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Characterization of α -synuclein oligomers

Implications for Lewy Body Disorders

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Abstract

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Parkinson's disease, dementia with Lewy bodies and multiple system atrophy are disorders featuring accumulation of Lewy bodies in brain. The main component of these large insoluble intracellular inclusions is the presynaptic protein alpha-synuclein (α -synuclein). It is generally believed that α -synuclein monomers adopt an abnormal conformation that favors the formation of soluble oligomers or protofibrils and, eventually, insoluble fibrils depositing as Lewy bodies. Notably, the intermediately sized oligomers/protofibrils seem to have particular neurotoxic effects. Several factors may influence the formation of α -synuclein oligomers/protofibrils, e.g. the reactive aldehydes 4-hydroxy-2-nonenal (HNE) and 4-oxo-2-nonenal (ONE) formed during oxidative stress.

The overall aims of this thesis were to investigate biophysical and biochemical properties of *in vitro* generated α -synuclein oligomers, characterize their functional effects on cell and animal disease models as well as to explore whether their formation could be prevented in a cell culture model for oligomerization.

Here, it was found that α -synuclein rapidly formed oligomers after incubation with both ONE and HNE. The resulting oligomers were stable and did not continue to form insoluble fibrils. By comparing HNE- and ONE induced α -synuclein oligomers biochemically they were both found to exhibit extensive β -beta sheet structure and had a molecular size of ~2000 kDa. However, they differed in morphology; the ONE induced α -synuclein oligomers described round amorphous species whereas the HNE induced α -synuclein oligomers appeared as elongated protofibril-like structures. Both these oligomers were cell internalized to varying degrees and induced toxicity in neuroblastoma cells.

In addition, the ONE induced α -synuclein oligomers seemed to initiate aggregation of monomeric α -synuclein *in vitro*, but failed to do so *in vivo*.

Finally, treatment of α -synuclein overexpressing cells with monoclonal antibodies specific for α -synuclein significantly reduced aggregation and lowered levels of the protein, suggesting increased turnover in these cells.

To conclude, this thesis has characterized different oligomeric α -synuclein species, which may have properties similar to soluble species central to the pathogenesis of Parkinson's disease and other disorders with α -synuclein pathology. For therapeutic strategies it is important to selectively target such harmful protein species and avoid interaction with other forms of α -synuclein, which may have vital physiological cellular functions.

Keywords: Parkinson's disease; Alpha-synuclein; Lewy bodies; Oligomers; Reactive aldehydes; monoclonal antibody

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”Visdom är inte en produkt av skolning, utan ett livslångt försök att skaffa sig det.”

Albert Einstein

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List of Papers

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

- I **Näsström T**, Wahlberg T, Karlsson M, Nikolajeff F, Lannfelt L, Ingelsson M, Bergström J. (2009) The lipid peroxidation metabolite 4-oxo-2-nonenal cross-links α -synuclein causing rapid formation of stable oligomers. *Biochemical and Biophysical Research Communications*, 378:872-6
- II **Näsström T**, Fagerqvist T, Barbu M, Karlsson M, Nikolajeff F, Kasrayan A, Ekberg M, Lannfelt L, Ingelsson M, Bergström J. (2011) The lipid peroxidation products 4-oxo-2-nonenal and 4-hydroxy-2-nonenal promote the formation of alpha-synuclein oligomers with distinct biochemical, morphological and functional properties. *Free Radical Biology and Medicine*, 50:428-37
- III Fagerqvist T, **Näsström T**, Sahlin C, Tucker S, Lindström V, Ingvast S, Karlsson M, Nikolajeff F, Schell H, Outeiro T.F, Kahle P.J, Lannfelt L, Ingelsson M, Bergström J. *In vitro* and *in vivo* seeding effects of alpha-synuclein oligomers. *Manuscript*
- IV **Näsström T**, Gonçalves S, Sahlin C, Nordström E, Screpanti Sundquist V, Lannfelt L, Bergström J, Outeiro T, Ingelsson M. Antibodies against alpha-synuclein reduce oligomerization in living cells. *PLoS ONE*, 6:e27230

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Abbreviations

α, β, γ -synuclein	Alpha, beta, gamma-synuclein
A β	Amyloid-beta
AD	Alzheimer's disease
AFM	Atomic force microscopy
AMPA	Alpha-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid
A β PP	Amyloid-beta precursor protein
BiFC	Bimolecular fluorescence complementation
BSA	Bovine serum albumin
C (1,2,3,4)	Carbon
CD	Circular dichroism
CHIP	Carboxyl terminus of hsp70 interacting protein
CJD	Creutzfeld-Jacob's disease
CMA	Chaperone-mediated autophagy
CNS	Central nervous system
CSF	Cerebrospinal fluid
DLB	Dementia with Lewy bodies
DJ-1	Parkinson's disease protein 7
ELISA	Enzyme linked immunosorbent assay
ER	Endoplasmatic reticulum
EM	Electron microscopy
FABP	Fatty-acid-binding protein
GFP	Green fluorescent protein
HEK	Human embryonic kidney cells
HNE	4-hydroxy-2-nonenal
HRP	Horseradish peroxidase
HSP 70	Heat shock protein 70
LB	Lewy body
L-DOPA	L-3,4-dihydroxyphenylalanine
LN	Lewy neurite
LRRK2	Leucine-rich repeat kinase 2
LTP	Long term potentiation
MMP	Metalloproteinase
MSA	Multiple system atrophy
MTT	3-(4,5-Dimethylthiazol-2-yl)-2,5 diphenyltetrazolium bromide
NACP	Non amyloid component precursor
NMDA	N-Methyl-D-aspartic acid
NMR	Nuclear magnetic resonance
ONE	4-oxo-2-nonenal
PCA	Protein complementation assay

PD	Parkinson's disease
PDB	The Protein Data Bank
PINK1	Serine/threonine-protein kinase 1
PK	Proteinase K
PRKN	Parkin
PrP ^C	Cellular prion protein
PrP ^{Sc}	Scrapie prion protein
PVDF	Polyvinylidene fluoride
ROS	Reactive oxygen species
SDS-PAGE	Sodium dodecyl sulfate polyacrylamide gel electrophoresis
SEC-HPLC	Size exclusion-high performance liquid chromatography
SNARE	<i>N</i> -ethylmaleimide-sensitive factor attachment protein complex
SNCA	Synuclein, alpha
SNCB	Synuclein, beta
ThS	Thioflavin S
ThT	Thioflavin T
TRIM21	Tripartite motif-containing 21
UCHL1	Ubiquitin thiolesterase 1
UV	Ultraviolet

Introduction

Background

In “An essay of the shaking palsy” [1] from 1817, James Parkinson described a disease that 60 years later would be named after him. In the essay, he described six individuals with impaired motor skills, speech difficulties and tremor. In the 1950’s, the underlying biochemical deficiency of dopamine in the brains of Parkinson’s disease (PD) patients was described and its precursor L-3, 4-dihydroxyphenylalanine (L-DOPA) became a clinical treatment in 1967 [2, 3].

Almost 100 years after Parkinson had published his findings, the German neurologist Friedrich Lewy identified abnormal protein deposits in the PD brain, which later became known as Lewy bodies (LBs) [4]. However, the biochemical nature of the deposits remained unknown until 1997 when Maria Spillantini and colleagues found that their main component was the presynaptic protein alpha-synuclein (α -synuclein) [5]. In addition, LB brain pathology was also found in dementia with Lewy bodies (DLB) and multiple system atrophy (MSA), two other neurodegenerative diseases [6, 7]. All disorders with deposition of α -synuclein in the brain are commonly referred to as α -synucleinopathies.

Parkinson’s disease

Parkinson’s disease is characterized by degeneration of dopaminergic neurons in the brain. The lack of dopamine results in disproportionate neurotransmission and a predominance of acetyl cholinergic activity, leading to a clinical picture with varying degrees of rigidity, resting tremor and hypokinesia [8]. Altered cognition is also often seen and patients may develop dementia in late stages of the disease [9, 10].

A majority of PD cases are idiopathic, but about 5% of all cases are known to have dominantly or recessively inherited disease forms that arise from mutations in specific genes [11]. However, additional disease genes remain to be identified and a number of risk genes have been suggested (for an updated overview of PD risk genes, please visit <http://www.pdgene.org/>). In addition, environmental factors that may cause PD have been suggested, such as pesticides [12] and heavy metals [13] and also traumatic brain injury [14].

Although PD is a chronic and progressive disorder, symptomatic treatments, such as L-DOPA and deep brain stimulation, are successful to alleviate symptoms. L-DOPA, a prodrug to dopamine, can enter the central nervous system (CNS) where it is converted to dopamine, which in turn can compensate for the loss of neurotransmission. Because PD is a progressive neurodegenerative disease, L-DOPA is only efficient for a limited amount of time. With prolonged L-DOPA treatment, side effects such as dyskinesia and motor fluctuations are becoming common [15].

Dementia with Lewy bodies

Dementia with Lewy bodies is a neurodegenerative disease considered to account for up to 20% of late onset dementia [16, 17]. Clinical hallmarks of the disease are dementia, fluctuating cognition, visual hallucination and parkinsonian symptoms such as postural instability [18]. The disease is characterized by loss of a wide range of neurons, including acetylcholine and dopamine producing cells [18]. Furthermore, 50-60% of Alzheimer's disease (AD) patients present with LB pathology [19]. For DLB, similar medications as for AD and PD are sometimes efficient, i.e. cholinesterase inhibitors/ N-Methyl-D-aspartic acid (NMDA)-receptor antagonists and L-DOPA, respectively.

Multiple system atrophy

Multiple system atrophy is characterized by signs of autonomic dysfunction together with cerebellar ataxia or parkinsonism [20]. Neuropathologically, the disorder features neuronal loss in the cerebellum, pons, inferior olivary nuclei, basal ganglia and spinal cord. In addition, filamentous glial cytoplasmic inclusions of aggregated α -synuclein can be found [6, 21]. The MSA patients with parkinsonism respond well to L-DOPA treatment in the beginning of the disease. However, as the dopaminergic neurons die, the therapeutic effects wear off [22]. In addition, other symptoms, such as orthostatic hypotension, are treated with non-pharmacological means, e.g. with compression stockings and / or an increased intake of fluid and salt [23].

Neuropathology – Lewy bodies and Lewy neurites

In the PD brain, LBs and Lewy neurites (LN) are found in the substantia nigra of the midbrain (*Figure 1*). As the disease progresses, protein deposits can also be found in other brain areas such as the olfactory bulb, the limbic system and neocortex [24]. However, the findings of LB-like inclusions of α -

synuclein in colonic biopsies in PD [25-27] has suggested that the pathological events in PD occur also in other parts of the nervous system, such as the enteric plexus of the gastrointestinal system [28]. Patients with DLB also display pathology in the midbrain but mainly show LBs in neocortical areas [29, 30]. The topographical distribution of LBs determines the nature of the symptoms, e.g. DLB patients with visual hallucinations typically have LBs in the temporal lobe [31]. Ultrastructurally, LBs describe round eosinophilic cytoplasmic large inclusions 5-25 μm in size composed of a halo of radiating fibrils with a less defined core [32]. Moreover, LBs mainly consist of ubiquitinated, phosphorylated (at Serine 129) and nitrosylated fibrillar forms of full-length α -synuclein [5, 33, 34] whereas also C-terminally truncated (1-110 and 1-119) can be identified [7, 35]. In addition, more than 300 other proteins have been found to be co-deposited in LBs [36].

The number of LBs do not correlate well with either the severity or duration of dementia in DLB [37]. Although it has been suggested that 3-4% of the neurons in the PD substantia nigra harbor LBs, the number of affected cells is relatively constant regardless of disease stage [38]. Thus, the pathological significance and potential biological function of LBs are unclear. A possible, albeit controversial, explanation for the presence of LBs in surviving neurons is that they represent a depot for misfolded α -synuclein, and as such are protective [39]. Such a theory is further supported by the observation that LB-containing neurons appear healthier than adjacent neurons [40].

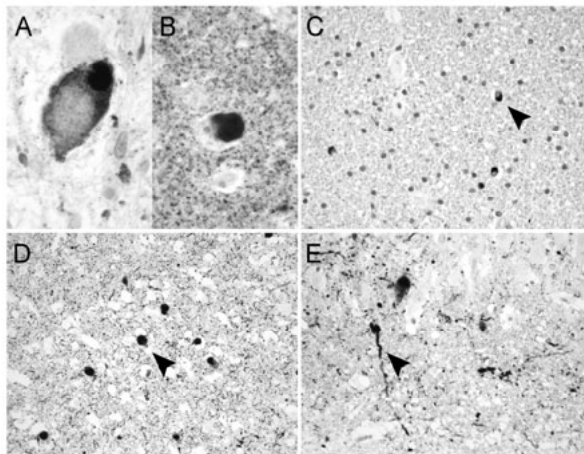


Figure 1. Immunohistochemistry showing Lewy bodies in PD (A and B), MSA (C) and DLB (D) as well as Lewy neurites in DLB (E). Lewy bodies are shown as black dense inclusions residing inside the neurons (A, B) and oligodendrocytes (C). A Lewy neurite is presented (arrow) as a red-brown elongated structure containing aggregated α -synuclein (E). Pictures A and B are taken at 40x magnification and C, D and E at 20x magnification. Images provided by Hannu Kalimo.

Alpha-synuclein

The main component of LBs and LNs is α -synuclein, a 140 amino acid long protein (*Figure 2*) mainly expressed in the CNS with its primary localization in presynaptic nerve terminals [41]. The exact physiological role of α -synuclein is unclear although some reports suggest that it is involved in neurotransmitter regulation [5]. Other data suggest that α -synuclein promotes soluble *N*-ethylmaleimide-sensitive factor attachment protein receptor (SNARE)-complex assembly by binding to phospholipids via its N-terminus and to synaptobrevin-2 via its C-terminus [42]. Furthermore, α -synuclein has been shown to interact with several proteins implicated in signal transduction, mitochondrial function, regulation of oxidative stress and in the ubiquitin-proteasome system [43, 44].

Alpha-synuclein was first described when a short peptide, the non-amyloid component (NAC), was purified from AD plaques [45]. The study found that this peptide was derived from a larger precursor, the non-amyloid component precursor (NACP), which now is known to be identical to human α -synuclein. Although this finding probably could be explained by the presence of LBs or LNs in the sample preparation [46], there is an ongoing debate whether α -synuclein still may be a component of amyloid- β plaques.

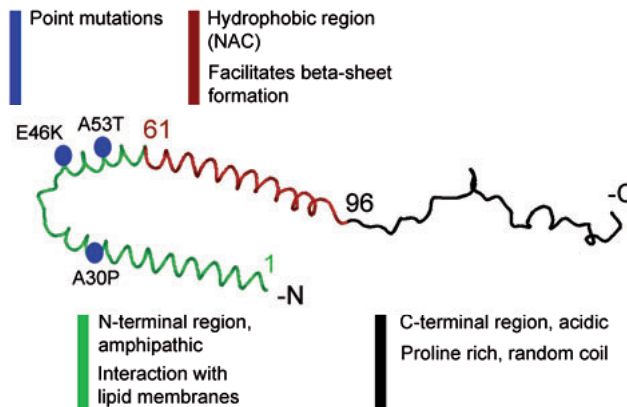


Figure 2. Nuclear magnetic resonance (NMR) prediction of human α -synuclein structure. The amino-terminal of the protein is highly conserved, includes a number of imperfect repeats (KTKEGV) and is associated with lipid binding (1-60). Also located in the N-terminal region are the mutations linked to either early-onset PD (A30P, A53T) or DLB (E46K). The middle part consists of hydrophobic residues and is also known as the non amyloid component (NAC) region (61-95), which is believed to facilitate α -synuclein aggregation and β -sheet formation. The carboxy-terminal region includes a high proportion of acidic residues and is characterized by a random coil structure (96-140). (NMR prediction from the protein data bank (PDB); 1xq8, original structural data provided by [47]).

Beta- and γ -synuclein are other members of the synuclein protein family. All synucleins have a variable number of imperfect repeats in their amino-terminus, a more hydrophobic middle region and a predominance of acidic residues in their carboxy-terminus [48]. Beta-synuclein has 134 amino acids and differs from α -synuclein in the middle region whereas the 127 amino acid γ -synuclein lacks the C-terminal part of α - and β -synuclein [49, 50].

Due to overall homologies and overlapping functionality for proteins in the synuclein family, α -synuclein knock-out mice have been described to be as viable and fertile as wild-type mice, in spite of decreased levels of striatal dopamine [51]. In addition, there is little difference in neurotransmitter release between α -synuclein knock-out mice and wild-type mice, providing a further indication of the functional redundancy between the different synucleins [52].

Certain features are unique for the synucleins. For example, the highly conserved repeat of the N-terminal classifies α -synuclein as an apolipoprotein-type A2 helix that facilitates binding to phospholipid vesicles [53] (*Figure 2*). Interestingly, α -synuclein has been shown to act as a fatty-acid-binding protein (FABP) [54], perhaps serving as a transporter of fatty acids from the cytosol to the lipid membrane, or vice versa, a process also known as collisional transfer [55].

Alpha-synuclein is an intrinsically unstructured protein, mostly due to its overall hydrophilicity and the high net negative charge of its C-terminus, giving the molecule high flexibility [34]. The aggregation propensity of α -synuclein is unique among the synucleins, as neither β - nor γ -synuclein are prone to form fibrils [49]. The part associated with aggregation is located in the mid region, i.e. the NAC-region, of the protein [56]. Both β and γ -synuclein have been demonstrated to inhibit α -synuclein aggregation *in vitro* [49]. Furthermore, β -synuclein has also been shown to inhibit α -synuclein aggregation *in vivo* [57]. Also, an increased expression of α - relative to β -synuclein has been reported in DLB brains compared to healthy controls, indicating that α -synuclein aggregation may be promoted when a critical ratio between the two synucleins is exceeded [58]. In addition, point mutations in the *SNCB* (Synuclein, beta) gene have been found in two unrelated DLB cases, further implicating the possible protective role of β -synuclein in LB disorders [59]. Indeed, *in vitro* experiments have shown that, by adding β -synuclein to α -synuclein in a 1:1 ratio, the aggregation of α -synuclein is delayed [49].

Recently it has been suggested that α -synuclein exists endogenously as a physiologically folded tetramer [60]. It was described that the α -synuclein tetramer was stable in solution and did not undergo aggregation over time compared to the monomer. In fact, most of the *in vitro* results in the literature are based upon the use of recombinant α -synuclein, which may not provide the best structural structure and function of the protein.

