Master's Thesis

α-Synuclein aggregation in Parkinson's disease

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List of abbreviations

Aβ	Amyloid-β
AD	Alzheimer's disease
ADL	Activities of daily living
ALS	Amyotropic lateral sclerosis
APP	Amyloid-β precursor protein
CNS	Central nervous system
COMT	Catechol-*o*-methyltransferase
DA	Dopamine
DBS	Deep brain stimulation
DLB	Dementia with Lewy bodies
DM2	Type II diabetes mellitus
IAPP	Islet amyloid polypeptide
LB	Lewy body
L-Dopa	Levodopa
LRRK2	Leucine-rich repeat kinase 2
MAO	Monoaminoxidase
MSA	Multiple system atrophy
NFT	Neurofibrillary tangle
NMDA	N-methyl d-aspartate
PD	Parkinson's disease
PHF	Paired helical filament
POPC	1-palmitoyl-2-eleoyl-sn-glycero-3-phosphocholine
PrP	Prion protein
PrPC	Benign prion protein
PrPSc	Malignant prion protein
TSE	Transmissible spongiform encephalopathy
UPS	Ubiquitin-proteasome system.

Summary

Parkinson's disease (PD) is a slowly progressive disease that affects approximately 6,3 million individuals worldwide. The disease manifests itself with both motor symptoms and non-motor symptoms, the latter of which influence the quality of life at most. Furthermore, mortality rates in PD patients are relatively high, compared to those of the general population. At present, it is still not possible to delay or to cease the progressive neuronal cell death that is associated with PD. Therapies that are currently used only diminish the severity of the symptoms and have no effect on the cause of the disease.

Histopathologically, PD is characterized by a progressive decreased amount of dopaminergic neurons in the brain, which ultimately leads to dopamine deficiency, deterioration of the affected brain areas and impaired brain functioning. Furthermore, Lewy bodies evolve in the damaged dopaminergic neurons, with α-synuclein as their major component. Although the exact function of α-synuclein is largely unknown, it is associated with neuronal development, regulation of synaptic plasticity and the regulation of dopamine release.

Up to now, the exact pathogenesis of PD is unknown. Although several PD-associated gene mutations have been identified, these seem to account for only a small part of all PD cases. One of the most promising indications in unraveling the pathogenesis of PD is the potential role of the α-synuclein gene (*SNCA*). In this gene, PD associated point-mutations, as well as gene duplications and triplications have been identified. Besides the presence of *SNCA* mutations in PD patients, α-synuclein is the main component of Lewy bodies, and α-synuclein aggregation is found in all PD patients. Although it is still not completely clear what causes this protein to aggregate, these findings implicate there is an important role for α-synuclein in the development and progression of PD. In addition, α-synuclein also seems to be involved in the development of several other human neurodegenerative diseases, including dementia with Lewy bodies (DLB), multiple system atrophy (MSA) and amyotrophic lateral sclerosis (ALS), making it a promising protein for further research.

α-Synuclein aggregation that is associated with PD, shows great resemblance with amyloidoses. Amyloidoses are a group of protein folding diseases that are accompanied by failure of the organs that are involved and include Alzheimer's disease (AD), diabetes mellitus type II (DM2) and prion disease. In PD, similar deposits to those in amyloidoses have been found. Interestingly, in contrast to other amyloid diseases, these deposits are exclusively located intracellular.

All of the diseases described in this thesis, including PD, are associated with the formation of protein oligomers, that eventually develop into aggregates. However, the oligomers are the toxic components, rather than the protein aggregates. Although several factors have been described that can induce protein misfolding and aggregation, the motive for the formation of oligomers and aggregates is still unknown. Furthermore, it is still undefined to what extent the oligomers are involved in cell death of their target cells. Therefore, extensive research is necessary to each of the described diseases. However, since the diseases show great resemblance, studies on one disease can also be informative for the other diseases, as well as for other amyloidoses that have not been described in this thesis.

