflammatory markers were used in paper IV to evaluate the effect of the antibody treatment in transgenic mice.

**Intracerebral injection (paper II)**

In paper II the potential seeding effect of ONE-induced oligomers *in vivo* where studied in 13 months old (Thy1)-h[A30P]α-synuclein transgenic mice that were injected intracerebrally with two different doses of oligomer. The total injected volume was 2 µl, using a Hamilton syringe connected to a pump, with an injection speed of 0.2 µl/min. Both the volume and injection speed is crucial to ensure a consistent and reliable injection. To further ensure the accuracy the procedure was monitored with a microscope to ensure that there was no fluid leakage. After the injection the needle was left in place for five minutes, this allows the injected fluid to diffuse into the brain tissue. Thereafter the syringe-pump was started again and the needle was slowly withdrawn. The stereological coordinates for the injections measured from the bregma were: 1.2 mm mediolateral, 0.7 mm dorsoventral and 0 mm anteroposterior. The injection site, neocortex, was chosen because it normally does not contain proteinase K-resistant α-synuclein pathology but robust amounts of monomeric α-synuclein. In so doing, any staining present after proteinase K-treatment in this area could be expected to have arisen from newly seeded aggregates of α-synuclein.

**LDH- and MTT assay (paper I and II)**

To study potential toxic effects of α-synuclein oligomers two different assays were used; the LDH (lactate dehydrogenase) assay [261] was used in paper II to measure the membrane integrity of H4 neuroglioma cells after addition of ONE-induced α-synuclein oligomers. The LDH assay fluorescently measures the release of lactate dehydrogenase indicative of cells with a damaged cell membrane. Lactate, NAD+ and resazurin are supplied to the cellmedia, firstly lactate is converted to pyruvate by LDH. This in turn converts resazurin to the fluorresent resorufin that is proportional
to the amount of released LDH. In paper I the MTT assay [262, 263] was used as a measure of cell viability in a SH-SY5Y neuroblastoma cell line. Viable cells reduce the MTT salt to a purple formazan product which then can be used to calculate the relative degree of viable cells.

Passive immunization (paper IV)

In previous immunization studies α-synuclein has been targeted either non-specifically or with antibodies directed against the C-terminus [221, 223, 225, 226]. In paper IV, antibodies binding preferably to oligomeric species of α-synuclein were administered to transgenic mice with α-synuclein pathology. The selectivity of the antibodies is important since oligomers are considered to be the main neurotoxic species. In a previous passive immunization study it was shown that antibodies could pass the BBB and reduce both behavioral dysfunction and α-synuclein accumulation [225]. The mechanisms for antibody mediated removal of α-synuclein in the brain are not fully understood but have been suggested to involve the autophagy-lysosomal pathway [264]. Antibodies can pass the BBB, but only to a relative low degree, ~0.1 % [225]. How the antibodies pass the BBB is not known but one suggested mechanism involves passive transcytosis. The antibodies present in the blood are nonspecifically captured in endocytic vesicles, together with for example nutrients or iron, and transported through the cell and are then released inside the brain [265]. It has been shown that antibodies can also exit the brain [266] which is an important feature if the antibody is to be used for clearance of α-synuclein pathology. Even though α-synuclein is an intracellular protein there are proposed mechanisms for how the antibody could bind to and/or remove α-synuclein aggregates. The antibodies could exert their effect intracellularly, possibly by entering the cell and thereafter localize to the lysosomes [225]. On the other hand, several studies suggest that both monomers and oligomers of α-synuclein can be excreted from cells and are present in the extracellular environment, [146, 150, 197, 226] thereby providing an opportunity for the antibody to clear the extracellular α-synuclein. Interestingly, extracellular treatment with an oligomer-selective antibody in cells did reduce the intracellular presence of α-synuclein [255].

Proteinase K-treatment and urea stability (paper I)

In paper I the use of proteinase K and urea provided valuable information of the compactness and stability of the ONE- and HNE-induced oligomers. Proteinase K is a stable serine protease with broad substrate specificity which digests soluble α-synuclein but not insoluble (fibrillar) forms [117]
and can therefore provide insight to the compactness of different aggregates. Urea is a denaturant and breaks non-covalent bonds in proteins. This can then be used to test the stability of proteins, and in contrast to proteinase K, it breaks down larger aggregates without cleaving peptide bonds.

PK-PET-blot immunohistochemical staining (paper II)

In paper II, the brain tissue from the mice injected intracerebrally with ONE-induced oligomers were analyzed with the PK-PET-blot method (proteinase K paraffin-embedded-tissue blot), as previously described [119]. The aim was to see if the injected oligomers had caused any seeding effect, recruiting monomers to form larger aggregates. Brains were cut in 6 µm sections and mounted on a nitrocellulose membrane and subsequently treated with 50 µg/ml of proteinase K. Immunostaining was made with an α-synuclein antibody. It has previously been described that proteinase K digests soluble α-synuclein but not the more insoluble species, for example fibrils. With the PK-PET-blot method treatment with proteinase K is used to reveal more α-synuclein pathology compared to standard immunohistochemical methods, both in human and transgenic mouse brain [94, 120].