Genetics

It is generally assumed that both PD and DLB are caused by a combination of genetic and environmental factors in the context of the ageing brain. Although most cases of both disorders are sporadic, numerous genes, in which mutations cause either autosomal dominant or autosomal recessive inherited disease forms, have been described. Among these, *SNCA* (Synuclein, alpha (non A4 component of amyloid precursor)), *PRKN* (Parkin) and *LRRK2* (Leucine-rich repeat kinase 2) [61-66] are the most prevalent (for a list of disease genes, see *Table 1*). Overt α -synuclein pathology seem to be always found in subjects with *SNCA* and *LRRK2* mutations, but only in a minority of brains from *PRKN* mutation carriers [67, 68].

Table 1. List of known genes and identified disease loci linked to either autosomal dominant or autosomal recessive PD.

Locus	Chromosome	Gene	Inheritance
PARK1	4q21.3	SNCA	Autosomal dominant
PARK2	6q25.2-27	PARKIN	Autosomal recessive
PARK3	2p13	UNKNOWN	Autosomal dominant
PARK4	4p15	UNKNOWN	Autosomal dominant
PARK5	4p14	UCHL1	Autosomal dominant
PARK6	1p35-36	PINK1	Autosomal recessive
PARK7	1p36	DJ-1	Autosomal recessive
PARK8	12p11.2-q13.1	LRRK2	Autosomal dominant
PARK9	1p36	ATP13A2	Autosomal recessive
PARK10	1p32	UNKNOWN	Unknown

The α -synuclein gene *SNCA* has been mapped to the 4q21 region of chromosome 4 [69] (*Table 1*). The first mutation, A53T, was identified in Greek and Italian families in 1997 and was found to cause a dominantly inherited form of early onset PD [61]. Two more *SNCA* point mutations, A30P and E46K (*Figure 2*), were subsequently described to cause dominant forms of early-onset PD and DLB, respectively [70-72]. A further indication of the link between α -synuclein and PD was discovered when duplications and triplications of the wild-type gene were reported in a family in southern Sweden as well as in several other families world-wide [73, 74] (*Figure 2*). Interestingly, the 50% increase in gene dose for duplications was found to cause a typical PD phenotype, whereas the 100% increase seen with triplications was reported to result in a more severe phenotype with dementia, i.e. DLB.

Alpha-synuclein aggregation

In neurodegenerative diseases, misfolded proteins are the basic elements of the pathological deposits found intra- or extracellularly in the brain [75, 76]. In addition, general mechanisms that link early misfolded proteins to final insoluble deposits found in the brain have been suggested [77, 78] (*Figure 3*). For example, a study using the amyloid-beta ($A\beta$) peptide has shown that the initial stages of aggregation might involve formation of a pentamer/hexamer, which can continue to form larger oligomers or protofibrils [79].

For α -synuclein, the aggregation starts with a restructuring of the molecule from a natively unfolded monomer to a partially folded form, leading to self-assembly and formation of oligomers and protofibrils [80]. In addition, these species may recruit more monomers that finally results in the formation of insoluble fibrils [77, 80, 81]. A critical parameter for α -synuclein aggregation is the local protein concentration. Therefore, possible underlying mechanisms could include increased expression [82] or decreased degradation of α -synuclein [83] or a combination thereof. Furthermore, it has been shown that non-specific molecular crowding can accelerate α -synuclein fibrillization [84-86].

Moreover, point-mutations have been demonstrated to increase aggregation or oligomerization of α -synuclein [49, 87, 88]. Expression of the A30P mutant in living cells was shown to result in an increased level of higher molecular weight species (e.g. oligomers and protofibrils) as compared to wild-type α -synuclein [89]. Multiplication of the *SNCA* gene was found to promote oligomer formation, causing binding to cytoskeletal components and damaging of mitochondrial and cellular membranes [73]. In addition, oligomeric species of wild-type and mutant A53T α -synuclein have been observed in brain tissue from α -synuclein transgenic mice and PD patients [90]. Furthermore, elevated levels of soluble oligomers have also been detected in post-mortem brain extracts from DLB patients as compared to control brains [91].

The folding of a polypeptide into its unique three dimensional conformation is a complex process that is insufficiently understood [92]. It involves the search for the lowest possible energy state, i.e. a conformation of the molecule that is the most thermodynamically stable under physiological conditions. After synthesis of proteins on the ribosome, the folding process is initiated in different cellular compartments such as the cytoplasm, the endoplasmic reticulum (ER) and mitochondria. Correctly folded molecules are further transported within the cell to perform their proper biological function. However, if the protein is incorrectly folded, it is recognized and tagged with ubiquitin molecules for lysosomal and proteasomal degradation [93, 94] (*Figure 3*).

In vitro studies have shown that degradation of α -synuclein occur via the proteasome and that it is mediated by the carboxyl terminus of the HSP70-

interacting protein (CHIP) [95]. In addition, accumulating evidence suggests that α -synuclein is mainly degraded via lysosomal pathways and, in particular, with the help of macroautophagy [96] and chaperone-mediated autophagy (CMA) [97]. However, in contrast to monomers, oligomers and larger aggregates of α -synuclein were found to block proteasomal degradation [98, 99]. On the molecular level, α -synuclein is cleaved by metalloproteinase- (MMP) 1, 2, 3, 9 and 14, with the most efficient cleaving performed by MMP-3 [100].

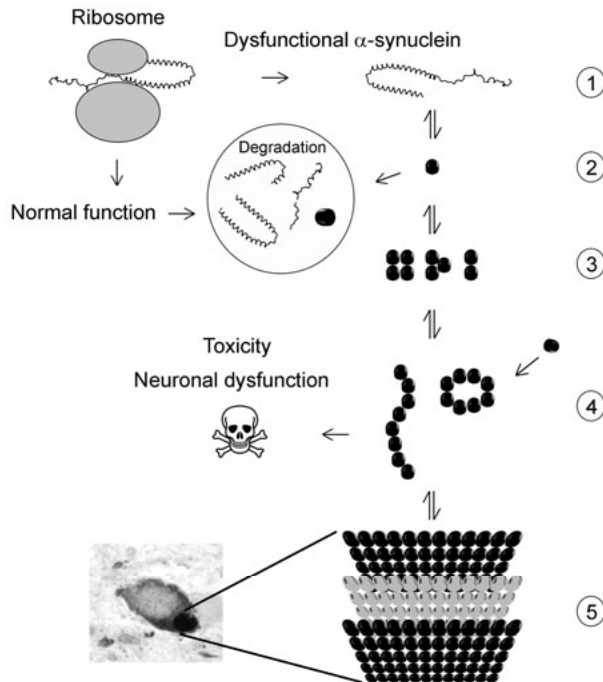


Figure 3. Alpha-synuclein misfolding, aggregation and degradation. Alpha-synuclein is synthesized, performs its normal function and is then degraded via lysosomal and proteasomal pathways. However, the natively unfolded monomer (1) can adopt a partially folded conformation (2), leading to self-aggregation and the formation of soluble intermediate species such as dimers, trimers or tetramers (3). In addition, these species can continue into soluble oligomers and protofibrils (4) that are believed to act as seeds for the recruitment of other α -synuclein molecules, which eventually form long insoluble fibrils (5).

Alpha-synuclein oligomers/protofibrils

There is growing evidence that α -synuclein oligomers, intermediate soluble forms in the aggregation pathway, have neurotoxic properties [89, 101, 102]. Such oligomeric forms of α -synuclein seem to be present within and outside of neurons [103, 104]. Thus, the intra and extracellular levels of oligomers are

highly likely to be in equilibrium with each other via various secretion mechanisms. In fact, oligomers of α -synuclein have been detected both in cerebrospinal fluid (CSF) and plasma from PD patients and their levels possibly correlate to the disease [105, 106]. Functional studies in transgenic mice have shown that α -synuclein oligomers are generated after lentiviral overexpression of monomeric protein and that the oligomers induce selective cell toxicity as shown by the loss of dopaminergic neurons [107, 108]. However, to track intermediate species *in vitro* during the aggregation process is a challenging task since these forms exist only during a very short period of time, after which they readily continue into insoluble fibrils. In order to overcome this, the use of chemicals to trap or isolate these forms can be used. The use of stabilizing molecules or oligomer inducing elements to generate intermediate forms of α -synuclein have been well documented [76, 101, 102, 109-122] (Table 2). Moreover, it has been shown that the induced oligomers can exhibit different functional properties, either causing or inhibiting toxicity to cells [101, 109], highlighting the structural complexity of the oligomerization process.

Table 2. Modifiers known to either cause oligomerization of α -synuclein by cross-linking mechanisms or by promoting aggregation.

Modifier	Promotion of aggregation	Cross-linking	Reference
Baicalein		X	Zhu <i>et al.</i> 2004
EtOH	X		Danzer <i>et al.</i> 2007
Ca ²⁺	X		Lowe <i>et al.</i> 2004
Transglutaminase		X	Konno <i>et al.</i> 2005
Disulfide bridges		X	Jiang <i>et al.</i> 2007
Dithyrosine		X	Souza <i>et al.</i> 2000
Dopamine		X	Cappai <i>et al.</i> 2005
DTT + metal ions	X		Cole <i>et al.</i> 2005
EGCG	X		Ehrnhoefer <i>et al.</i> 2008
Gangliosides	X		Martinez <i>et al.</i> 2007
DTBP		X	Cole <i>et al.</i> 2002
Nicotine	X		HONG <i>et al.</i> 2009
Nitration		X	Uversky <i>et al.</i> 2005
Rifampicin		X	Li <i>et al.</i> 2005
HNE	X		Qin <i>et al.</i> 2007

Oxidative stress

The human brain is highly vulnerable to oxidative stress or, more specifically, to reactive oxygen species (ROS). This vulnerability could be explained by the brain's high usage of oxygen, relatively low antioxidant defences and high content of polyunsaturated lipids [123, 124]. In addition, transition metals such as iron, copper and zinc are present in relatively high amounts and mediate catalyzing production of ROS [125]. Moreover, mitochondria, which play an important role in apoptotic events, also have a significant role in ROS metabolism linking oxidative stress to cell death, a key feature in neurodegenerative diseases [126].

One of the central events in the process of neurodegeneration-related oxidative stress is a chemical chain reaction known as lipid peroxidation. This sequence of events features the ROS attack and degradation of polyunsaturated lipids in lipoparticles and lipid bilayers (*Figure 4*). Among the end products, reactive aldehydes such as 4-oxo-2-nonenal (ONE) and 4-hydroxy-2-nonenal (HNE) have also been shown to readily modify proteins *in vitro* [127-129]. Importantly, increased levels of HNE modified proteins have been detected in nigral neurons of PD brains [110]. Moreover, HNE has been found to induce oligomerization of α -synuclein and cause toxicity to dopaminergic neurons *in vitro* [130]. More recent data also suggest that HNE induced α -synuclein oligomers may impair long term potentiation (LTP) by inducing changes in α -amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid (AMPA) receptor composition [Maiolino et al. submitted].

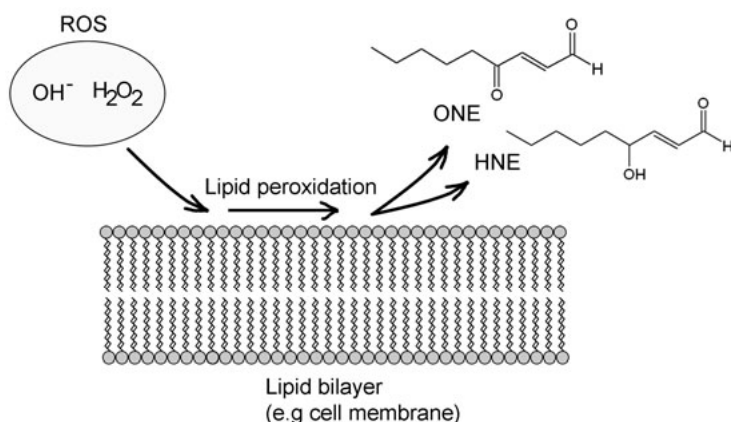


Figure 4. Reactive oxygen species (ROS) attack on a lipid bilayer. Neurodegeneration-associated oxidative stress is featured by a chemical chain reaction known as lipid peroxidation. This reaction features ROS attack on double bonds in polyunsaturated lipids. Reactive aldehydes, such as 4-oxo-2-nonenal (ONE) and 4-hydroxy-2-nonenal (HNE), are examples of end-products of this reaction.

The molecular structures of ONE and HNE are almost identical, with the only difference being that ONE features a carbonyl group at the C4 position whereas HNE has a hydroxyl group at the same site [131] (*Figure 5*). The primary reactions of ONE and HNE with amines are known as Michael addition and Schiff base formation. Both these reactions are reversible with the use of reducing agents such as Sodium Borohydride (NaBH_4) [131]. In addition, the C1 carbon (of the aldehyde group) in ONE is responsible for a Schiff base formation with nucleophiles, whereas the C2 or C3 carbon (the double bond) is responsible for Michael addition.

Moreover, ONE has been described as being more reactive with protein nucleophiles and more neurotoxic [132] as compared to HNE. The electrophilic HNE mainly shows Michael addition reactivity with nucleophilic amino acid residues at position C3. The highest affinity for HNE at this site is seen for cysteine, followed by histidine, lysine and arginine respectively. In contrast, the bifunctional electrophile ONE reactivity order is: cysteine Michael addition > lysine Schiff base formation > histidine Michael addition > arginine/lysine Michael addition [131] (*Figure 5*).

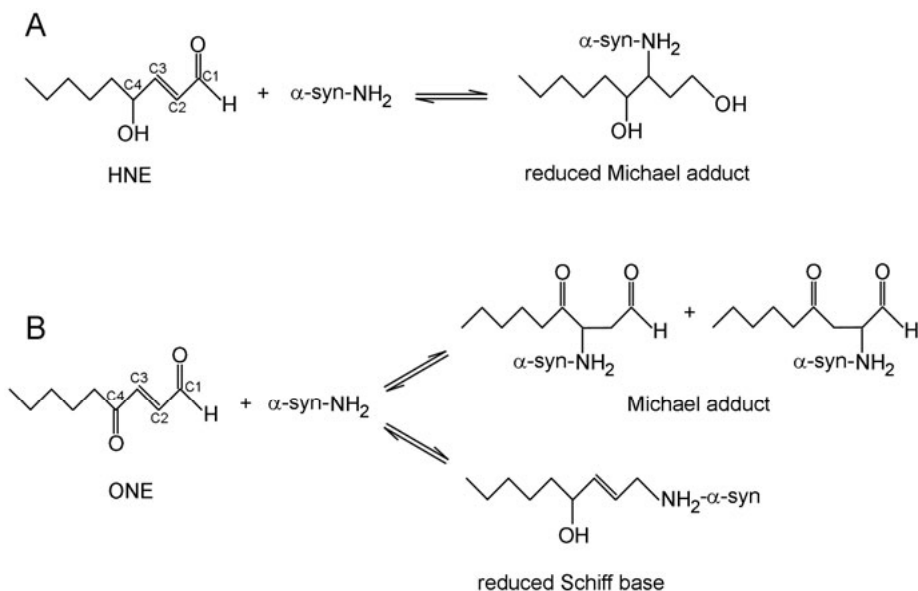


Figure 5. Primary reactions for HNE (A) and ONE (B). These two aldehydes feature similar molecular structures. However, ONE has a carbonyl group at the C4 position whereas HNE has a hydroxyl group at the same site. The primary reactions of ONE and HNE with amines are Michael addition and Schiff base formation. These two reactions are reversible via sodium borohydride (NaBH_4). In addition, the C2 or C3 carbon (the double bond) in ONE is responsible for Michael addition with nucleophiles and the C1 carbon (the aldehyde group) is responsible for a Schiff base formation. HNE has mainly shown to react with nucleophilic amino acid residues with Michael addition.

Alpha-synuclein lacks both arginine and cysteine residues, resulting in 16 possible sites (1 histidine, 15 lysine) for adduct formation with ONE and HNE. It has been shown that ONE favors Schiff-base formation with Lysine residues and Michael addition with histidine residues [131, 132]. In contrast, reactions with HNE on lysine and histidine residues have shown mainly to exhibit Michael addition. (*Figure 5*)

The role for reactive aldehydes in α -synuclein misfolding may be of importance also *in vivo*. Firstly, besides being highly toxic themselves, lipid peroxidation metabolites can readily interact with proteins and alter their normal function [132-134]. Secondly, protein adducts (e.g. oligomers) generated after reactive aldehyde modification of α -synuclein have been shown to be very stable and can induce cell damage [130].

Alpha-synuclein and cellular dysfunction

To date, there is evidence to suggest that protein-mediated dysfunction in neurodegenerative diseases is due to loss of that protein's physiological function, gain of a toxic function, or a combination of both. For example, accumulating data from studies with α -synuclein suggest a gain of toxic function after conversion of monomers to oligomers both *in vitro* [87] and *in vivo* [108]. Alpha-synuclein has also been proven to, directly or indirectly, induce cellular dysfunction by inhibiting proteasomal or lysosomal degradation [135-137], Golgi fragmentation [138], ER stress, mitochondrial cell death [139] or the formation of pores in cell membranes [140, 141]. As α -synuclein has been shown to accumulate in cell bodies, synapses and neuronal processes in the PD brain, it is likely that these accumulations are accompanied by altered neurotransmission [142]. Indeed, α -synuclein overexpressing mice displayed decreased neurotransmitter release accompanied with abnormal size and shape of the synaptic vesicles [143].

The tau protein is another example of a protein aggregating in neurodegenerative disorders. The normal function of tau is to stabilize microtubules [144]. However, in the AD brain tau is hyperphosphorylated, which can lead to its detachment from microtubules and thereby a loss of function [145]. Thus, the concentration of soluble cytoplasmic tau is increased, resulting in aggregation and formation of insoluble filaments as tangles, a key feature in AD [146]. In addition, *in vitro* generated oligomers of tau have been shown to be neurotoxic, suggesting that tau pathology can also cause a gain of toxic function [147].

Propagation of alpha-synuclein pathology

The concept of “seeding” of brain pathology was first observed for Creutzfeldt-Jacob’s disease (CJD) and other prion disorders [148-150]. Since then it has been established that the cellular prion protein (PrP^C) can change its conformation to a β -sheet rich form (PrP^{Sc}) that in turn can act as a seed to affect other PrP^C molecules to adopt this pathological conformation [151].