1. Parkinson's disease

1.1 Introduction to Parkinson's disease

Parkinson's disease (PD) was first described by James Parkinson (1755-1824), a British political agitator and geologist [1]. Although there is no evidence of any medical education, he was also working as a surgeon. Furthermore, Parkinson was a writer of popular medical articles [1, 2]. In 1817, he wrote '*Essay on the Shaking Palsy*', in which he described the condition that is now known as Parkinson's disease [2]. In 1824, Parkinson suffered from a stroke and died at age 69.

PD is, after Alzheimer's disease (AD), the second most common neurodegenerative human disease [1, 3]. PD is a progressive disorder, characterized by slowness of movement (bradykinesia), an increase in muscle tone that causes resistance to passive movement (rigidity), tremors, and postural instability [4]. There are two types of PD; early-onset PD and late-onset PD, the latter of which includes 85% of all disease cases. Early-onset PD is also referred to as familial PD, while late-onset PD is also called idiopathic PD [5]. Since age is an important risk factor for the development of PD, it is primarily a disease of the elderly [6]. The age of onset of PD is usually over 60 years. However, approximately one in ten patients are younger than 50 years at the time of diagnosis [7, 8].

Based on studies conducted from 1994 to 2008, approximately 6,3 million people suffer from this disease worldwide [8]. In a study published in 2004, the prevalence of PD was determined in two Spanish populations. The total prevalence of PD was 1500 per 100,000 persons. The prevalence per age group is presented in Table 1 [9]. These numbers are comparable to those found in other studies in European countries [9-11].

Table 1. The prevalence of PD in different age groups [9].

Age group	Prevalence per 100,000
Total population	1500
65-74 years	400
75-84 years	4700
> 85 years	2900

In 2009, a paper was published, describing a 15-year prospective study to provide insight in PD incidence. The researchers found an incidence of 263 per 100,000 person-years, with a slightly higher incidence in men compared to woman. The mean age at time of diagnosis was 78.5 years and the incidence decreased over 85 years of age [12]. The incidence determined in this study, is comparable to PD incidence measured in other European and American studies, conducted in the period from 2000 to 2009 (Table 2) [12-15].

Table 2. Incidence of PD in recent population-based longitudinal studies [12].

Year	Study reference	Country	Population age	Screening phase	Follow-up (years)	Person-years at risk	Incidence per 100000
2000	ILSA8	Italy	65–84	Yes	3	12140	326
2004	NEDICES6	Spain	>65	Yes	3	11110	235.9
2004	Rotterdam7	The Netherlands	>55	Yes	6	38422	170
2006	Aberdeen29	UK	All ages	>65	1.5	148600	22.4
2009	PHS30	USA	40–84	No	23	467316	120.48
	PAQUID	France	>65	Yes	15	25820	263.4

Mortality rates in patients with PD are relatively high, compared to those of the general population [16, 17]. In early onset PD, where the disease begins to develop before age 50, the average total life expectancy is shorter, compared to that of the general population (Fig. 1). However, these differences become smaller as the age of onset increases [18].