SDS-PAGE, western blot and dot blot (paper I, II and III)

With the sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) method proteins can be separated according to size. The proteins are denaturated with SDS and heat; SDS gives the proteins a negative net charge which renders it possible for the proteins to migrate when an electrical current is applied. Because of the denaturation the proteins will have a similar unfolded structure and will migrate in the gel only according to their size. The proteins are visualised by the western blot method. First the proteins are transferred onto a nitrocellulose or a polyvinylidene fluoride (PVDF) membrane which is incubated with primary antibody raised against the protein of interest, for example α-synuclein. A fluorescently tagged secondary antibody is applied and binds to the primary antibody. The protein is then detected in a scanner which excites the fluorophore and detects its emitted light. This technique was used in paper I to better understand the stability of the ONE- and HNE-induced oligomers and in paper II to measure the levels of α-synuclein in cell lysates. To study the compactness of the newly generated oligomers in paper I the dot blot method was used after proteinase K-treatment. The same principal for detection is used in the
dot blot method as in western blot but here the proteins are not separated according to their size, rather the method is useful for determining if the protein of interest is present or not in the sample. In paper III, dot blot with subsequent western blot was utilized to determine if the newly generated oligomer-selective antibodies bound other amyloidogenic proteins, such as Aβ or tau.

SEC-HPLC (paper I)

In paper I size exclusion-high performance liquid chromatography (SEC-HPLC) was utilized to characterize the molecular weights of the ONE- and HNE-induced oligomers. The method separates proteins according to their molecular size by a retention mechanism. The column is packed with porous particles in which small proteins enters and therefore moves more slowly through the column. Larger proteins are excluded from entering the porous particles and moves quickly along the column. The retention time of small proteins are therefore longer than for larger proteins [267]. The molecular weight of the samples is determined by the use of globular protein standards with known molecular weights.

Sequential extraction and subcellular fractionation (paper III and IV)

One way of studying α-synuclein aggregates in brain homogenates is by dividing them in different fractions according to their solubility. In paper III and IV the levels of oligomers/protofibrils were measured in different fractions. The mouse brain tissue is mechanically homogenized in TBS buffer and after centrifugation at 16 000 x g the soluble α-synuclein will be present in the supernatant. The resultant pellet is re-homogenized in 0.5 % Triton buffer and centrifuged yielding Triton-soluble α-synuclein, which could be the membrane associated species. Finally, the pellet is re-suspended in 1 % SDS buffer and after centrifugation the supernatant contains SDS-soluble α-synuclein (figure 16). In paper III, the tissue was subcellularly fractionated to investigate the presence of oligomers/protofibrils in different cellular compartments, such as the ER, mitochondria and the nucleus [105]. In this method the different fractions are generated based on their size rather than solubility by centrifugation.
Figure 16. Sequential extraction of brain homogenate from (Thy-1)-h[A30P] α-synuclein transgenic mice. The mouse brain tissue is mechanically homogenized in TBS buffer followed by centrifugation at 16 000 x g for 1 h resulting in a TBS pellet and a TBS-soluble α-synuclein fraction. The TBS pellet is then re-suspended in Triton buffer and the same procedure is repeated. In the final step, the Triton pellet is resuspended in SDS buffer and centrifuged resulting in a fraction containing SDS-soluble α-synuclein.
Jordens befolkning ökar ständigt och tack vare bättre levnadsvanor och mer avancerad sjukvård lever vi allt längre. Därför tilltar också kraven på vård och behandling mot åldrandets sjukdomar.

Arbetet i avhandlingen handlar om proteinet alfa-synuklein som normalt finns i hjärnan, men som av någon okänd anledning ändrar form, ansamlas till långa olösliga proteintrådar, som med tiden resulterar i så kallade Lewykroppar, och orsakar en förlust av nervceller i hjärnan. Denna process anses vara en bakomliggande faktor till utvecklingen av både Parkinsons sjukdom och Lewy body demens. Nervcellsdöden i parkinsonhjärnan leder till en gradvis försämring av bland annat rörelseförmåga och minne. Idag kan symptomen bara lindras och det finns inget botemedel mot sjukdomen. I Sverige är ca 25 000 personer drabbade och sjukdomen startar oftast kring 50-års ålder. Lewy body demens kännetecknas av minnesstörningar och synhallucinationer och är den näst vanligaste demenssjukdomen efter Alzheimers sjukdom.