Studies with the A β peptide have suggested a similar transmission of pathology. When transgenic A β PP brain homogenate and AD brain homogenate were intracerebrally injected into brains of transgenic mice, endogenous A β was deposited in the hippocampus and neocortex [152, 153].

Similar results have been described for the tau protein, where wild-type tau expressing mice were injected with brain homogenate containing filamentous aggregates of tau [154]. The injected material induced tau pathology, which in turn was shown to spread to other parts of the brain.

It has been suggested from humans [155] as well as from animal- [156] and cell- [104] models that α -synuclein pathology can be transmitted from neuron to neuron. Evidence suggest that α -synuclein can translocate into neighboring neurons via endocytosis [157, 158]. In addition, propagation of intracellular α -synuclein was shown to be induced by extracellular addition of oligomeric forms of α -synuclein with a distinct morphology [159]. However, fibrils of α -synuclein have also been shown to induce seeding [160].

In experimental treatment programs, embryonic mesencephalic neurons have been transplanted into the striatum of PD patients. The grafts were shown to survive up to 16 years with detectable neuromelanin in the transplanted neurons [155, 161, 162]. However, post-mortem examination of the grafted brains revealed that α -synuclein pathology was present in the transplants, suggesting that the pathology had spread from the host into the grafted neurons [155, 161]. The grafts were shown to contain both LBs and LNs, i.e. similar to the host tissue pathology. Although protein spreading is the most likely explanation for the presence of α -synuclein deposits in the grafted neurons, it cannot be excluded that other factors, such as oxidative stress, growth factors and inflammation, may have influenced the formation of pathology in the transplanted neurons [163].

Immunotherapy

Immunotherapy is a promising disease modifying strategy for neurodegenerative disorders. It was demonstrated that active vaccination with aggregated A β in A β PP transgenic mice prevented younger mice from developing A β pathology and reduced insoluble A β in the brains of older animals [164]. These biochemical findings were accompanied by other reports, describing prevention of age-dependent behavioral impairment and

memory loss after active vaccination [165, 166]. In a human clinical trial (AN1792) based on immunization with A β fibrils, there was less plaque burden and also evidence of reduced cognitive decline in patients producing high titers of A β antibodies [167, 168]. However, the trial was halted due to the occurrence of meningoencephalitis, in a small number of patients [169].

Also passive immunization (i.e. delivery of A β antibodies) has been evaluated on A β PP transgenic mice models. Such treatment has shown similar effects as active immunization and can avoid the proinflammatory T-cell response and thereby hopefully give fewer side effects [170, 171].

The use of immunotherapy as a disease modifying strategy has also been studied in animal models for LB disease. In one study, α -synuclein transgenic mice were actively immunized with recombinant α -synuclein, resulting in a significant reduction of aggregated α -synuclein in neurons and synapses [172]. In a recent study with passive immunization, monoclonal α -synuclein antibodies could translocate into the CNS of transgenic mice, bind to α -synuclein intracellular inclusions and facilitate their degradation via lysosomes [173].

There are a few suggested mechanisms for the clearance of protein deposits associated with immunotherapy. One possible mechanism could be that the pathology is cleared via microglia and Fc-receptor mediated phagocytosis. Another mechanism could be a more direct effect of the antibodies, inhibiting further aggregation due to antibody binding or, simply, dissociation of the fibrils [174].

Aims

Overall aim

The overall aim of this thesis was to characterize early events of α -synuclein aggregation with a particular focus on the formation of oligomers and protofibrils. Also, we wanted to investigate biochemical and functional effects of such protein forms *in vitro* and *in vivo* and, finally, explore whether oligomerization could be prevented in a cellular model.

Specific aims

- I To investigate structural implications of the molecular interactions between ONE and α -synuclein.
- II To evaluate and compare biochemical, biophysical and functional features of ONE and HNE induced α -synuclein oligomers.
- III To investigate potential pathology propagating effects of ONE induced α -synuclein oligomers *in vitro* and *in vivo*.
- IV To explore whether monoclonal α -synuclein antibodies could inhibit protein oligomerization in a cell culture model.

Results and discussion

Characterization of *in vitro* generated alpha-synuclein oligomers

For **Paper I** and **II**, we sought to investigate the biochemical and biophysical properties of *in vitro* generated α -synuclein oligomers, using several established methods.

After incubation with ONE for 18 h at 37 °C the α -synuclein monomers were consumed into a larger oligomeric form corresponding to a molecular weight of ~2000 kDa (**paper I, figure 2 and paper II, figure 1**), as analyzed by size exclusion-high performance liquid chromatography (SEC-HPLC) (*Figure 6A*). The HNE modified α -synuclein generated under the same conditions was found to elute with a similar retention time, corresponding to oligomeric species with an apparent molecular weight of ~2100 kDa (*Figure 6B*). However, ONE modified α -synuclein eluted as a slightly broader peak as compared to HNE modified α -synuclein, indicating that the ONE induced oligomers comprised a more heterogeneously-sized population.

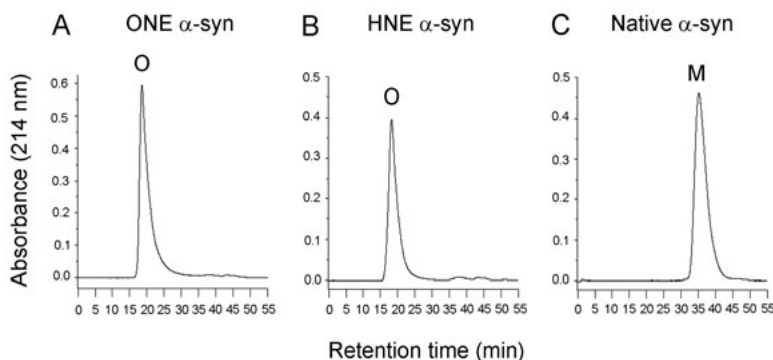


Figure 6. Size-exclusion chromatography shows ONE (A) or HNE (B) induced α -synuclein oligomers and α -synuclein monomers (C). The α -synuclein oligomers (A and B) eluted at a molecular weight of ~2000 kDa. The α -synuclein monomer (C) eluted at a molecular weight of ~50 kDa

Native α -synuclein eluted as a monomeric peak corresponding to a molecular weight of ~47 kDa (*Figure 6C*). Monomeric α -synuclein is known to elute at a higher molecule weight compared to its sequence determined mass which is 14460 Da. This size difference can be explained by the effect on its Stokes

radius since α -synuclein has been defined as having a natively unfolded structure [175]. On the other hand, α -synuclein has recently been suggested to exist as a physiologically folded tetramer, eluting at 58 kDa [60]. However, as evidenced by the characteristic minimum at 198 nm, our native α -synuclein displayed a random coil structure in the far-UV circular dichroism spectra. This is in contrast to the α -helical secondary structure reported by Bartels and colleagues [60], and thus it is likely that the peak for native α -synuclein in SEC-HPLC represents monomeric protein.

For comparison, the interactions between ONE and the other members of the synuclein family were also investigated. Native β - and γ -synuclein eluted as single monomeric peaks with a molecular weight of \sim 50 kDa and \sim 47 kDa, respectively. In comparison to α -synuclein, both β - and γ -synuclein were found to oligomerize to a lesser extent when incubated with the same concentration of ONE. When ONE modification was carried out in Tris/NaCl buffer, only γ -synuclein formed oligomeric species to some extent, whereas β -synuclein almost exclusively eluted as a monomeric form. The two γ -synuclein oligomeric peaks corresponded in size to \sim 1850 kDa and \sim 850 kDa, respectively.

Taken together, α -synuclein was more prone to form larger oligomeric species, as compared to β - and γ -synuclein, further implicating α -synuclein's inherent ability to self-assemble [49]. In a control experiment, BSA was modified by ONE using a similar molar excess, but no oligomeric forms could be detected by SEC-HPLC analysis (data not shown). These results illustrate that oligomerization is dependent on the structure of the protein and is not a general phenomenon.

Furthermore, the far-UV circular dichroism spectrum displayed a major conformational change in the secondary structure of α -synuclein after incubation with ONE and HNE, revealing an extensive β -sheet formation as evident by its negative minimum near 217 nm (**paper I, figure 4 and paper II, figure 2**).

After incubation with ONE, large non-hollow structures 4–8 nm in height and 40–80 nm in width were formed as demonstrated by atomic force microscopy (AFM) (**paper I, figure 3 and paper II, figure 3**) (*Figure 7*). In sharp contrast, HNE modified α -synuclein oligomers had a different morphology and appeared as curved protofibril-like rods with dimensions of 2–4 nm in height and 100–200 nm in length. Moreover, the morphology of the HNE induced α -synuclein oligomers differed compared to what has been described in the literature, highlighting the heterogeneity of species formed by such modifications [130] (*Figure 7*).

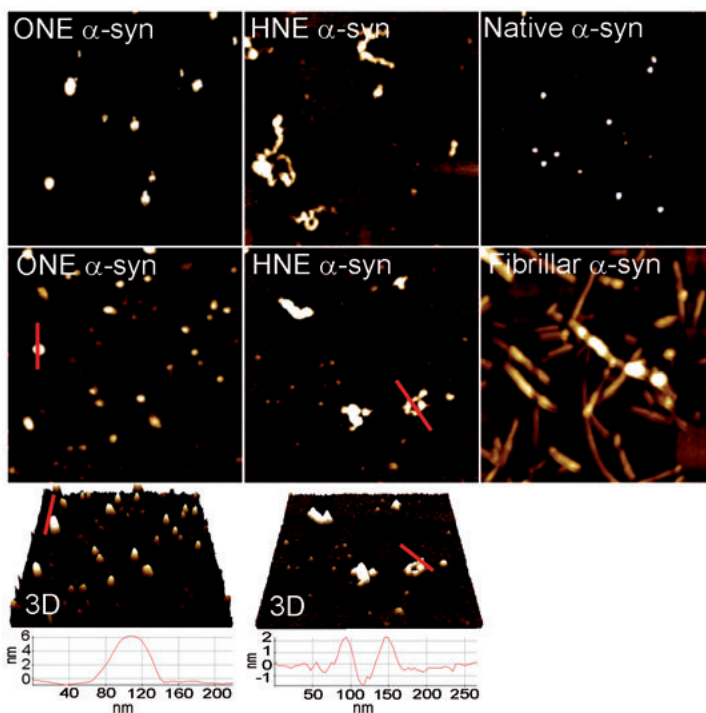


Figure 7. Atomic force microscopy analyses of α -synuclein. ONE induced α -synuclein oligomers were amorphous and round with dimensions of 4-8 nm in height and 40–80 nm in width. In contrast, HNE induced α -synuclein oligomers exhibited protofibril-like morphology with dimensions of 2-4 nm in height and 100–200 nm in length. Monomeric α -synuclein displayed round smaller species with dimensions between 1-2 nm in height and 20-30 nm in diameter. Fibrillar α -synuclein aged for 4 d displayed typical amyloid-like fibrils with dimensions of up to 1 μ m in length, 6-12 nm in height and up to 30 nm in width. Scan size 1 μ m.

The AFM analyses for monomeric α -synuclein (**paper I, figure 3** and **paper II, figure 3**) revealed a more homogenous appearance of small round structures with dimensions of about 20-30 nm in width and 1-2 nm in height. The identification of α -synuclein monomers with AFM has been previously described, albeit with less resolution of the acquired images [176, 177]. Although it is debatable whether a monomer of α -synuclein is within the detection boundaries of the AFM system, it is suggested that the interaction between α -synuclein and the sample surface (e.g. the mica substrate) slightly exaggerates its molecular size as compared to its predicted size in solution [130].

To study the stability of ONE and HNE induced α -synuclein oligomers, the samples were also analyzed by Western blot (WB) (**paper I, figure 4** and **paper II, figure 5**). After SDS-treatment of the ONE induced α -synuclein oligomers, a band exceeding 300 kDa, and not entering the stacking gel, was observed. A weak high molecular weight smear (100-300 kDa) was also

detected following increased exposure. In sharp contrast, HNE induced α -synuclein oligomers treated with SDS displayed an extensive series of bands including monomeric and multimeric forms of the protein, as well as high molecular weight bands exceeding 100 kDa. In agreement with these findings ONE has been shown to be a more potent cross-linker than HNE because of its carbonyl group instead of a hydroxyl group at the C4 position [132]. Under these denaturing conditions, monomeric α -synuclein was observed as a single band at 16 kDa.

To further test the structural stability, oligomeric samples were subjected to increasing concentrations of urea (**paper II, figure 5**). After the urea treatment of the ONE induced α -synuclein oligomers, one band that did not enter the stacking gel was seen at all tested concentrations. In addition, when the urea concentration was increased, a high molecular weight smear of α -synuclein species at ~150-300 kDa was observed. In contrast, urea treatment of HNE induced α -synuclein oligomers revealed greater urea sensitivity, indicated by a strongly labeled high molecular weight smear (75-300 kDa) and a monomeric protein band. This effect increased with higher concentrations of urea.

Finally, to study the compactness of the generated α -synuclein oligomers, modified samples were treated with proteinase K (PK) and analyzed by WB (**paper II, figure 5**). Unexpectedly, HNE modified α -synuclein showed greater stability towards PK digestion than ONE modified α -synuclein, as evident by WB analysis. Notably, in the HNE modified samples the most SDS sensitive material (i.e. low molecular weight α -synuclein) became degraded and disappeared from the immunoblot with increasing PK concentrations. At the highest PK concentration only a high molecular weight smear (~150-300 kDa) was observed. For ONE modified α -synuclein, a band above 300 kDa and a faint high molecular weight smear (~150-300 kDa) was observed at low PK concentrations, whereas the immunoreactivity was totally abolished at the highest concentration. In addition, dot blot analysis of PK-treated ONE or HNE induced α -synuclein oligomers verified a greater PK-resistance for HNE induced α -synuclein oligomers (**paper II, figure 5**).

Taken together, these findings indicate that although HNE induced α -synuclein oligomers were found to be less covalently cross-linked and less SDS and urea- stable, at least parts of the oligomeric structure were more compact and less accessible to PK digestion, as compared to ONE induced α -synuclein oligomers.

Alpha-synuclein aggregation

To study aggregation characteristics of α -synuclein, a Thioflavin T (ThT) kinetic assay was employed (**paper I, figure 4; paper II, figure 4 and paper III, figure 2**) (*Figure 8*).

For the ONE induced α -synuclein oligomers, the ThT assay showed a weak increase in fluorescence which coincided with the appearance of oligomeric species, as verified by SEC-HPLC and AFM analyses (**paper I, figures 2, 3 and paper II, figures 1, 3**). In addition, ThT measurements of the HNE induced α -synuclein oligomers exhibited a higher ThT signal compared to ONE modified α -synuclein (**paper II, figure 4**). Prolonged incubation of ONE and HNE- modified α -synuclein gave no indications that the oligomers were on a fibrillogenic pathway, in concordance with properties of other reported off pathway oligomers [178-180]. On the other hand, monomeric α -synuclein aggregated for five days resulted in a high ThT fluorescence, indicating the formation of amyloid-like fibrils (**paper I, figure 4 and paper II, figure 4**).

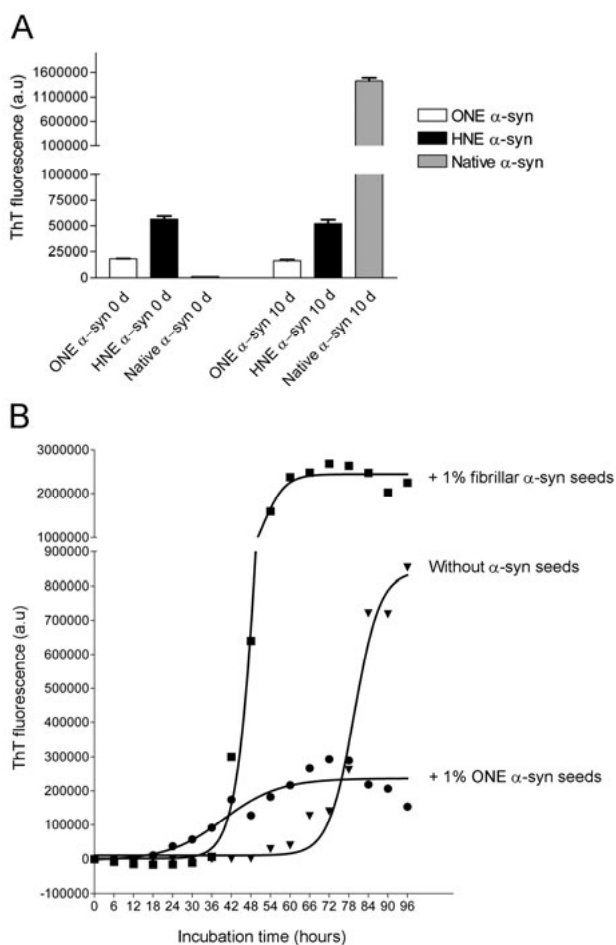


Figure 8. Aggregation profiles of different species of α -synuclein, as determined by the Thioflavin T assay. When ONE or HNE induced oligomers were incubated for 10 d under aggregation promoting conditions, the samples did not continue to form larger aggregates, as shown by moderate ThT signals that did not change during the experiment (A). Native monomeric α -synuclein formed amyloid-like fibrils as evident by a large increase in ThT signal after 10 d of incubation (A). Furthermore, to study potential seeding effects of the ONE induced α -synuclein oligomers, monomeric α -synuclein were incubated with either 1% α -synuclein oligomers or 1% sonicated α -synuclein fibrils (B). The ThT profile indicated an early increase in aggregation of α -synuclein with 1% ONE α -synuclein oligomer seeds at 24 h compared both to preparations seeded with 1% fibrils or preparations without seeds (B).

To study potential pathology-propagating effects of α -synuclein, ONE induced oligomers were added to monomeric α -synuclein preparations (**paper III, figure 2**). Interestingly, an earlier start of the aggregation of the monomeric α -synuclein was seen after addition of 1% ONE induced α -synuclein oligomers, as compared to non seeded samples and samples seeded with fibrillar α -

synuclein. However, the aggregation rate of the oligomer seeded samples was slower compared to samples seeded with fibrillar α -synuclein and non seeded samples. In agreement with these observations, *in vitro* generated α -synuclein oligomers have been described to propagate aggregation, acting as a nucleus for the initiation of the process [159]. In the current study, the oligomer seeded samples ended on a much lower final ThT signal as compared to non seeded samples and samples seeded with fibrillar α -synuclein. Furthermore, when 1% HNE induced α -synuclein oligomers were added to monomeric α -synuclein there was no seeding effect (data not shown).