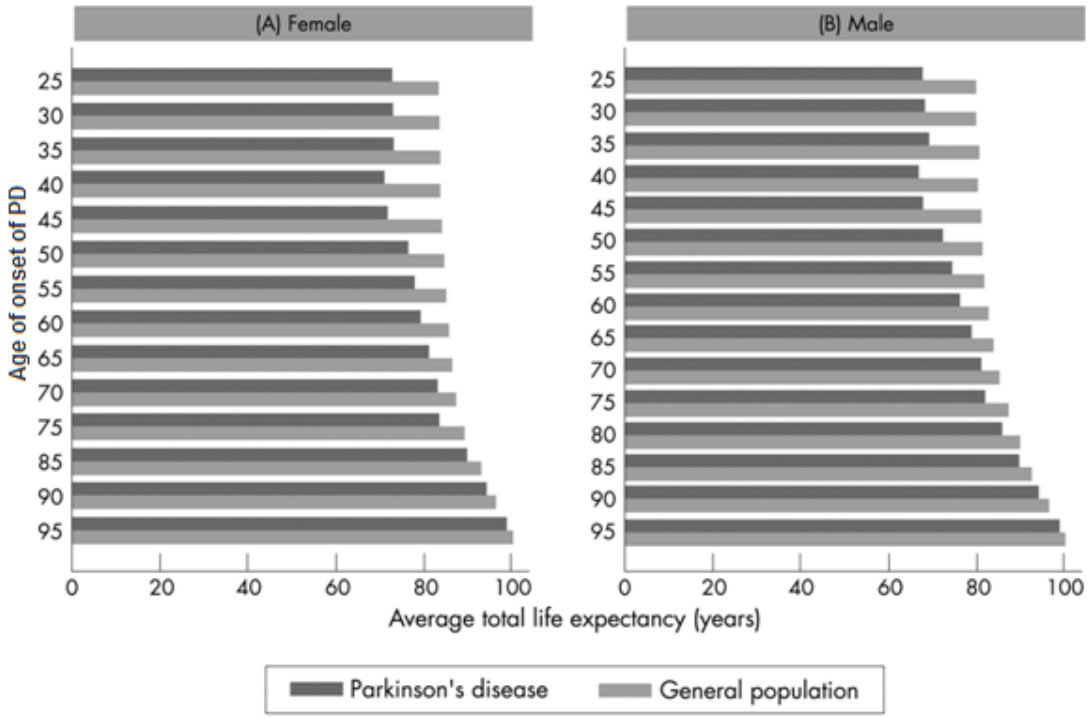

Figure 1*. *Average anticipated age at death for patients with PD. *The differences between PD patients and the general population become smaller as the age of onset of PD increases [adapted from 18].*

Multiple studies have revealed that the increase in mortality was associated with disease duration, male sex, older age, postural instability, gait difficulty (difficulties in achieving movement) and the development of dementia [16, 17]. Although studies are contradictory, most studies show a

higher incidence in men [17]. In a Norwegian study, Alves et al demonstrated a 58% higher age standardised incidence in man compared to women [19]. A possible explanation might be that female steroid hormones protect women from developing neurological conditions. However, there seem to be no gender associated differences in PD incidence in Asian countries. These findings imply that female steroid hormones are not the only factor involved in the differences in developing PD between genders [19].

Lo et al found that cognitive impairment within two years of PD diagnosis was the strongest predictor of mortality, showing a two-fold increase in mortality risk. The more severe the cognitive impairment, the higher the mortality risk. Furthermore, hallucinations that occur early in the development of PD seem to be associated with an increased mortality risk [16]. Other studies have revealed that malignancies, ischaemic heart disease, pneumonia and cerebrovascular diseases are common causes of death in PD patients [16, 20-24]. During the progression of PD, dysphagia (difficulty swallowing) is likely to develop [23, 25]. Aspiration while eating can result in aspiration pneumonia, and is accompanied by a high mortality risk ratio [16, 20-24].

1.2 Symptoms and disease progression

Parkinson's disease is a slowly progressive condition. It may take 20 years to develop and is characterized by various symptoms, which can be divided into motor symptoms and non-motor symptoms [13]. Patients with PD live through several disease stages. In the beginning, the disease manifests as indefinable non-motor symptoms [26], such as subtle cognitive deficits. These include impaired problem solving capacities and defective planning and organization, as well as impaired learning skills and impaired memory [27, 28].

In the next phase, the characteristic motor symptoms develop. Motor-symptoms in PD involve bradykinesia, cogwheel rigidity (abnormal muscle stiffness that goes along with uncontrolled movements), postural instability, and rest tremor [26, 29, 30]. As a result of the developing motor symptoms, the patient's sleep pattern changes [26]. Sleeping disorders evolve, which include difficulties falling asleep, frequent awakenings, night-time incontinence, night-time confusion, and daytime sleepiness [27, 31]. In some patients, anxiety and depression evolve in this stage, which become more present as the disease progresses [26, 32]. Other symptoms many PD patient's have to manage are a loss of initiative and assertiveness, disturbed mood regulation, a lack of emotional reactivity and painful sensory complaints, caused by muscle cramps or tightness [32, 33].

In the final stage of the disease, patients may suffer from apathy and psychosis, and eventually become demented [26]. Psychosis seems to be the most challenging non-motor symptom [27, 34], and approximately one-third of patient's with PD suffer from it [35]. Psychosis mainly develops during cognitive deterioration and usually manifests as vivid dreams, hallucinations and delusions. Both the development of psychosis and dementia has a substantial impact on the patients quality of life and are major risk factors for nursing home placement [27, 34-36].