Vid Parkinsons sjukdom och Lewy body demens finns så kallade Lewykroppar i hjärnans nervceller som huvudsakligen består av aggregerat alfa-synuklein. Aktuell forskning tyder på att det framför allt är mellanstora lösliga förstådier till Lewykroppar, s.k. oligomerer, som är skadliga för nervcellen. Det finns årtliga former av både Parkinsons sjukdom och Lewy body demens där man har kunnat visa att patienterna bär på skadliga förändringar i arvsmassan för alfa-synuklein. Dessa årtliga former är ovanliga men de har gett oss viktig kunskap om sjukdomsmechanismerna, t.ex. att en ökad produktion av alfa-synuklein i hjärnan är skadligt och att förhöjda nivåer av oligomerer är särskilt skadligt för nervcellerna.

Målet med denna avhandling var att studera egenskaperna hos de skadliga oligomererna och utveckla antikroppar som endast känner igen dessa former.

I avhandlingens första arbete undersöktes egenskaperna hos två olika typer av oligomerer som var stabiliserade med två aldehyder, substanser som bildas i hjärnan vid oxidativ stress. Den ena typen, ONE-oligomeren, hade en rund, kompakt form och visade sig vara veckad annorlunda än HNE-oligomeren, som hade en mer avlång form. Båda typerna visade sig vara skadliga då de tillsattes till nervcellslänkande celler. De båda oligomertyperna hade dock olika känslighet för nedbrytning vilket vi tror kan vara relaterat till de olika kemiska bindningar som uppstår inom oligomeren.
Alfa-synuklein patologi kan sprida sig mellan nervceller i hjärnan och oligomererna verkar vara en del i denna spridning. Man tror också att oligomerer kan få normalt alfa-synuklein att klumpa ihop sig och bilda både nya skadliga oligomerer och andra större aggregat. I avhandlingens andra arbete undersökte vi denna mekanism och fann att ONE-oligomeren ökade bildningen av aggregat i provrörsförsök. När oligomererna tillsattes till celler eller applicerades i mössens hjärnor kunde vi dock inte se denna effekt. Däremot verkar det som att oligomererna påverkar antingen produktionen eller nedbrytningen av alfa-synuklein inuti cellen, vilket i sig kan leda till cellskador om detta pågår under en längre tid.

Att kunna mäta nivåer av alfa-synuklein oligomerer hos patienter kan visa sig vara värdefullt för att få en ökad förståelse för sjukdomsprocesserna, för att tidigt kunna diagnostisera sjukdomarna och för att utvärdera nya behandlingsmetoder. Antikroppar är kroppens eget försvar mot skadliga ämnen, men även laboratoriefremställda antikroppar används allt mer som läkemedel mot en rad sjukdomar. Genom att rikta antikropparna mot de skadliga proteinformerna kan man avlägsna eller oskadliggöra proteinet i fråga, vilket kan bromsa eller bota sjukdomen.

Vi framställde antikroppar som specifikt känner igen oligomerer och egenskaperna hos dessa beskrivs i avhandlingens tredje arbete. Dessa antikroppar känner igen relevant patologi i hjärnan hos avlidna patienter med Parkinsons sjukdom och Lewy body demens och med hjälp av dessa antikroppar har vi utvecklat en metod som med stor säkerhet och känslighet kan mäta mängden av alfa-synuklein oligomerer. Vi använde möss som är genetiskt förändrade att producera alfa-synuklein och som bildar Lewykroppslignande förändringar i hjärnan samt utvecklar Parkinsonliknande symptom. Med hjälp av dessa möss och vår nya mätmetod kunde vi följa hur nivåerna av oligomerer förändrades med sjukdomsutvecklingen. Dels kunde vi se att mängden oligomerer ökar i hjärnan ju sjukare mössen blev. En annan viktig egenskap hos antikropparna var att de kunde upptäcka patologi i hjärnan hos möss tidigare än traditionella antikroppar riktade mot alfa-synuklein.

I avhandlingens fjärde arbete undersökte vi om oligomer-antikropparna, liknande de som beskrivits i arbete tre, kunde användas för att minska mängden alfa-synuklein patologi hos möss som har Parkinsonliknande symptom. Liknande studier, men med antikroppar riktade mot ett annat protein, beta-amyloid, har tidigare utförts på människor som är drabbade av Alzheimers sjukdom och har visat lovande resultat. Efter att mössen behandlats med antikropp varje vecka i drygt tre månader kunde vi se en sänkning av mängden skadliga oligomerer i de möss som fått antikroppsbehandlingen, jämfört med de möss som inte fått någon behandling. Antikroppsbefandlingen verkade dessutom vara relativt säker då mössen inte fick någon ökad inflammation i hjärnan.
Sammanfattningsvis har denna avhandling främst beskrivit egenskaper hos alfa-synuklein oligomerer, skadliga proteinformer som är centrala i utvecklingen av Parkinsons sjukdom och Lewy body demens. Antikroppar som specifikt binder till oligomerer har framställts och genom antikroppsbehandling visats sig kunna minska nivåerna av dessa skadliga proteiner i centrala nervsystemet i en musmodell för dessa sjukdomar.
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