As measured by AFM, α -synuclein aggregated (without α -synuclein seeds) for five days showed typical amyloid-like fibrils with dimensions of 30 nm in width and 200-800 nm in length. Interestingly, the fibrils formed with the ONE induced α -synuclein oligomers had reduced height and width compared to fibrils seeded with either 1% fibrils or fibrils formed without seed.

There are numerous reports describing AFM imaging of *in vitro* generated α -synuclein fibrils, all suggesting different sizes of the aggregated species [181-186]. These discrepancies can be explained, firstly, by the effect of different pre-treatment methods before analysis and, secondly, (and perhaps more importantly) by which aggregational state that the samples had adopted in the preceding *in vitro* aggregation process.

To also investigate potential seeding effects of the ONE induced α -synuclein oligomers on living cells, we adopted the BiFC assay [187] (**Methodological considerations, figure 12**). This technique allows the study of protein oligomerization in cells by expressing two non-fluorescent green fluorescent protein (GFP) fragments fused to α -synuclein. When brought together, the two fragments reconstitute and form a complete GFP molecule, emitting fluorescence [89]. Thus, intracellular dimerization/early oligomerization can be efficiently monitored with the BiFC assay. The α -synuclein-hemi:GFP constructs were expressed in neuroglioma cells followed by addition of either ONE induced α -synuclein oligomers or PBS, after which aggregation (as measured by GFP fluorescence) could be monitored at different time points (**paper III, figure 3**). In PBS treated cells expression of α -synuclein-hemi:GFP resulted in green fluorescence starting at 6 h with increasing intensity up to 24 h. In contrast, α -synuclein-hemi:GFP expressing cells treated with ONE induced α -synuclein oligomers displayed green fluorescence signal at the 4 h time point but the fluorescence was undetectable at the later time points.

The effect of the ONE induced α -synuclein oligomers on the intracellular dimerization/oligomerization pattern is intriguing. However, experimental evidence indicate that α -synuclein aggregation can be reversed using remodelling agents [180], thus suggesting dissociation of the generated α -synuclein aggregates. In **Paper II (figure 8)** it was demonstrated that cell-uptake of ONE induced α -synuclein oligomers was first detected after four h of incubation. This suggests that the loss of GFP fluorescence, observed here

at time points after 4 h, most likely depends on internalization of ONE induced α -synuclein oligomers. Alternatively, the ONE induced α -synuclein oligomers interact with the respective α -synuclein hemi:GFP molecules, thereby interfering with the reconstitution of the GFP molecule.

In a previous seeding study, it was shown that the morphology of the α -synuclein oligomer was important for both cell uptake and seeding propensity [101]. In addition, the authors could demonstrate formation of inclusions in the oligomer treated cells. In yet another study, extracellular addition of α -synuclein oligomers to primary neurons in culture resulted in large cell-associated aggregates within 4 h [159]. The results from these two studies were in contrast to the current work, as it was not able to detect any inclusions within the cells.

In order to explore possible seeding effects *in vivo*, ONE induced α -synuclein oligomers were intracerebrally injected in (Thy1)-h [A30P] α -synuclein transgenic mice (**paper III, figure 4**). Two different doses of α -synuclein (40 or 400 ng, diluted in wild-type mouse brain homogenate), as well as brain homogenate only, were injected in three groups of five-eight mice. For months after injection, the mice were sacrificed. Five to ten sections around the injection area were stained with the PK-PET-blot method [188]. Briefly, this technique features the addition of a tissue slice to a nitrocellulose membrane followed by PK treatment. The mice in the control group displayed PK resistant α -synuclein in or around the injected area for only one of eight animals. For the mice injected with the low dose of ONE induced α -synuclein oligomers PK resistant α -synuclein was detected at or around the injection site in three out of seven animals. Mice injected with the high dose of ONE induced α -synuclein oligomers did not display PK-resistant α -synuclein material at or around the injection site in any of the animals.

The neocortex was chosen as injection site, as this area normally contains very few PK-resistant aggregates but still displays an overexpression of human A30P α -synuclein [189, 190]. Thus, for seeding purposes, this area should have contained robust amounts of non aggregated monomeric α -synuclein accessible for recruitment into fibrils.

In **paper II (figure 5)** we could show that the ONE induced α -synuclein oligomers were degraded by treatment with PK. Because of the low amount of oligomers injected into the mice as well as the PK sensitivity of the seeding material, it would not have been possible to observe the injected oligomers using the PK-PET-blot method. We could therefore conclude that any potential PK resistant staining observed at or around the injection site would come from newly formed aggregates of α -synuclein due to seeding effects.

We chose the four month incubation time between injection and analysis based on the results from a separate study in which A β brain homogenate was administered into the brain of transgenic A β PP mice. In that study, no apparent seeding of pathology was seen one month after the injection, but robust seeding could be demonstrated after four to five months [152]. The use

of synthetic oligomeric species alone failed to induce A β deposition in that study, implying that other factors present in the brain may be necessary for seeding/propagation of pathology. In addition, the multimeric A β species used in that study were perhaps not stable enough and became consequently degraded as they were injected into the living mouse brain [152]. In contrast, we showed that the ONE induced α -synuclein oligomers were stable and not on the pathway towards becoming fibrils (**paper I, figure 4 and paper II, figure 4**).

Functional effects of alpha-synuclein oligomers

To explore functional cell biological aspects, human neuroblastoma cells were subjected to aldehyde induced α -synuclein oligomers followed by various analyses (**paper II, figure 7 and 8**).

To investigate potential toxic effects of ONE or HNE induced α -synuclein oligomers, SH-SY5Y neuroblastoma cells were exposed to modified samples in a dose-dependent manner (**paper II, figure 7**). Both ONE and HNE induced α -synuclein oligomers led to reduced cell viability compared to native (i.e. monomeric) preparations, starting at 5 nM and the reduction increased with higher oligomer concentrations. For example, a significant difference ($p < 0.05$) could be observed at 5 nM for ONE modified α -synuclein compared to monomeric protein, whereas both ONE and HNE modified samples showed significant decrease ($p < 0.01$) in cell viability at 50 nM. These results are consistent with previous observations. In one study, HNE modified α -synuclein was shown to cause a decrease in neurotransmitter uptake, although only at the higher concentration of 5 μ M [130].

Our study is the first report showing that ONE induced α -synuclein oligomers cause cell toxicity. However, these findings could also be said to contradict results from previous studies, in which other α -synuclein off-pathway oligomers were shown to be non toxic [109, 179]. Possibly, this discrepancy could imply that α -synuclein oligomers formed as a result of lipid peroxidation are more toxic than other types of α -synuclein off pathway oligomers. In addition, the observed toxic effects were fairly pronounced, as the ONE induced α -synuclein oligomers caused cell toxicity at lower concentrations than what has been described even for on pathway oligomers formed by other amyloidogenic proteins. For example, A β was found to cause cell toxicity at 500 nM [191] whereas transthyretin was cell toxic at 1 μ M [192]. Furthermore, formaldehyde-induced tau aggregates (2 μ M), which morphologically resembled the ONE induced α -synuclein oligomers, also gave rise to a reduction in cell viability [193]. In our study, no significant difference in toxicity between ONE and HNE induced oligomer treated cells could be detected.

To study the interaction and potential cellular uptake of monomeric and oligomeric forms of α -synuclein, the SH-SY5Y cell line was treated at different time points (**paper II, figure 8**). Native and ONE or HNE induced α -synuclein oligomers conjugated with a fluorescent dye were added to the cells and incubated for 4 h in a concentration dependent manner to determine the ratio of protein uptake. By fluorescence microscopy, cells treated with dye-labeled ONE induced α -synuclein oligomers showed occasional small round inclusions in the cell. In contrast, cells incubated with fluorescently labeled HNE induced α -synuclein oligomers displayed a high degree of interaction with most of the cells. Fluorescently labeled monomeric α -synuclein could be observed throughout the whole soma and inside the nucleus of treated cells. The intracellular localization of the internalized protein was not entirely surprising as α -synuclein is known to be largely abundant in the cytosol [194] as well as in the nucleus [195, 196] of neurons in the mammalian CNS. Furthermore, α -synuclein has been described to be associated with the cell membrane [194]. However, the current study did not seek to answer whether the exogenously added α -synuclein was translocated to the cell membrane, and therefore we cannot further strengthen those observations. In the current study, all tested species of α -synuclein were internalized to varying degrees. However, these results are in contrast to another study which could not demonstrate internalization of either pre-formed fibrils or monomers of α -synuclein using a HEK-293 cell line [160].

A growing body of evidence suggests that both monomers and oligomers of α -synuclein can pass cell membranes [158, 197]. Furthermore, translocation of α -synuclein into cells has been shown to occur rapidly, with detectable protein levels already within five minutes [198]. However, the mechanism of α -synuclein uptake remains unknown.

Inhibition of alpha-synuclein aggregation

To investigate potential inhibiting effects on α -synuclein aggregation by monoclonal antibodies, H4 neuroglioma cells overexpressing α -synuclein were treated for different lengths of time and the rate of aggregation was monitored using the BiFC assay (**paper IV, figure 5**) (*Figure 9*).

Forty-eight hours after transfection of the α -synuclein hemi:GFP constructs, green fluorescence could be detected in the cell soma and the nuclei in approximately 50% of the cells, indicating α -synuclein dimerization/oligomerization. These results are in line with previous work, suggesting that different subcellular locations provide distinct environments for dimerization/oligomerization [89]. Furthermore, results from **Paper III (figure 3)** indicate that the expression of these α -synuclein hemi:GFP constructs occurs rapidly, as verified by GFP detection between the 0 h and 4

h sampling time points. However, the exact levels of α -synuclein were not quantified.

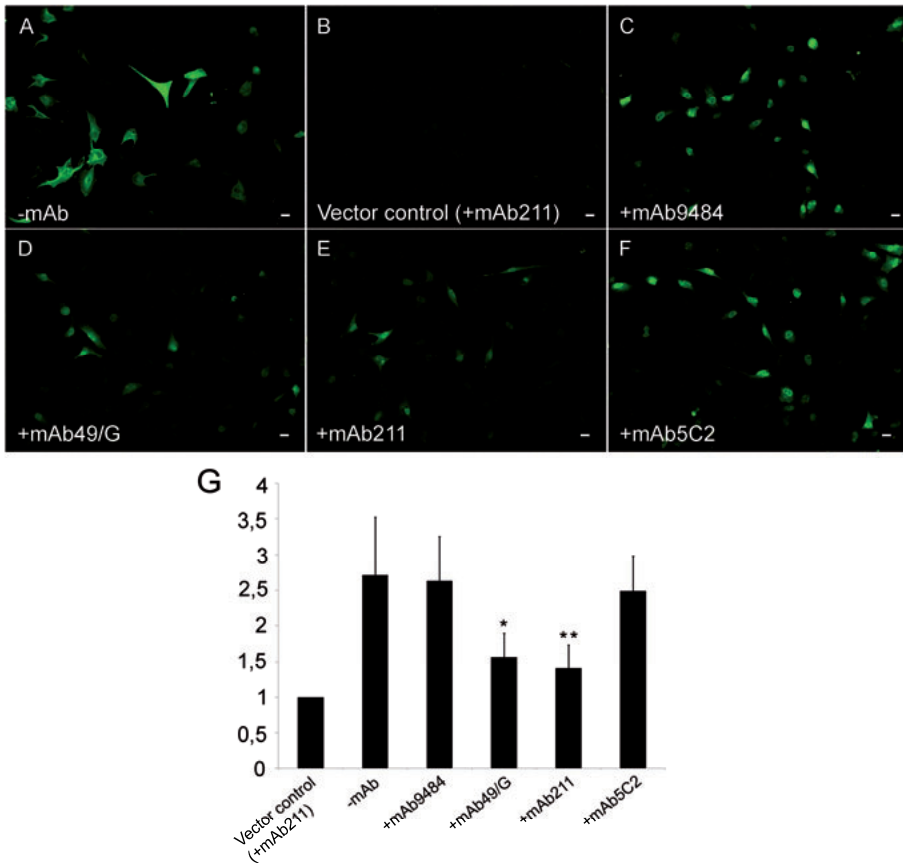


Figure 9. *Alpha-synuclein dimerization/oligomerization, as shown by GFP fluorescence reconstitution.* After forty-eight hours of incubation non-treated cells (-mAb) displayed robust GFP fluorescence in the cell soma and nucleus (A and G). In contrast, cells treated (+mAb) with the α -synuclein oligomer selective antibodies mAb49/G and mAb211 demonstrated a significant reduction in GFP fluorescence compared to non-treated cells (-mAb) (* $p < 0.05$, ** $p < 0.01$) (1.4- and 1.5-fold over expression to vector controls respectively) revealing less dimerization/oligomerization (Fig. D, E and G). Cells treated with the N-terminal α -synuclein mAb5C2 antibody did not show any reduction (2.5-fold over expression to vector controls) in GFP fluorescence, suggesting no effect on the formation of dimers/oligomers (Fig. F and G). With the monoclonal antibody mAb9484 against GAPDH, no apparent effect (2.6-fold over expression to vector controls) on dimerization/oligomerization was detected (Fig. C and G). 20x magnification. Scale bar 20 μ m.

The fluorescence in α -synuclein hemi:GFP transfected cells corresponded to a robust increase in signal intensity (2.7-fold overexpression to vector controls). Furthermore, in parallel experiments, α -synuclein antibodies were added to the

cell media immediately after transfection. The α -synuclein oligomer selective antibody mAb49/G and the C-terminal antibody mAb211 reduced the GFP fluorescence significantly (1.4- and 1.5-fold overexpression as compared to vector controls, respectively, $p < 0.05$, $p < 0.01$). In contrast, when the α -synuclein mid-region antibody mAb5C2 raised against the non-A β component (NAC) region was added to cells, there was no reduction (2.5-fold overexpression to vector controls) of GFP fluorescence. In agreement with these findings, the C-terminal of α -synuclein has also previously been shown to be an effective target for reduction of aggregation by expression of single chain fragments i.e. intrabodies [199]. Also, we (**paper II, figure 6**) and others [200] have described that oligomers and fibrils of α -synuclein expose C-terminal epitopes and α -synuclein antibodies directed against such epitopes are more efficient in clearing α -synuclein pathology in transgenic mice [173]. Along the same lines, the lack of effect on lowering dimer/oligomer levels for the 5C2 antibody in the current study could be explained by the fact that its hydrophobic NAC-region epitope (61-95) is hidden in the oligomeric core [56]. The inhibiting effect on oligomerization by mAb49/G was to some extent expected, as we believe that this antibody recognizes an epitope exclusively present in the oligomeric structure of α -synuclein.

To assess potential antibody-related toxicity (**paper IV**), the area of the cell nucleus was measured and analyzed by ImageJ from the mAb5C2 and mAb9484 treated cells. As measured by fluorescence microscopy, α -synuclein hemi:GFP transfected cells treated with antibodies appeared smaller in size compared to untreated cells. However, as compared to non treated cells (-mAb) there was no difference in the area of the cell nuclei, suggesting normal nuclear processes and thus no signs of pycnosis (*Figure 10*).

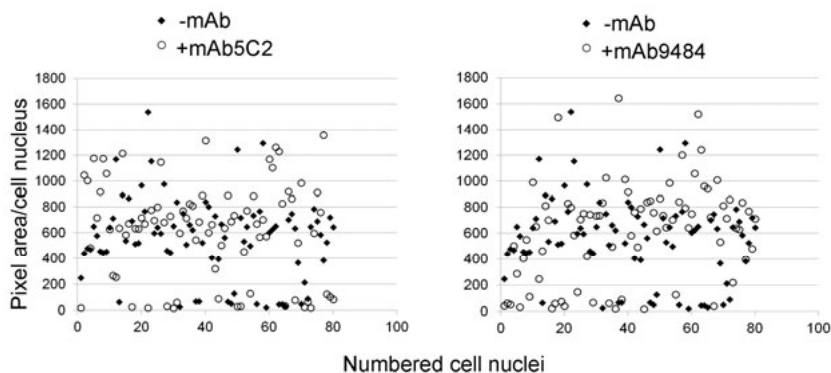


Figure 10. Representation of individual cell nuclei areas from antibody treated and non treated cells. As revealed by image analysis, cells treated with the α -synuclein antibody mAb5C2 and the GAPDH antibody mAb9484 display similar cell nuclei areas as compared to non treated cells, suggesting normal nuclear processes and few signs of pycnosis.

The use of immunotherapeutic strategies to clear (or perhaps prevent) abnormal protein aggregates has emerged as a promising tool for treatment of neurodegenerative disorders. Also, disorders with aggregated α -synuclein may be targeted with immunotherapy and active immunization in transgenic mice has indeed been shown to reduce the accumulation of α -synuclein in brain [172]. Although in that study it was proposed that immunization result in degradation of α -synuclein via the lysosomal pathway, it is still largely unknown by which mechanisms intraneuronal α -synuclein aggregates can be cleared [172].

To further investigate antibody effects on α -synuclein oligomerization, we utilized sandwich ELISA (**Methodological considerations, figure 13**). Levels of α -synuclein were measured in cell lysates and conditioned media from wells under the various experimental conditions (**paper IV, figure 6**). Separate studies have shown that passive immunization with A β and α -synuclein antibodies on cell and animal models could clear protein pathology, probably via autophagy [173, 201]. Along the same lines, our results suggest that the levels of α -synuclein were decreased both in cell lysate and conditioned media after antibody treatment, indicating an increased degradation of the α -synuclein species.

The current study revealed that the N-terminal specific mAb5C2 antibody influenced α -synuclein levels in both cell lysate and conditioned media without affecting oligomer formation. This discrepancy can perhaps be explained by the fact that mAb5C2 failed to affect dimerization/oligomerization of α -synuclein but could still bind to the monomeric α -synuclein hemi:GFP in which the NAC region is exposed. Thus, this antibody could facilitate protein degradation, explaining the decreased total α -synuclein levels seen in the ELISA. However, the oligomer-selective mAb49/G (**paper IV, figure 2**) and C-terminal mAb211 antibodies should be more beneficial as suitable antibody candidates, by their higher affinity for pathological α -synuclein aggregates instead of the monomeric physiological form.

It is debatable whether extracellularly administered antibodies can pass the cell membrane and affect intracellular pathology. However, an antibody utilized in cancer research has been shown to effectively bind to its target after cell internalization [202]. In addition, more recent work showed that an antibody directed against A β PP can maintain its structure and remain associated with its target after internalization [201]. In the present study we could detect α -synuclein antibodies within the cells (**paper IV, figure 4**) after 48 h of incubation and find that they co-occurred with the α -synuclein dimers/oligomers (**paper IV, figure 3**). These results are in agreement with findings from a previous study that extracellular administered antibodies generated against the C-terminal of α -synuclein were localized to large inclusions in the cell soma [173]. Possibly, the effects seen in both this and previous studies may be explained by antibody uptake via particular receptors.

For example, the IgG-receptor tripartite motif-containing 21 (TRIM21) was shown to mediate antibody internalization followed by transferring of the antibody-antigen complex to the proteasome for degradation [203].

The current study revealed that α -synuclein can be targeted intracellularly. However, aggregated soluble species may also be possible to target outside the cell. Indeed, several recent studies on cells and transgenic mice have indicated cell-to-cell propagation of α -synuclein pathology [101, 156, 157]. In addition, neuropathological analyses of PD brains that had been transplanted with fetal mesencephalic dopaminergic neurons displayed LBs in the grafted cells, suggesting a similar propagation mechanism in the human brain [155].

Concluding remarks

In 1997, Spillantini and colleagues identified that the main component of Lewy bodies, the pathological hallmarks in disorders like PD and DLB, was the presynaptic protein α -synuclein. Since then, research has focused on elucidating which role α -synuclein play in the pathogenesis of such disorders. A mechanism for the dysfunction and loss of cells may be the formation of intermediately sized aggregated forms of α -synuclein. Such oligomers or protofibrils may have direct toxic effects on the neurons.

The focus of this thesis was to characterize oligomers/protofibrils of α -synuclein in order to understand the underlying mechanisms of how they are formed, what cellular effects they have, how they may propagate pathology and how they could be targeted for immunotherapeutic approaches.

Reactive aldehydes such as 4-hydroxy-2-nonenal (HNE) and 4-oxo-2-nonenal (ONE) were used to promote and stabilize *in vitro* generated α -synuclein oligomers. Upon formation, the oligomers were characterized based on size, morphology, structure and stability using biophysical and biochemical methods. It was found that both HNE and ONE induced large soluble α -synuclein oligomers that did not continue into fibrils. In addition, functional studies showed that the oligomers could get internalized into cultured cells. They were also found to cause cell toxicity in neuron-like cells.

Secondly, potential seeding effects of the α -synuclein oligomers were studied using kinetic assays as well as in cell- and animal models. When a small amount of oligomers were added to freshly monomeric preparations of α -synuclein, the aggregation started earlier as compared to controls suggesting a seeding effect of the α -synuclein oligomers. By adding the oligomers to α -synuclein overexpressing cells it was found that they affect early aggregation, indicating a direct binding to the expressed protein and that they act as a nucleus or a seed for further aggregation. Also, the oligomers were injected into the cerebral cortex of α -synuclein transgenic mice to study potential *in vivo* seeding effects. The results showed a detectable seeding effect in the group injected with 40 ng of α -synuclein.

In the last part of this thesis, oligomer selective α -synuclein antibodies were used to study potential inhibition of protein aggregation using a cell model. Here, it was shown that early α -synuclein dimerization/oligomerization could be reduced by treatment with such antibodies as well as with antibodies against the C-terminal part of α -synuclein. In addition, lowered amounts of α -synuclein were found both in the cell lysate and in cultured media of antibody

treated cells, suggesting increased degradation in treated cells. Furthermore, by following the extracellular antibody uptake, it was shown that the antibodies localized to inclusions of α -synuclein, as evidenced by co-occurrence with a reporter (GFP) molecule.

To conclude, this thesis has characterized different oligomeric α -synuclein species, which may have properties similar to soluble species central in the pathogenesis of PD and other disorders with α -synuclein pathology. In addition, using similar forms of α -synuclein oligomers, the pharmaceutical company BioArctic Neuroscience AB has developed oligomer selective α -synuclein antibodies as potential drug candidates for treatment of Lewy body disorders. For therapeutic strategies it should be of importance to selectively target such harmful protein species and thereby avoid interaction with other forms of α -synuclein, which may have vital physiological cellular functions. Such antibodies are currently under evaluation for treatment of α -synuclein pathology in transgenic mice models.

Methodological considerations

Alpha-synuclein transgenic mice (Paper III)

In **Paper III**, the homozygous (Thy-1)-h [A30P] α -synuclein transgenic mouse model was used to study potential propagation of pathology. This model develops age-dependent impairment in fear conditioning behavior accompanied with phosphorylated α -synuclein pathology [204]. The brainstem and spinal cord stain positively for Thioflavin S (ThS). In detail, these mice develop proteinase K resistant pathology at 12 months of age, predominantly in brainstem, midbrain and spinal cord. In addition, there is evidence of astrogliosis in the same brain regions. These mice have a low density of α -synuclein pathology in the substantia nigra, mostly due to the choice of promoter (Thy-1). Moreover, there is little or no evidence of atrophy in the brains of these mice. At 17 months of age, they display locomotor difficulties compared to wild-type mice [189]. Furthermore, the mice die prematurely at an average age of 18 ± 3 months.

Atomic force microscopy (Paper I, II and III)

To characterize the morphology of α -synuclein species generated after ONE and HNE modification, we employed AFM, a high-resolution scanning microscopy imaging technique that uses a mechanical probe to “feel” the surface of a sample at the nanoscale level. However, the technique can also be used in a force mode where the interaction forces between a ligand and its receptor can be measured [205]. The probe is made up of a cantilever with a sharp silicon based tip. As the cantilever is scanned over the surface and the tip comes into proximity with samples, van der Waals forces, chemical bonding and electrostatic forces cause a deflection of the probe to adjust to a normal position. A laser beam pointed at the probe then records this adjustment and an image of the surface can be calculated [206] (*Figure 11*).

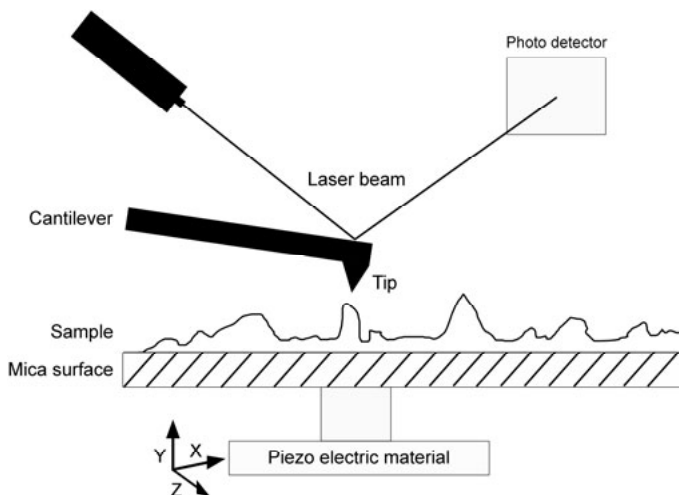


Figure 11. A schematic representation of the principle of atomic force microscopy. The cantilever with a sharp tip is moved over the surface. As the tip comes into proximity of a sample, forces between the tip and the surface will cause the cantilever to deflect to adjust to the normal position. A laser beam pointed at the cantilever is reflected to a photo detector which records this adjustment and an image can be calculated. A piezo electric scanner is employed to move the surface in three directions (x, y and z) for extreme precision.

All AFM experiments in this thesis were carried out by analyzing the sample after it had dried completely on the surface. The rationale for using this approach was that the species analyzed in this system were water soluble. However, AFM can successfully analyze particles in solution [207]. In fact, it is likely that by measuring samples in solution, more representative images can be generated as a non-physiological (e.g. drying) environment might affect the morphology and size of the analyzed species. Although AFM can be a more sensitive method than electron microscopy (EM), it requires several scans on different places of the mica surface, to enable representative images of the sample. In contrast, EM enables scan of larger area but lack high-resolution at the nanoscale.

Bimolecular fluorescence complementation assay (Paper III and IV)

The need for tools to study the interactions between aggregation prone proteins is essential to gain increased knowledge of the early pathogenic events in neurodegeneration [208]. To study α -synuclein aggregation in cell culture, bimolecular fluorescence complementation (BiFC) [89], also known as protein complementation (PCA), was used [187]. This technique is based upon the simultaneous transfection of two different α -synuclein hemi:GFP

fragments (with GFP's N and C-terminal halves, respectively) Fluorescence arises only when a reconstitution of the GFP-halves occur, i.e. after dimerization/oligomerization of α -synuclein [89] (*Figure 12*).

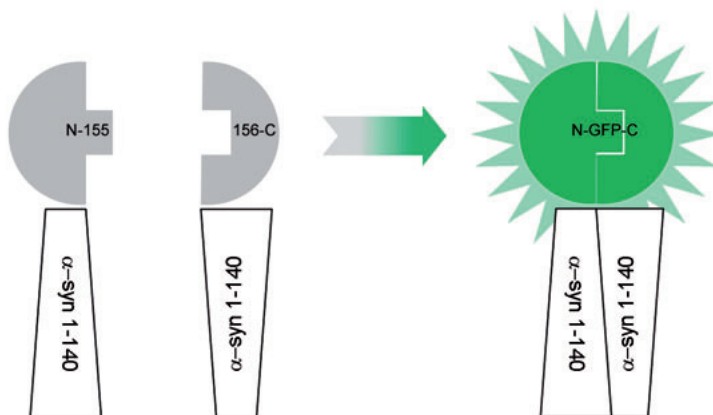


Figure 12. Schematic picture of the bimolecular fluorescence complementation (BiFC) assay. Complementation of two hemi-GFP-fragments each fused to full-length α -synuclein form a complete fluorescent GFP-molecule only upon dimerization/oligomerization of α -synuclein.

By using the BiFC system, the localization of dimerized/oligomerized α -synuclein has been determined to be in the cell soma, as well as the nucleus, when expressed in H4 neuroglioma cells and HEK-293 [89, 156]. Moreover, control experiments have been carried out to ensure that it is not the GFP-fragments that are responsible for driving the observed interactions [89]. Furthermore, it is important to remember that, by using this system, α -synuclein most likely adopts a different conformation due to the addition of the GFP fragments. Thus, it is highly likely that the resultant protein will have different aggregation properties as compared to unmodified α -synuclein. For example, it has been shown that, by adding another protein fragment to the C-terminus of α -synuclein, the inherent propensity to form inclusions in cultured cells can get increased [209].

Dot blot (Paper II)

The dot blot method was used to study the compactness of the ONE and HNE induced α -synuclein oligomers [210]. This technique is a simplified version of western blot, where the molecules first have been separated with gel electrophoresis and then probed with a specific antibody. In dot blot, the denaturation step is removed and an appropriate amount of the sample is dotted onto a membrane, followed by detection of a specific antibody. Thus,

this technique only provides information on whether the targeted molecules are present or not in the samples and no data on neither the size nor the amount of the detected molecules. In addition, it enables detection of all molecules in the sample, including molecules too large to enter a gel system and therefore cannot be electrophoretically separated.

Enzyme linked immunosorbent assay (Paper II, III and IV)

Enzyme linked immunosorbent assay (ELISA) is an established method to detect and quantify for instance peptides and proteins [211]. This method uses the interaction between an antibody and an antigen to measure the presence of a particular molecule, regardless of whether it is targeted in solution or bound to a surface [212]. The interaction of antibody and antigen is typically revealed by an anti-Ig antibody coupled to an enzyme, e.g. Horseradish peroxidase (HRP). This interaction can in turn be detected by adding a substrate for HRP that is converted to a signal that can be read using spectroscopic methods.

In **Paper II** an indirect ELISA was used to study the surface-exposed epitopes of the ONE and HNE induced α -synuclein oligomers. In this setting, the antigen (i.e. α -synuclein) is coated to the bottom of the microtiter plate. Next, the excess of antigen is removed and an antibody specific α -synuclein for the target is incubated on the plate. To reveal the interaction, an anti- α -synuclein antibody conjugated with HRP is added followed by a substrate to develop the assay.

Inhibition ELISA was used in **Paper IV** for characterization of α -synuclein specific antibodies. Briefly, this immunoassay starts with incubation of the antigen and the antibody in solution. Next, the antigen-antibody complex is transferred to the plate followed by the same antibody conjugated to HRP. Thus, the more non-bound antibody there is in the sample solution (which can bind to the antigen coated plate) the less chance there is for the enzyme conjugated antibody to bind and thus “inhibit” the antigen on the plate.

In **Paper IV**, a sandwich ELISA was used [213]. This variant features the coating of a capture antibody to a surface followed by addition of the antigen. A detection antibody conjugated to HRP is added and the resultant signal can be measured with spectrophotometric methods (*Figure 13*).

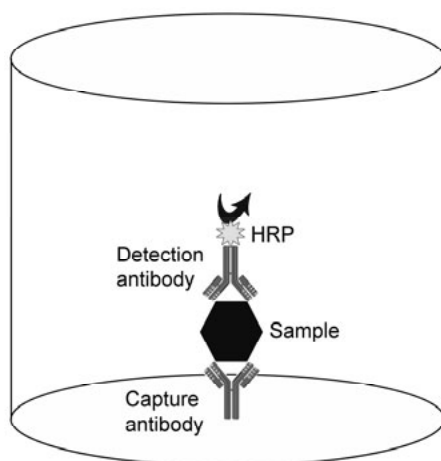


Figure 13. Schematic representation of a sandwich ELISA. A capture antibody is coated to the bottom of an ELISA plate. Next, the sample (black) is incubated to the well followed by a separate washing step. Finally a detection antibody conjugated to HRP is added to the well and a substrate for the enzyme is added. The enzyme converts the substrate to produce a colored product which can be spectrophotometrically measured.

Far-UV circular dichroism (Paper I and II)

Far-UV circular dichroism (CD) spectroscopy was used to investigate structural implications of ONE and HNE-modification of α -synuclein. With this technique, the use of polarized light can determine important properties of the secondary structure of proteins. The far ultraviolet (UV) spectra over proteins can be used to determine alpha-helix, beta-turn, random coil and beta-sheet conformation. The alpha-helix conformation has a far-UV CD spectra of a double minimum at 208 nm and 222 nm. The beta-turn exhibits a minimum at 220 nm, the random coil at a minimum of 200 nm and the beta-sheet at 218 nm [214-216].

Immunostaining and optical microscopy (Paper II, III and IV)

In **Paper II**, the use of fluorescently tagged ONE and HNE induced α -synuclein oligomers or antibodies specific for α -synuclein was used to investigate the extracellular effects on SH-SY5Y neuroblastoma cells. By exciting the fluorophore conjugated to the α -synuclein oligomers, the potential interaction or uptake of α -synuclein can be traced by the resultant emitted light using epifluorescence microscopy [217]. Secondly, fluorescently tagged antibodies specific for α -synuclein can be probed to trace the oligomers inside

the cell. However, this setting only answers where a specific target (i.e. α -synuclein) is in the two-dimensional plane and does not provide reliable information on the subcellular localization of the target. To correctly trace the three-dimensional position of protein in a cell, confocal microscopy is a more suitable method [218]. This microscopy technique enables tracing of the light that arises from just one plane. By creating optical sections from the top to the bottom of a specimen, a three-dimensional image can be generated and the subcellular location of a protein can be traced to its exact position. In addition, the use of subcellular tracers, e.g. antibodies specific for β -actin, give an even more exact position of the traced protein. In **Paper IV**, Cy3 conjugated antibodies were used to follow cellular translocation of monoclonal α -synuclein antibodies. In more detail, H4 cells were treated for 48 h with the α -synuclein antibodies, and after fixation of the cells, only anti- α -synuclein antibodies conjugated with Cy3 were used for immunostaining. Thus, staining signals should only arise from the internalized α -synuclein antibodies.

MTT assay (Paper II)

To investigate the functional properties of α -synuclein oligomers, the 3-(4, 5-Dimethylthiazol-2-yl)-2, 5-diphenyltetrazolium bromide (MTT) viability assay was used [219, 220]. The reduction of the yellow salt MTT in living cells yields an insoluble purple formazan product. After dissolving the purple product, the degree of reduced MTT can be calculated as the relative degree of number of viable cells. In **Paper II**, the MTT assay was used to study potential toxic effects of α -synuclein by exposing SH-SY5Y neuroblastoma cells to α -synuclein oligomers and monomers in various doses [221-223].

Proteinase K treatment and urea stability (Paper II and III)

To study the compactness and stability of the different α -synuclein forms analyzed in this thesis, proteinase K (PK) and urea were used. Proteinase K is a broad spectrum serine protease that digests native proteins. Therefore, this enzyme can provide useful information regarding the compactness of different protein structures, especially of insoluble forms such as fibrils [224]. Moreover, α -synuclein fibrils also display PK resistance *in vitro* [225] and *in vivo* [190, 226]. When used in high concentrations, urea is a highly useful denaturant as it breaks non-covalent bonds in proteins. In contrast to PK, it breaks up larger protein structures into smaller units without cleaving peptide bonds, providing information regarding the stability of different protein filaments.

Therefore, in **Paper II**, the combination of PK and urea treatment provided information that gave a better understanding of the structural differences between the ONE induced and HNE induced α -synuclein oligomers.

In **Paper III**, PK was used to isolate insoluble forms of α -synuclein from the monomeric overexpressed α -synuclein in the brains of the transgenic mice [227]. In detail, because these mice display a low amount of insoluble α -synuclein accompanied by a robust monomeric expression, it is essential to eliminate PK sensitive material (i.e. monomers) for accurate detection.

SDS-PAGE and immunoblot (Paper I and II)

For determination of stability of the oligomeric α -synuclein species, sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) was used. SDS-PAGE separates proteins in a polymer gel according to their size. Here, the separating method is based upon an initial denaturation by heat and SDS treatment of the proteins. In addition, SDS is bound along the peptide chains which give the proteins a net negative charge. Finally, the samples are subjected to an electric field and the proteins will migrate in the gel only according to their size. Because the proteins will have a similar denatured structure and charge, small proteins will migrate faster than large proteins. After separation, the proteins are visualized by different staining techniques, e.g. silver staining.

For detection of migrated proteins in a SDS-PAGE, western blot can be used [228, 229]. With this technique, the proteins are subjected to an electric field, in which they migrate from the SDS-PAGE gel to a nitrocellulose or a polyvinylidene fluoride (PVDF) membrane. Next, the proteins are visualized by adding a primary antibody generated against the target. The primary antibody is targeted with a HRP conjugated secondary antibody (**Paper IV**). Finally, the protein is detected by adding a substrate for HRP and the resulting chemiluminescence can be read. However, in **Paper I and II** a fluorescently tagged secondary antibody was used. For this detection, a fluorophore is excited and a scanner detects its emitted light.

Size exclusion chromatography (Paper I and II)

To be able to separate the different components generated by the interaction of ONE and HNE on α -synuclein, size exclusion-high performance liquid chromatography (SEC) was used. Size-exclusion chromatography separates components according to size based upon their hydrodynamic range. Briefly, the samples are run through a column, the stationary phase, containing a matrix that constitutes a polymer material (e.g. agarose). When the samples are run through the column, small components enter the pores of the matrix

whereas large components elute mostly in the mobile phase. Because smaller components enter more pores, the retention time will be longer than for the larger molecules. The elution volume of the components is then logarithmically proportional to their hydrodynamic range (*Figure 14*).

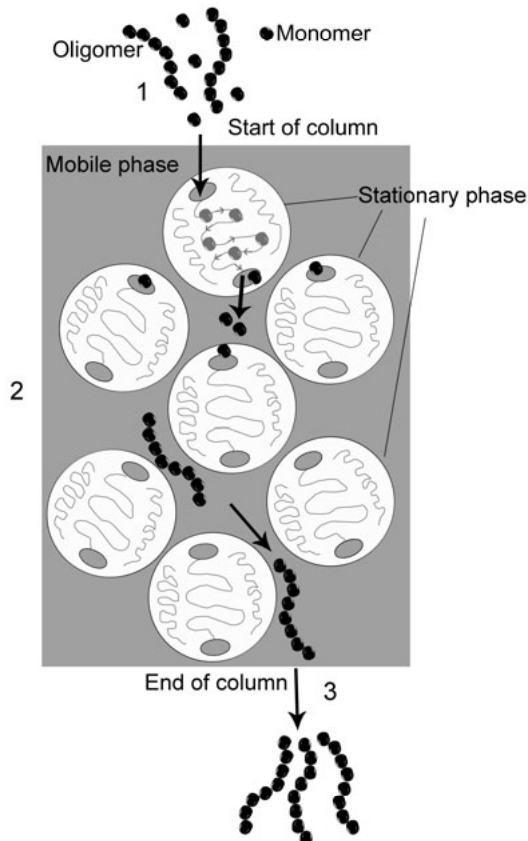


Figure 14. Scheme representing the principle of size-exclusion chromatography analysis where molecules are separated based upon their size. For example, a mix of molecules containing both monomers and oligomers are run through a column with a polymer material (1). The monomers can enter pores of the matrix (the stationary phase), whereas the larger oligomers will migrate mainly in the mobile phase (2). The retention time will thereby be longer for monomers than for larger molecules (3).

To determine the size of a particular molecule using SEC, molecular weight standards with pre-determined sizes are run in parallel with the experimental sample. This is commonly achieved using globular weight standards. However, because α -synuclein lacks a proper folded structure, it will occupy a larger space (i.e. larger hydrodynamic radius) in the column compared to its theoretical size. Alpha-synuclein has been extensively analyzed with SEC,

both as its native form, as in higher multimeric forms and as post-translationally modified species [120, 130, 230]. In **Paper I** and **II**, the α -synuclein monomer eluted at ~ 50 kDa, corresponding roughly to a trimer of the protein. However recent data suggest that α -synuclein occurs physiologically as a folded tetramer with a size of ~ 58 kDa [60].

Thioflavin T kinetic assay (Paper I, II and III)

In Paper I and II Thioflavin T was used to monitor the aggregation pathway of α -synuclein *in vitro*. Thioflavin T is a benzothiazole salt that has the ability to bind to beta-sheets in fibril material. When this compound binds to beta-sheet structures, the dye shifts in its emission spectra and the presence of fibrils can be detected by exciting the fluorophore at 445 nm and read its emitted light at 485 nm.

Populärvetenskaplig sammanfattning

Många av oss känner kanske till den flerfaldige världsmästaren i boxning Cassius Clay, mer känd som Muhammad Ali eller skådespelaren Michael J Fox, känd från storfilmen "Tillbaka till framtiden" och tv-serien "Spin City". Ali och Fox har en sak gemensamt, de är båda drabbade av Parkinsons sjukdom.

Parkinsons sjukdom är en progressiv, neurologisk sjukdom som kan drabba både män och kvinnor, oftast vid 50-60 års ålder även om vissa ärftliga fall kan leda till sjukdom redan vid 30 års ålder. I Sverige beräknas bortåt 25 000 personer ha sjukdomen. Symptomen utgörs av bl.a. stelhet, skakningar och rörelsefattigdom. Idag finns ingen bot mot Parkinsons sjukdom, men symptomatisk behandling med framförallt L-DOPA, som i hjärnan omvandlas till dopamin, har visat sig vara effektiv under tidiga sjukdomsstadier. I Parkinsonhjärnan förtvinar vissa nervceller i mellanhjärnan som producerar signalsubstansen dopamin, vilket förklarar varför L-DOPA har sin goda effekt. Diagnosen är klinisk, d.v.s. baseras på själva sjukhistorien och en detaljerad neurologisk undersökning. Avbildning av vissa hjärnområden kan dessutom ge stöd för diagnosen. Utöver Parkinsons sjukdom finns två andra relaterade sjukdomar, som också leder till liknande rörelsestörningar. Lewy body demens kännetecknas vanligtvis även av minnesproblem och synhallucinationer, medan patienter med multipel systematrofi ofta har balansproblem och autonoma rubbningar, såsom blodtrycksfall.

Vid obduktion av hjärnor med dessa sjukdomar kan man med hjälp av mikroskop se stora olösliga aggregat av proteinet alfa-synuklein, s.k. Lewykroppar. Dessa antas störa nervcellernas funktion, men på senare år har studier visat att det verkar vara lösliga förstadier till Lewykroppar, s.k. oligomerer eller protofibriller av alfa-synuklein, som skadar hjärnan och leder till nervcellsförlust. Den normala, biologiska funktionen för alfa-synuklein är delvis fortfarande oklar, men proteinet finns inuti nervceller och antas vara involverat i nervsignaler. Vid vissa ärftliga former av både Parkinsons sjukdom och Lewy body demens har man kunnat påvisa mutationer i genen för alfa-synuklein. Dessa mutationer har visats antingen leda till ökade mängder alfa-synuklein eller till förändrade egenskaper hos proteinet. Framför allt har man experimentellt kunnat påvisa att dessa mutationer leder till en ökad bildning av just de mer skadliga oligomererna/protofibrillerna.

I denna avhandling har alfa-synukleinoligomerer studerats i detalj. Genom utveckling av olika laborativa protokoll har de kunnat framställas i provrör

och sedan analyserats med olika metoder. Vidare har deras inverkan på odlade celler och på en musmodell av sjukdomarna undersökts.

I de två första studierna framställdes två olika typer av alfa-synukleinoligomerer i provrör, genom påverkan av aldehyderna 4-hydroxy-2-nonenal och 4-oxo-2-nonenal, substanser som bildas vid oxidativ stress. Oligomererna karaktäriserades med biofysiska och biokemiska metoder i syfte att kartlägga deras struktur, storlek, utseende, stabilitet och benägenhet att bilda aggregat. Vidare tillsattes dessa oligomerer till nervceller i syfte att studera deras inverkan på cellernas funktioner. Resultaten pekar på relativt stora strukturella skillnader mellan de båda typerna av alfa-synukleinoligomerer. Däremot visade sig båda typerna ha en skadlig inverkan på odlade celler.

Aktuell forskning tyder på att aggregat av alfa-synuklein kan sprida sig mellan nervceller, och kanske även mellan olika delar av hjärnan, genom att sjukligt förändrat alfa-synuklein ”smittar” normala alfa-synuklein molekyler. Sådana effekter skulle kunna uppstå genom just oligomerer. I avhandlingens tredje arbete studerades därför om oligomerer av alfa-synuklein kan leda till bildning av aggregat. Vi undersökte proteinet i provrör, odlade celler och i hjärna på en musmodell för sjukdomarna. Vi kunde då se att oligomererna ökade bildningen av alfa-synuklein aggregat i provrör och dessutom föreföll påverka bildningen av aggregat i celler, men vi kunde inte se någon tydligt effekt på mushjärna.

Tidigare forskning har banat väg för immunterapi mot hjärnsjukdomar där behandling med antikroppar mot ett annat protein, beta-amyloid, visat lovande resultat vid kliniska studier på patienter med Alzheimers sjukdom. I det fjärde och avslutande arbetet ville vi därför undersöka om även aggregering av alfa-synuklein kan förhindras med immunterapi. Olika antikroppar mot alfa-synuklein tillsattes odlade celler som bildar oligomerer. Resultaten tyder på att antikropparna framgångsrikt kunde förebygga bildning av alfa-synukleinoligomerer och dessutom kunde reducera mängden av alfa-synuklein i cellerna.

Sammanfattningsvis har denna avhandling undersökt grundläggande frågor kring uppkomsten av centrala sjukliga förändringar i hjärnan vid Parkinsons sjukdom och relaterade tillstånd. Sådan kunskap kan visa sig vara viktig inför utvecklingen av både ny diagnostik och effektiv behandling av sjukdomarna.

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Uppsala i oktober 2011

A handwritten signature in black ink, appearing to read 'Thomas Näsström'. The signature is stylized with a large, sweeping initial 'T' and a long horizontal stroke at the end.

Thomas Näsström

References

1. Parkinson, J., *An essay on the shaking palsy*. 1817. J Neuropsychiatry Clin Neurosci, 2002. **14**(2): p. 223-36; discussion 222.
2. Hornykiewicz, O., *L-DOPA: from a biologically inactive amino acid to a successful therapeutic agent*. Amino Acids, 2002. **23**(1-3): p. 65-70.
3. Yahr, M.D., et al., *Treatment of parkinsonism with levodopa*. Arch Neurol, 1969. **21**(4): p. 343-54.
4. Lewy, F.H., *Paralysis agitans*, in *Handbuch der Neurologie*. 1912. p. 920-933.
5. Spillantini, M.G., et al., *Alpha-synuclein in Lewy bodies*. Nature, 1997. **388**(6645): p. 839-40.
6. Wakabayashi, K., et al., *Alpha-synuclein immunoreactivity in glial cytoplasmic inclusions in multiple system atrophy*. Neurosci Lett, 1998. **249**(2-3): p. 180-2.
7. Baba, M., et al., *Aggregation of alpha-synuclein in Lewy bodies of sporadic Parkinson's disease and dementia with Lewy bodies*. Am J Pathol, 1998. **152**(4): p. 879-84.
8. Jankovic, J., *Parkinson's disease: clinical features and diagnosis*. J Neurol Neurosurg Psychiatry, 2008. **79**(4): p. 368-76.
9. Buter, T.C., et al., *Dementia and survival in Parkinson disease: a 12-year population study*. Neurology, 2008. **70**(13): p. 1017-22.
10. Hely, M.A., et al., *The Sydney multicenter study of Parkinson's disease: the inevitability of dementia at 20 years*. Mov Disord, 2008. **23**(6): p. 837-44.
11. Lesage, S. and A. Brice, *Parkinson's disease: from monogenic forms to genetic susceptibility factors*. Hum Mol Genet, 2009. **18**(R1): p. R48-59.
12. Franco, R., et al., *Molecular mechanisms of pesticide-induced neurotoxicity: Relevance to Parkinson's disease*. Chem Biol Interact, 2010. **188**(2): p. 289-300.
13. de Lau, L.M. and M.M. Breteler, *Epidemiology of Parkinson's disease*. Lancet Neurol, 2006. **5**(6): p. 525-35.
14. Critchley, M., *Medical aspects of boxing, particularly from a neurological standpoint*. Br Med J, 1957. **1**(5015): p. 357-62.
15. Hauser, R.A., *Early pharmacologic treatment in Parkinson's disease*. Am J Manag Care, 2010. **16 Suppl Implications**: p. S100-7.

16. Campbell, S., S. Stephens, and C. Ballard, *Dementia with Lewy bodies: clinical features and treatment*. *Drugs Aging*, 2001. **18**(6): p. 397-407.
17. McKeith, I.G., *Spectrum of Parkinson's disease, Parkinson's dementia, and Lewy body dementia*. *Neurol Clin*, 2000. **18**(4): p. 865-902.
18. Hanson, J.C. and C.F. Lippa, *Lewy body dementia*. *Int Rev Neurobiol*, 2009. **84**: p. 215-28.
19. McKeith, I.G., et al., *Diagnosis and management of dementia with Lewy bodies: third report of the DLB Consortium*. *Neurology*, 2005. **65**(12): p. 1863-72.
20. Graham, J.G. and D.R. Oppenheimer, *Orthostatic hypotension and nicotine sensitivity in a case of multiple system atrophy*. *J Neurol Neurosurg Psychiatry*, 1969. **32**(1): p. 28-34.
21. Papp, M.I., J.E. Kahn, and P.L. Lantos, *Glial cytoplasmic inclusions in the CNS of patients with multiple system atrophy (striatonigral degeneration, olivopontocerebellar atrophy and Shy-Drager syndrome)*. *J Neurol Sci*, 1989. **94**(1-3): p. 79-100.
22. Swan, L. and J. Dupont, *Multiple system atrophy*. *Phys Ther*, 1999. **79**(5): p. 488-94.
23. Wenning, G.K. and N. Stefanova, *Recent developments in multiple system atrophy*. *J Neurol*, 2009. **256**(11): p. 1791-808.
24. Braak, H., et al., *Staging of brain pathology related to sporadic Parkinson's disease*. *Neurobiol Aging*, 2003. **24**(2): p. 197-211.
25. Shannon, K.M., et al., *Alpha-synuclein in colonic submucosa in early untreated Parkinson's disease*. *Mov Disord*, 2011.
26. Lebouvier, T., et al., *Colonic biopsies to assess the neuropathology of Parkinson's disease and its relationship with symptoms*. *PLoS One*, 2010. **5**(9): p. e12728.
27. Braak, H., et al., *Gastric alpha-synuclein immunoreactive inclusions in Meissner's and Auerbach's plexuses in cases staged for Parkinson's disease-related brain pathology*. *Neurosci Lett*, 2006. **396**(1): p. 67-72.
28. Hawkes, C.H., K. Del Tredici, and H. Braak, *Parkinson's disease: a dual-hit hypothesis*. *Neuropathol Appl Neurobiol*, 2007. **33**(6): p. 599-614.
29. Hamilton, R.L., *Lewy bodies in Alzheimer's disease: a neuropathological review of 145 cases using alpha-synuclein immunohistochemistry*. *Brain Pathol*, 2000. **10**(3): p. 378-84.
30. Lippa, C.F., et al., *Lewy bodies contain altered alpha-synuclein in brains of many familial Alzheimer's disease patients with mutations in presenilin and amyloid precursor protein genes*. *Am J Pathol*, 1998. **153**(5): p. 1365-70.
31. Harding, A.J., G.A. Broe, and G.M. Halliday, *Visual hallucinations in Lewy body disease relate to Lewy bodies in the temporal lobe*. *Brain*, 2002. **125**(Pt 2): p. 391-403.

32. Spillantini, M.G., et al., *alpha-Synuclein in filamentous inclusions of Lewy bodies from Parkinson's disease and dementia with lewy bodies*. Proc Natl Acad Sci U S A, 1998. **95**(11): p. 6469-73.
33. Lowe, J., et al., *Ubiquitin is a common factor in intermediate filament inclusion bodies of diverse type in man, including those of Parkinson's disease, Pick's disease, and Alzheimer's disease, as well as Rosenthal fibres in cerebellar astrocytomas, cytoplasmic bodies in muscle, and mallory bodies in alcoholic liver disease*. J Pathol, 1988. **155**(1): p. 9-15.
34. Uversky, V.N., *Neuropathology, biochemistry, and biophysics of alpha-synuclein aggregation*. J Neurochem, 2007. **103**(1): p. 17-37.
35. Lewis, K.A., et al., *Abnormal neurites containing C-terminally truncated alpha-synuclein are present in Alzheimer's disease without conventional Lewy body pathology*. Am J Pathol, 2010. **177**(6): p. 3037-50.
36. Crowther, R.A., et al., *Synthetic filaments assembled from C-terminally truncated alpha-synuclein*. FEBS Lett, 1998. **436**(3): p. 309-12.
37. Harding, A.J. and G.M. Halliday, *Cortical Lewy body pathology in the diagnosis of dementia*. Acta Neuropathol, 2001. **102**(4): p. 355-63.
38. Greffard, S., et al., *A stable proportion of Lewy body bearing neurons in the substantia nigra suggests a model in which the Lewy body causes neuronal death*. Neurobiol Aging, 2008. **31**(1): p. 99-103.
39. Lee, H.G., et al., *Emerging evidence for the neuroprotective role of alpha-synuclein*. Exp Neurol, 2006. **200**(1): p. 1-7.
40. Tompkins, M.M. and W.D. Hill, *Contribution of somal Lewy bodies to neuronal death*. Brain Res, 1997. **775**(1-2): p. 24-9.
41. Jakes, R., M.G. Spillantini, and M. Goedert, *Identification of two distinct synucleins from human brain*. FEBS Lett, 1994. **345**(1): p. 27-32.
42. Burre, J., et al., *Alpha-synuclein promotes SNARE-complex assembly in vivo and in vitro*. Science, 2010. **329**(5999): p. 1663-7.
43. Dev, K.K., et al., *Part II: alpha-synuclein and its molecular pathophysiological role in neurodegenerative disease*. Neuropharmacology, 2003. **45**(1): p. 14-44.
44. Zhou, Y., et al., *Analysis of alpha-synuclein-associated proteins by quantitative proteomics*. J Biol Chem, 2004. **279**(37): p. 39155-64.
45. Ueda, K., et al., *Molecular cloning of cDNA encoding an unrecognized component of amyloid in Alzheimer disease*. Proc Natl Acad Sci U S A, 1993. **90**(23): p. 11282-6.
46. Culvenor, J.G., et al., *Non-Abeta component of Alzheimer's disease amyloid (NAC) revisited. NAC and alpha-synuclein are not associated with Abeta amyloid*. Am J Pathol, 1999. **155**(4): p. 1173-81.
47. Ulmer, T.S., et al., *Structure and dynamics of micelle-bound human alpha-synuclein*. J Biol Chem, 2005. **280**(10): p. 9595-603.
48. George, J.M., *The synucleins*. Genome Biol, 2002. **3**(1): p. REVIEWS3002.

49. Uversky, V.N., et al., *Biophysical properties of the synucleins and their propensities to fibrillate: inhibition of alpha-synuclein assembly by beta- and gamma-synucleins*. J Biol Chem, 2002. **277**(14): p. 11970-8.
50. Lavedan, C., *The synuclein family*. Genome Res, 1998. **8**(9): p. 871-80.
51. Abeliovich, A., et al., *Mice lacking alpha-synuclein display functional deficits in the nigrostriatal dopamine system*. Neuron, 2000. **25**(1): p. 239-52.
52. Nemani, V.M., et al., *Increased expression of alpha-synuclein reduces neurotransmitter release by inhibiting synaptic vesicle recluster after endocytosis*. Neuron, 2010. **65**(1): p. 66-79.
53. Perrin, R.J., et al., *Interaction of human alpha-Synuclein and Parkinson's disease variants with phospholipids. Structural analysis using site-directed mutagenesis*. J Biol Chem, 2000. **275**(44): p. 34393-8.
54. Sharon, R., et al., *alpha-Synuclein occurs in lipid-rich high molecular weight complexes, binds fatty acids, and shows homology to the fatty acid-binding proteins*. Proc Natl Acad Sci U S A, 2001. **98**(16): p. 9110-5.
55. Storch, J. and A.E. Thumser, *The fatty acid transport function of fatty acid-binding proteins*. Biochim Biophys Acta, 2000. **1486**(1): p. 28-44.
56. Giasson, B.I., et al., *A hydrophobic stretch of 12 amino acid residues in the middle of alpha-synuclein is essential for filament assembly*. J Biol Chem, 2001. **276**(4): p. 2380-6.
57. Hashimoto, M., et al., *beta-Synuclein inhibits alpha-synuclein aggregation: a possible role as an anti-parkinsonian factor*. Neuron, 2001. **32**(2): p. 213-23.
58. Rockenstein, E., et al., *Altered expression of the synuclein family mRNA in Lewy body and Alzheimer's disease*. Brain Res, 2001. **914**(1-2): p. 48-56.
59. Ohtake, H., et al., *Beta-synuclein gene alterations in dementia with Lewy bodies*. Neurology, 2004. **63**(5): p. 805-11.
60. Bartels, T., J.G. Choi, and D.J. Selkoe, *alpha-Synuclein occurs physiologically as a helically folded tetramer that resists aggregation*. Nature, 2011. **477**(7362): p. 107-10.
61. Polymeropoulos, M.H., et al., *Mutation in the alpha-synuclein gene identified in families with Parkinson's disease*. Science, 1997. **276**(5321): p. 2045-7.
62. Kitada, T., et al., *Mutations in the parkin gene cause autosomal recessive juvenile parkinsonism*. Nature, 1998. **392**(6676): p. 605-8.
63. Leroy, E., et al., *The ubiquitin pathway in Parkinson's disease*. Nature, 1998. **395**(6701): p. 451-2.
64. Valente, E.M., et al., *Hereditary early-onset Parkinson's disease caused by mutations in PINK1*. Science, 2004. **304**(5674): p. 1158-60.

65. Bonifati, V., et al., *Mutations in the DJ-1 gene associated with autosomal recessive early-onset parkinsonism*. Science, 2003. **299**(5604): p. 256-9.
66. Zimprich, A., et al., *Mutations in LRRK2 cause autosomal-dominant parkinsonism with pleomorphic pathology*. Neuron, 2004. **44**(4): p. 601-7.
67. Alegre-Abarrategui, J., et al., *LRRK2 is a component of granular alpha-synuclein pathology in the brainstem of Parkinson's disease*. Neuropathol Appl Neurobiol, 2008. **34**(3): p. 272-83.
68. Farrer, M., et al., *Lewy bodies and parkinsonism in families with parkin mutations*. Ann Neurol, 2001. **50**(3): p. 293-300.
69. Chen, X., et al., *The human NACP/alpha-synuclein gene: chromosome assignment to 4q21.3-q22 and TaqI RFLP analysis*. Genomics, 1995. **26**(2): p. 425-7.
70. Kruger, R., et al., *Ala30Pro mutation in the gene encoding alpha-synuclein in Parkinson's disease*. Nat Genet, 1998. **18**(2): p. 106-8.
71. Zarranz, J.J., et al., *The new mutation, E46K, of alpha-synuclein causes Parkinson and Lewy body dementia*. Ann Neurol, 2004. **55**(2): p. 164-73.
72. Fuchs, J., et al., *Phenotypic variation in a large Swedish pedigree due to SNCA duplication and triplication*. Neurology, 2007. **68**(12): p. 916-22.
73. Singleton, A.B., et al., *alpha-Synuclein locus triplication causes Parkinson's disease*. Science, 2003. **302**(5646): p. 841.
74. Ibanez, P., et al., *Causal relation between alpha-synuclein gene duplication and familial Parkinson's disease*. Lancet, 2004. **364**(9440): p. 1169-71.
75. Caughey, B. and P.T. Lansbury, *Protofibrils, pores, fibrils, and neurodegeneration: separating the responsible protein aggregates from the innocent bystanders*. Annu Rev Neurosci, 2003. **26**: p. 267-98.
76. Dobson, C.M., *Principles of protein folding, misfolding and aggregation*. Semin Cell Dev Biol, 2004. **15**(1): p. 3-16.
77. Jarrett, J.T. and P.T. Lansbury, Jr., *Seeding "one-dimensional crystallization" of amyloid: a pathogenic mechanism in Alzheimer's disease and scrapie?* Cell, 1993. **73**(6): p. 1055-8.
78. Hardy, J.A. and G.A. Higgins, *Alzheimer's disease: the amyloid cascade hypothesis*. Science, 1992. **256**(5054): p. 184-5.
79. Bitan, G., et al., *Amyloid beta -protein (Abeta) assembly: Abeta 40 and Abeta 42 oligomerize through distinct pathways*. Proc Natl Acad Sci U S A, 2003. **100**(1): p. 330-5.
80. Uversky, V.N., J. Li, and A.L. Fink, *Evidence for a partially folded intermediate in alpha-synuclein fibril formation*. J Biol Chem, 2001. **276**(14): p. 10737-44.
81. Conway, K.A., J.D. Harper, and P.T. Lansbury, Jr., *Fibrils formed in vitro from alpha-synuclein and two mutant forms linked to*

- Parkinson's disease are typical amyloid*. *Biochemistry*, 2000. **39**(10): p. 2552-63.
82. Nussbaum, R.L. and M.H. Polymeropoulos, *Genetics of Parkinson's disease*. *Hum Mol Genet*, 1997. **6**(10): p. 1687-91.
83. Shimura, H., et al., *Ubiquitination of a new form of alpha-synuclein by parkin from human brain: implications for Parkinson's disease*. *Science*, 2001. **293**(5528): p. 263-9.
84. Shtilerman, M.D., T.T. Ding, and P.T. Lansbury, Jr., *Molecular crowding accelerates fibrillization of alpha-synuclein: could an increase in the cytoplasmic protein concentration induce Parkinson's disease?* *Biochemistry*, 2002. **41**(12): p. 3855-60.
85. Minton, A.P., *Implications of macromolecular crowding for protein assembly*. *Curr Opin Struct Biol*, 2000. **10**(1): p. 34-9.
86. Uversky, V.N., et al., *Accelerated alpha-synuclein fibrillation in crowded milieu*. *FEBS Lett*, 2002. **515**(1-3): p. 99-103.
87. el-Agnaf, O.M. and G.B. Irvine, *Aggregation and neurotoxicity of alpha-synuclein and related peptides*. *Biochem Soc Trans*, 2002. **30**(4): p. 559-65.
88. Conway, K.A., et al., *Accelerated oligomerization by Parkinson's disease linked alpha-synuclein mutants*. *Ann N Y Acad Sci*, 2000. **920**: p. 42-5.
89. Outeiro, T.F., et al., *Formation of toxic oligomeric alpha-synuclein species in living cells*. *PLoS One*, 2008. **3**(4): p. e1867.
90. Sharon, R., et al., *The formation of highly soluble oligomers of alpha-synuclein is regulated by fatty acids and enhanced in Parkinson's disease*. *Neuron*, 2003. **37**(4): p. 583-95.
91. Paleologou, K.E., et al., *Detection of elevated levels of soluble alpha-synuclein oligomers in post-mortem brain extracts from patients with dementia with Lewy bodies*. *Brain*, 2009. **132**(Pt 4): p. 1093-101.
92. Dobson, C.M., *Protein folding and misfolding*. *Nature*, 2003. **426**(6968): p. 884-90.
93. Hershko, A. and A. Ciechanover, *The ubiquitin system*. *Annu Rev Biochem*, 1998. **67**: p. 425-79.
94. Pan, T., et al., *The role of autophagy-lysosome pathway in neurodegeneration associated with Parkinson's disease*. *Brain*, 2008. **131**(Pt 8): p. 1969-78.
95. Shin, Y., et al., *The co-chaperone carboxyl terminus of Hsp70-interacting protein (CHIP) mediates alpha-synuclein degradation decisions between proteasomal and lysosomal pathways*. *J Biol Chem*, 2005. **280**(25): p. 23727-34.
96. Webb, J.L., et al., *Alpha-Synuclein is degraded by both autophagy and the proteasome*. *J Biol Chem*, 2003. **278**(27): p. 25009-13.
97. Cuervo, A.M., et al., *Impaired degradation of mutant alpha-synuclein by chaperone-mediated autophagy*. *Science*, 2004. **305**(5688): p. 1292-5.
98. Lindersson, E., et al., *Proteasomal inhibition by alpha-synuclein filaments and oligomers*. *J Biol Chem*, 2004. **279**(13): p. 12924-34.

99. Emmanouilidou, E., L. Stefanis, and K. Vekrellis, *Cell-produced alpha-synuclein oligomers are targeted to, and impair, the 26S proteasome*. *Neurobiol Aging*, 2008. **31**(6): p. 953-68.
100. Sung, J.Y., et al., *Proteolytic cleavage of extracellular secreted {alpha}-synuclein via matrix metalloproteinases*. *J Biol Chem*, 2005. **280**(26): p. 25216-24.
101. Danzer, K.M., et al., *Different species of alpha-synuclein oligomers induce calcium influx and seeding*. *J Neurosci*, 2007. **27**(34): p. 9220-32.
102. Gouras, G.K., C.G. Almeida, and R.H. Takahashi, *Intraneuronal Abeta accumulation and origin of plaques in Alzheimer's disease*. *Neurobiol Aging*, 2005. **26**(9): p. 1235-44.
103. Klucken, J., et al., *Detection of novel intracellular alpha-synuclein oligomeric species by fluorescence lifetime imaging*. *FASEB J*, 2006. **20**(12): p. 2050-7.
104. Danzer, K.M., et al., *Heat-shock protein 70 modulates toxic extracellular alpha-synuclein oligomers and rescues trans-synaptic toxicity*. *FASEB J*, 2011. **25**(1): p. 326-36.
105. Tokuda, T., et al., *Detection of elevated levels of alpha-synuclein oligomers in CSF from patients with Parkinson disease*. *Neurology*, 2010. **75**(20): p. 1766-72.
106. El-Agnaf, O.M., et al., *Detection of oligomeric forms of alpha-synuclein protein in human plasma as a potential biomarker for Parkinson's disease*. *FASEB J*, 2006. **20**(3): p. 419-25.
107. Tsika, E., et al., *Distinct region-specific alpha-synuclein oligomers in A53T transgenic mice: implications for neurodegeneration*. *J Neurosci*, 2010. **30**(9): p. 3409-18.
108. Winner, B., et al., *In vivo demonstration that alpha-synuclein oligomers are toxic*. *Proc Natl Acad Sci U S A*, 2011. **108**(10): p. 4194-9.
109. Ehrnhoefer, D.E., et al., *EGCG redirects amyloidogenic polypeptides into unstructured, off-pathway oligomers*. *Nat Struct Mol Biol*, 2008. **15**(6): p. 558-66.
110. Yoritaka, A., et al., *Immunohistochemical detection of 4-hydroxynonenal protein adducts in Parkinson disease*. *Proc Natl Acad Sci U S A*, 1996. **93**(7): p. 2696-701.
111. Zhu, M., et al., *The flavonoid baicalein inhibits fibrillation of alpha-synuclein and disaggregates existing fibrils*. *J Biol Chem*, 2004. **279**(26): p. 26846-57.
112. Lowe, R., et al., *Calcium(II) selectively induces alpha-synuclein annular oligomers via interaction with the C-terminal domain*. *Protein Sci*, 2004. **13**(12): p. 3245-52.
113. Konno, T., et al., *Covalent blocking of fibril formation and aggregation of intracellular amyloidogenic proteins by transglutaminase-catalyzed intramolecular cross-linking*. *Biochemistry*, 2005. **44**(6): p. 2072-9.

114. Jiang, C. and J.Y. Chang, *Isomers of human alpha-synuclein stabilized by disulfide bonds exhibit distinct structural and aggregative properties*. *Biochemistry*, 2007. **46**(2): p. 602-9.
115. Souza, J.M., et al., *Dityrosine cross-linking promotes formation of stable alpha -synuclein polymers. Implication of nitrative and oxidative stress in the pathogenesis of neurodegenerative synucleinopathies*. *J Biol Chem*, 2000. **275**(24): p. 18344-9.
116. Cappai, R., et al., *Dopamine promotes alpha-synuclein aggregation into SDS-resistant soluble oligomers via a distinct folding pathway*. *FASEB J*, 2005. **19**(10): p. 1377-9.
117. Cole, N.B., et al., *Metal-catalyzed oxidation of alpha-synuclein: helping to define the relationship between oligomers, protofibrils, and filaments*. *J Biol Chem*, 2005. **280**(10): p. 9678-90.
118. Martinez, Z., et al., *GMI specifically interacts with alpha-synuclein and inhibits fibrillation*. *Biochemistry*, 2007. **46**(7): p. 1868-77.
119. Cole, N.B., et al., *Lipid droplet binding and oligomerization properties of the Parkinson's disease protein alpha-synuclein*. *J Biol Chem*, 2002. **277**(8): p. 6344-52.
120. Hong, D.P., A.L. Fink, and V.N. Uversky, *Smoking and Parkinson's disease: does nicotine affect alpha-synuclein fibrillation?* *Biochim Biophys Acta*, 2009. **1794**(2): p. 282-90.
121. Uversky, V.N., et al., *Effects of nitration on the structure and aggregation of alpha-synuclein*. *Brain Res Mol Brain Res*, 2005. **134**(1): p. 84-102.
122. Li, J., et al., *Rifampicin inhibits alpha-synuclein fibrillation and disaggregates fibrils*. *Chem Biol*, 2004. **11**(11): p. 1513-21.
123. Sayre, L.M., G. Perry, and M.A. Smith, *Oxidative stress and neurotoxicity*. *Chem Res Toxicol*, 2008. **21**(1): p. 172-88.
124. Gaeta, A. and R.C. Hider, *The crucial role of metal ions in neurodegeneration: the basis for a promising therapeutic strategy*. *Br J Pharmacol*, 2005. **146**(8): p. 1041-59.
125. Sayre, L.M., et al., *Metal ions and oxidative protein modification in neurological disease*. *Ann Ist Super Sanita*, 2005. **41**(2): p. 143-64.
126. Lin, M.T. and M.F. Beal, *Mitochondrial dysfunction and oxidative stress in neurodegenerative diseases*. *Nature*, 2006. **443**(7113): p. 787-95.
127. Esterbauer, H., R.J. Schaur, and H. Zollner, *Chemistry and biochemistry of 4-hydroxynonenal, malonaldehyde and related aldehydes*. *Free Radic Biol Med*, 1991. **11**(1): p. 81-128.
128. Montine, K.S., et al., *Isoprostanes and related products of lipid peroxidation in neurodegenerative diseases*. *Chem Phys Lipids*, 2004. **128**(1-2): p. 117-24.
129. Lee, S.H. and I.A. Blair, *Characterization of 4-oxo-2-nonenal as a novel product of lipid peroxidation*. *Chem Res Toxicol*, 2000. **13**(8): p. 698-702.
130. Qin, Z., et al., *Effect of 4-hydroxy-2-nonenal modification on alpha-synuclein aggregation*. *J Biol Chem*, 2007. **282**(8): p. 5862-70.

131. Sayre, L.M., et al., *Protein adducts generated from products of lipid oxidation: focus on HNE and one*. Drug Metab Rev, 2006. **38**(4): p. 651-75.
132. Lin, D., et al., *4-Oxo-2-nonenal is both more neurotoxic and more protein reactive than 4-hydroxy-2-nonenal*. Chem Res Toxicol, 2005. **18**(8): p. 1219-31.
133. Nieva, J., et al., *Lipid-derived aldehydes accelerate light chain amyloid and amorphous aggregation*. Biochemistry, 2008. **47**(29): p. 7695-705.
134. Siegel, S.J., et al., *The oxidative stress metabolite 4-hydroxynonenal promotes Alzheimer protofibril formation*. Biochemistry, 2007. **46**(6): p. 1503-10.
135. Stefanis, L., et al., *Expression of A53T mutant but not wild-type alpha-synuclein in PC12 cells induces alterations of the ubiquitin-dependent degradation system, loss of dopamine release, and autophagic cell death*. J Neurosci, 2001. **21**(24): p. 9549-60.
136. Tanaka, Y., et al., *Inducible expression of mutant alpha-synuclein decreases proteasome activity and increases sensitivity to mitochondria-dependent apoptosis*. Hum Mol Genet, 2001. **10**(9): p. 919-26.
137. Petrucelli, L., et al., *Parkin protects against the toxicity associated with mutant alpha-synuclein: proteasome dysfunction selectively affects catecholaminergic neurons*. Neuron, 2002. **36**(6): p. 1007-19.
138. Gosavi, N., et al., *Golgi fragmentation occurs in the cells with prefibrillar alpha-synuclein aggregates and precedes the formation of fibrillar inclusion*. J Biol Chem, 2002. **277**(50): p. 48984-92.
139. Smith, W.W., et al., *Endoplasmic reticulum stress and mitochondrial cell death pathways mediate A53T mutant alpha-synuclein-induced toxicity*. Hum Mol Genet, 2005. **14**(24): p. 3801-11.
140. Volles, M.J., et al., *Vesicle permeabilization by protofibrillar alpha-synuclein: implications for the pathogenesis and treatment of Parkinson's disease*. Biochemistry, 2001. **40**(26): p. 7812-9.
141. Lashuel, H.A., et al., *Alpha-synuclein, especially the Parkinson's disease-associated mutants, forms pore-like annular and tubular protofibrils*. J Mol Biol, 2002. **322**(5): p. 1089-102.
142. Roy, S., *The paradoxical cell biology of alpha-Synuclein*. Results Probl Cell Differ, 2009. **48**: p. 159-72.
143. Scott, D.A., et al., *A pathologic cascade leading to synaptic dysfunction in alpha-synuclein-induced neurodegeneration*. J Neurosci, 2010. **30**(24): p. 8083-95.
144. Mandelkow, E., et al., *Structural principles of tau and the paired helical filaments of Alzheimer's disease*. Brain Pathol, 2007. **17**(1): p. 83-90.
145. Amos, L.A., *Microtubule structure and its stabilisation*. Org Biomol Chem, 2004. **2**(15): p. 2153-60.

146. Ballatore, C., V.M. Lee, and J.Q. Trojanowski, *Tau-mediated neurodegeneration in Alzheimer's disease and related disorders*. Nat Rev Neurosci, 2007. **8**(9): p. 663-72.
147. Wille, H., et al., *Alzheimer-like paired helical filaments and antiparallel dimers formed from microtubule-associated protein tau in vitro*. J Cell Biol, 1992. **118**(3): p. 573-84.
148. Cobb, N.J. and W.K. Surewicz, *Prion diseases and their biochemical mechanisms*. Biochemistry, 2009. **48**(12): p. 2574-85.
149. Prusiner, S.B., *Novel proteinaceous infectious particles cause scrapie*. Science, 1982. **216**(4542): p. 136-44.
150. Gajdusek, D.C., *Spongiform virus encephalopathies*. J Clin Pathol Suppl (R Coll Pathol), 1972. **6**: p. 78-83.
151. Telling, G.C., *Transgenic mouse models of prion diseases*. Methods Mol Biol, 2008. **459**: p. 249-63.
152. Meyer-Luehmann, M., et al., *Exogenous induction of cerebral beta-amyloidogenesis is governed by agent and host*. Science, 2006. **313**(5794): p. 1781-4.
153. Kane, M.D., et al., *Evidence for seeding of beta -amyloid by intracerebral infusion of Alzheimer brain extracts in beta -amyloid precursor protein-transgenic mice*. J Neurosci, 2000. **20**(10): p. 3606-11.
154. Clavaguera, F., et al., *Transmission and spreading of tauopathy in transgenic mouse brain*. Nat Cell Biol, 2009. **11**(7): p. 909-13.
155. Li, J.Y., et al., *Lewy bodies in grafted neurons in subjects with Parkinson's disease suggest host-to-graft disease propagation*. Nat Med, 2008. **14**(5): p. 501-3.
156. Hansen, C., et al., *alpha-Synuclein propagates from mouse brain to grafted dopaminergic neurons and seeds aggregation in cultured human cells*. J Clin Invest, 2011. **121**(2): p. 715-25.
157. Desplats, P., et al., *Inclusion formation and neuronal cell death through neuron-to-neuron transmission of alpha-synuclein*. Proc Natl Acad Sci U S A, 2009. **106**(31): p. 13010-5.
158. Lee, H.J., et al., *Assembly-dependent endocytosis and clearance of extracellular alpha-synuclein*. Int J Biochem Cell Biol, 2008. **40**(9): p. 1835-49.
159. Danzer, K.M., et al., *Seeding induced by alpha-synuclein oligomers provides evidence for spreading of alpha-synuclein pathology*. J Neurochem, 2009. **111**(1): p. 192-203.
160. Luk, K.C., et al., *Exogenous alpha-synuclein fibrils seed the formation of Lewy body-like intracellular inclusions in cultured cells*. Proc Natl Acad Sci U S A, 2009. **106**(47): p. 20051-6.
161. Kordower, J.H., et al., *Lewy body-like pathology in long-term embryonic nigral transplants in Parkinson's disease*. Nat Med, 2008. **14**(5): p. 504-6.
162. Mendez, I., et al., *Dopamine neurons implanted into people with Parkinson's disease survive without pathology for 14 years*. Nat Med, 2008. **14**(5): p. 507-9.

163. Brundin, P., et al., *Research in motion: the enigma of Parkinson's disease pathology spread*. Nat Rev Neurosci, 2008. **9**(10): p. 741-5.
164. Schenk, D., et al., *Immunization with amyloid-beta attenuates Alzheimer-disease-like pathology in the PDAPP mouse*. Nature, 1999. **400**(6740): p. 173-7.
165. Morgan, D., et al., *A beta peptide vaccination prevents memory loss in an animal model of Alzheimer's disease*. Nature, 2000. **408**(6815): p. 982-5.
166. Janus, C., et al., *A beta peptide immunization reduces behavioural impairment and plaques in a model of Alzheimer's disease*. Nature, 2000. **408**(6815): p. 979-82.
167. Nicoll, J.A., et al., *Abeta species removal after abeta42 immunization*. J Neuropathol Exp Neurol, 2006. **65**(11): p. 1040-8.
168. Hock, C., et al., *Antibodies against beta-amyloid slow cognitive decline in Alzheimer's disease*. Neuron, 2003. **38**(4): p. 547-54.
169. Orgogozo, J.M., et al., *Subacute meningoencephalitis in a subset of patients with AD after Abeta42 immunization*. Neurology, 2003. **61**(1): p. 46-54.
170. Bard, F., et al., *Peripherally administered antibodies against amyloid beta-peptide enter the central nervous system and reduce pathology in a mouse model of Alzheimer disease*. Nat Med, 2000. **6**(8): p. 916-9.
171. Monsonogo, A., et al., *Microglia-mediated nitric oxide cytotoxicity of T cells following amyloid beta-peptide presentation to Th1 cells*. J Immunol, 2003. **171**(5): p. 2216-24.
172. Masliah, E., et al., *Effects of alpha-synuclein immunization in a mouse model of Parkinson's disease*. Neuron, 2005. **46**(6): p. 857-68.
173. Masliah, E., et al., *Passive immunization reduces behavioral and neuropathological deficits in an alpha-synuclein transgenic model of Lewy body disease*. PLoS One, 2011. **6**(4): p. e19338.
174. Weiner, H.L. and D. Frenkel, *Immunology and immunotherapy of Alzheimer's disease*. Nat Rev Immunol, 2006. **6**(5): p. 404-16.
175. Weinreb, P.H., et al., *NACP, a protein implicated in Alzheimer's disease and learning, is natively unfolded*. Biochemistry, 1996. **35**(43): p. 13709-15.
176. Wang, M.S., et al., *Curcumin reduces alpha-synuclein induced cytotoxicity in Parkinson's disease cell model*. BMC Neurosci, 2010. **11**: p. 57.
177. Zhu, M., J. Li, and A.L. Fink, *The association of alpha-synuclein with membranes affects bilayer structure, stability, and fibril formation*. J Biol Chem, 2003. **278**(41): p. 40186-97.
178. Hong, D.P., A.L. Fink, and V.N. Uversky, *Structural characteristics of alpha-synuclein oligomers stabilized by the flavonoid baicalein*. J Mol Biol, 2008. **383**(1): p. 214-23.
179. Zhou, W., et al., *Methionine oxidation stabilizes non-toxic oligomers of alpha-synuclein through strengthening the auto-inhibitory intramolecular long-range interactions*. Biochim Biophys Acta, 2010. **1802**(3): p. 322-30.

180. Bieschke, J., et al., *EGCG remodels mature alpha-synuclein and amyloid-beta fibrils and reduces cellular toxicity*. Proc Natl Acad Sci U S A, 2010. **107**(17): p. 7710-5.
181. Welander, H., et al., *Gelsolin co-occurs with Lewy bodies in vivo and accelerates alpha-synuclein aggregation in vitro*. Biochem Biophys Res Commun, 2011. **412**(1): p. 32-8.
182. Bruinsma, I.B., et al., *Inhibition of alpha-synuclein aggregation by small heat shock proteins*. Proteins, 2011. **79**(10): p. 2956-67.
183. Zhou, W., et al., *At low concentrations, 3,4-dihydroxyphenylacetic acid (DOPAC) binds non-covalently to alpha-synuclein and prevents its fibrillation*. J Mol Biol, 2009. **388**(3): p. 597-610.
184. Conway, K.A., J.D. Harper, and P.T. Lansbury, *Accelerated in vitro fibril formation by a mutant alpha-synuclein linked to early-onset Parkinson disease*. Nat Med, 1998. **4**(11): p. 1318-20.
185. Sweers, K., et al., *Nanomechanical properties of alpha-synuclein amyloid fibrils: a comparative study by nanoindentation, harmonic force microscopy, and Peakforce QNM*. Nanoscale Res Lett, 2011. **6**(1): p. 270.
186. Yagi, H., et al., *Amyloid fibril formation of alpha-synuclein is accelerated by preformed amyloid seeds of other proteins: implications for the mechanism of transmissible conformational diseases*. J Biol Chem, 2005. **280**(46): p. 38609-16.
187. Remy, I. and S.W. Michnick, *Clonal selection and in vivo quantitation of protein interactions with protein-fragment complementation assays*. Proc Natl Acad Sci U S A, 1999. **96**(10): p. 5394-9.
188. Dickson, D.W., *Misfolded, protease-resistant proteins in animal models and human neurodegenerative disease*. J Clin Invest, 2002. **110**(10): p. 1403-5.
189. Freichel, C., et al., *Age-dependent cognitive decline and amygdala pathology in alpha-synuclein transgenic mice*. Neurobiol Aging, 2007. **28**(9): p. 1421-35.
190. Neumann, M., et al., *Misfolded proteinase K-resistant hyperphosphorylated alpha-synuclein in aged transgenic mice with locomotor deterioration and in human alpha-synucleinopathies*. J Clin Invest, 2002. **110**(10): p. 1429-39.
191. Cizas, P., et al., *Size-dependent neurotoxicity of beta-amyloid oligomers*. Arch Biochem Biophys, 2010. **496**(2): p. 84-92.
192. Reixach, N., et al., *Tissue damage in the amyloidoses: Transthyretin monomers and nonnative oligomers are the major cytotoxic species in tissue culture*. Proc Natl Acad Sci U S A, 2004. **101**(9): p. 2817-22.
193. Nie, C.L., et al., *Amyloid-like aggregates of neuronal tau induced by formaldehyde promote apoptosis of neuronal cells*. BMC Neurosci, 2007. **8**: p. 9.
194. Lee, H.J., C. Choi, and S.J. Lee, *Membrane-bound alpha-synuclein has a high aggregation propensity and the ability to seed the*

- aggregation of the cytosolic form. *J Biol Chem*, 2002. **277**(1): p. 671-8.
195. Yu, S., et al., *Extensive nuclear localization of alpha-synuclein in normal rat brain neurons revealed by a novel monoclonal antibody*. *Neuroscience*, 2007. **145**(2): p. 539-55.
196. Kontopoulos, E., J.D. Parvin, and M.B. Feany, *Alpha-synuclein acts in the nucleus to inhibit histone acetylation and promote neurotoxicity*. *Hum Mol Genet*, 2006. **15**(20): p. 3012-23.
197. Lee, H.J., S. Patel, and S.J. Lee, *Intravesicular localization and exocytosis of alpha-synuclein and its aggregates*. *J Neurosci*, 2005. **25**(25): p. 6016-24.
198. Ahn, K.J., et al., *Amino acid sequence motifs and mechanistic features of the membrane translocation of alpha-synuclein*. *J Neurochem*, 2006. **97**(1): p. 265-79.
199. Zhou, C., et al., *A human single-chain Fv intrabody blocks aberrant cellular effects of overexpressed alpha-synuclein*. *Mol Ther*, 2004. **10**(6): p. 1023-31.
200. Gai, W.P., et al., *alpha-Synuclein fibrils constitute the central core of oligodendroglial inclusion filaments in multiple system atrophy*. *Exp Neurol*, 2003. **181**(1): p. 68-78.
201. Tampellini, D., et al., *Internalized antibodies to the Abeta domain of APP reduce neuronal Abeta and protect against synaptic alterations*. *J Biol Chem*, 2007. **282**(26): p. 18895-906.
202. Hagan, P.L., et al., *Tumor size: effect on monoclonal antibody uptake in tumor models*. *J Nucl Med*, 1986. **27**(3): p. 422-7.
203. Mallery, D.L., et al., *Antibodies mediate intracellular immunity through tripartite motif-containing 21 (TRIM21)*. *Proc Natl Acad Sci U S A*, 2010. **107**(46): p. 19985-90.
204. Schell, H., et al., *Nuclear and neuritic distribution of serine-129 phosphorylated alpha-synuclein in transgenic mice*. *Neuroscience*, 2009. **160**(4): p. 796-804.
205. Hinterdorfer, P. and Y.F. Dufrene, *Detection and localization of single molecular recognition events using atomic force microscopy*. *Nat Methods*, 2006. **3**(5): p. 347-55.
206. Allison, D.P., et al., *Atomic force microscopy of biological samples*. *Wiley Interdiscip Rev Nanomed Nanobiotechnol*, 2010. **2**(6): p. 618-34.
207. Yu, J., S. Malkova, and Y.L. Lyubchenko, *alpha-Synuclein misfolding: single molecule AFM force spectroscopy study*. *J Mol Biol*, 2008. **384**(4): p. 992-1001.
208. Goncalves, S.A., J.E. Matos, and T.F. Outeiro, *Zooming into protein oligomerization in neurodegeneration using BiFC*. *Trends Biochem Sci*, 2010. **35**(11): p. 643-51.
209. McLean, P.J., H. Kawamata, and B.T. Hyman, *Alpha-synuclein-enhanced green fluorescent protein fusion proteins form proteasome sensitive inclusions in primary neurons*. *Neuroscience*, 2001. **104**(3): p. 901-12.

210. Liu, Y., et al., *A novel molecular mechanism for nitrated {alpha}-synuclein-induced cell death*. J Mol Cell Biol, 2011. **3**(4): p. 239-49.
211. Engvall, E. and P. Perlmann, *Enzyme-linked immunosorbent assay (ELISA). Quantitative assay of immunoglobulin G*. Immunochemistry, 1971. **8**(9): p. 871-4.
212. Lequin, R.M., *Enzyme immunoassay (EIA)/enzyme-linked immunosorbent assay (ELISA)*. Clin Chem, 2005. **51**(12): p. 2415-8.
213. Englund, H., et al., *Sensitive ELISA detection of amyloid-beta protofibrils in biological samples*. J Neurochem, 2007. **103**(1): p. 334-45.
214. Kelly, S.M., T.J. Jess, and N.C. Price, *How to study proteins by circular dichroism*. Biochim Biophys Acta, 2005. **1751**(2): p. 119-39.
215. Greenfield, N.J., *Using circular dichroism spectra to estimate protein secondary structure*. Nat Protoc, 2006. **1**(6): p. 2876-90.
216. Greenfield, N.J., *Analysis of the kinetics of folding of proteins and peptides using circular dichroism*. Nat Protoc, 2006. **1**(6): p. 2891-9.
217. Bohmer, M. and J. Enderlein, *Fluorescence spectroscopy of single molecules under ambient conditions: methodology and technology*. Chemphyschem, 2003. **4**(8): p. 793-808.
218. Wilson, T., *Optical sectioning in fluorescence microscopy*. J Microsc, 2011. **242**(2): p. 111-6.
219. Berridge, M.V. and A.S. Tan, *Characterization of the cellular reduction of 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT): subcellular localization, substrate dependence, and involvement of mitochondrial electron transport in MTT reduction*. Arch Biochem Biophys, 1993. **303**(2): p. 474-82.
220. Mosmann, T., *Rapid colorimetric assay for cellular growth and survival: application to proliferation and cytotoxicity assays*. J Immunol Methods, 1983. **65**(1-2): p. 55-63.
221. Yuan, Y., et al., *Overexpressed alpha-synuclein regulated the nuclear factor-kappaB signal pathway*. Cell Mol Neurobiol, 2008. **28**(1): p. 21-33.
222. Kumar, B., et al., *Selenomethionine prevents degeneration induced by overexpression of wild-type human alpha-synuclein during differentiation of neuroblastoma cells*. J Am Coll Nutr, 2005. **24**(6): p. 516-23.
223. Liu, D.M., et al., *RNA interference mediated silencing of alpha-synuclein in MN9D cells and its effects on cell viability*. Neurosci Bull, 2008. **24**(2): p. 96-104.
224. Honda, H., et al., *Protease-resistant PrP and PrP oligomers in the brain in human prion diseases after intraventricular pentosan polysulfate infusion*. Neuropathology, 2011.
225. Miake, H., et al., *Biochemical characterization of the core structure of alpha-synuclein filaments*. J Biol Chem, 2002. **277**(21): p. 19213-9.
226. Tanji, K., et al., *Proteinase K-resistant alpha-synuclein is deposited in presynapses in human Lewy body disease and A53T alpha-synuclein transgenic mice*. Acta Neuropathol, 2010. **120**(2): p. 145-54.

227. Kahle, P.J., et al., *Subcellular localization of wild-type and Parkinson's disease-associated mutant alpha -synuclein in human and transgenic mouse brain*. J Neurosci, 2000. **20**(17): p. 6365-73.
228. Towbin, H., T. Staehelin, and J. Gordon, *Electrophoretic transfer of proteins from polyacrylamide gels to nitrocellulose sheets: procedure and some applications*. Proc Natl Acad Sci U S A, 1979. **76**(9): p. 4350-4.
229. Burnette, W.N., *"Western blotting": electrophoretic transfer of proteins from sodium dodecyl sulfate--polyacrylamide gels to unmodified nitrocellulose and radiographic detection with antibody and radioiodinated protein A*. Anal Biochem, 1981. **112**(2): p. 195-203.
230. Ahmad, A., *DnaK/DnaJ/GrpE of Hsp70 system have differing effects on alpha-synuclein fibrillation involved in Parkinson's disease*. Int J Biol Macromol, 2010. **46**(2): p. 275-9.

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