Although motor symptoms are mainly responsible for the morbidity of PD [37-39], the non-motor symptoms can be more disabling than the motor-symptoms [27, 33, 35]. As the disease progresses, the non-motor symptoms become more dominant and to a great extent determine the quality of life [4, 13, 26, 27].

1.3 Pathophysiological hallmarks

PD is associated with a progressive degeneration of dopaminergic neurons in the substantia nigra pars compacta (Fig. 2) and various other regions of basal ganglia, brainstem, autonomic nervous system and cerebral cortex. The loss of dopaminergic neurons results in dopamine deficiency in the striatum. This results in the characteristic PD symptoms, including tremor, rigidity, bradykinesia and postural disturbances [5, 6, 40-45]. Besides loss of dopaminergic neurons, also proteasomal dysfunction in the substantia nigra has been identified in PD patients [46]. The ubiquitin-proteasome system and autophagy-lysosome system are major intracellular cell-systems

for elimination of abnormal proteins. Alterations in the functioning of these systems seem to be important in the development of PD [42].

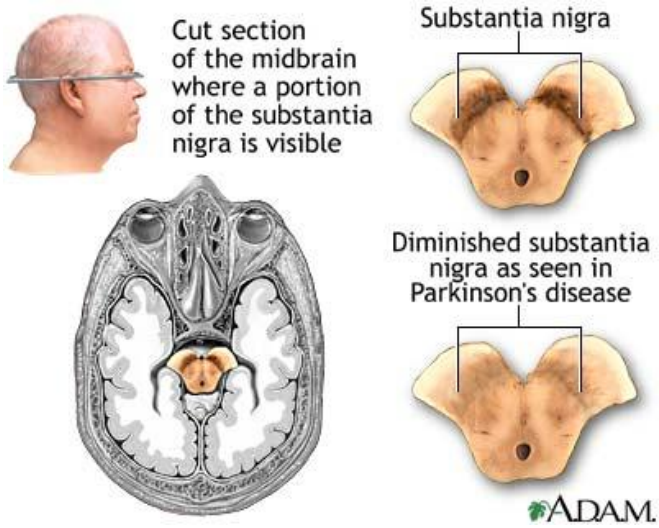

Figure 2. Schematic representation of the location of the substantia nigra in the human brain. *A decrease of dopaminergic neurons in, among others, the substantia nigra has been demonstrated in PD patients [47].*

A third characteristic of PD are the Lewy bodies (LBs) that are present in damaged dopaminergic neurons. Lewy bodies are intraneuronal inclusions that predominantly consist of α-synuclein and ubiquitin [42, 48, 49]. Although in PD patients LBs are mainly located in the brainstem, they are also found in the substantia nigra in the midbrain [41, 46]. There are two types of LBs: classic brainstem LBs, and cortical LBs. Classic brainstem LBs (Fig. 3A and B) are large spherical eosinophilic structures, located in dopaminergic neurons of the substantia nigra and locus coeruleus [49-51]. Classic brainstem LBs are enclosed by a characteristic clear ring [52]. Cortical LBs (Fig. 3C and D) are present in small neurons in the limbic and neocortal areas of the brain. Their size is smaller compared to classic LBs [49].

The presence of LBs is accompanied by deterioration of the affected brain areas [42, 48]. Although 90 proteins have been identified in brainstem LBs, their major components are a-synuclein, ubiquitin, phosphorylated neurofilaments, parkin, components of the ubiquitin-proteasome system, molecular chaperons and lipids [41, 42, 46, 50]. Also LRRK2 (leucine-rich repeat kinase 2) has been found in LBs and plays an important role in the development of LBs and the pathogenesis of PD [41].

De la Fuente-Fernandez et al found that, in the presence of LBs, there is an increase in neuronal death. This indicates a connection between LB formation and neuronal death [53]. However, if LBs would kill the neurons in which they are present, the amount of LBs would decrease over time. Greffard et al, showed that the amount of LBs found in the neurons of PD patients remains constant during the disease [54].

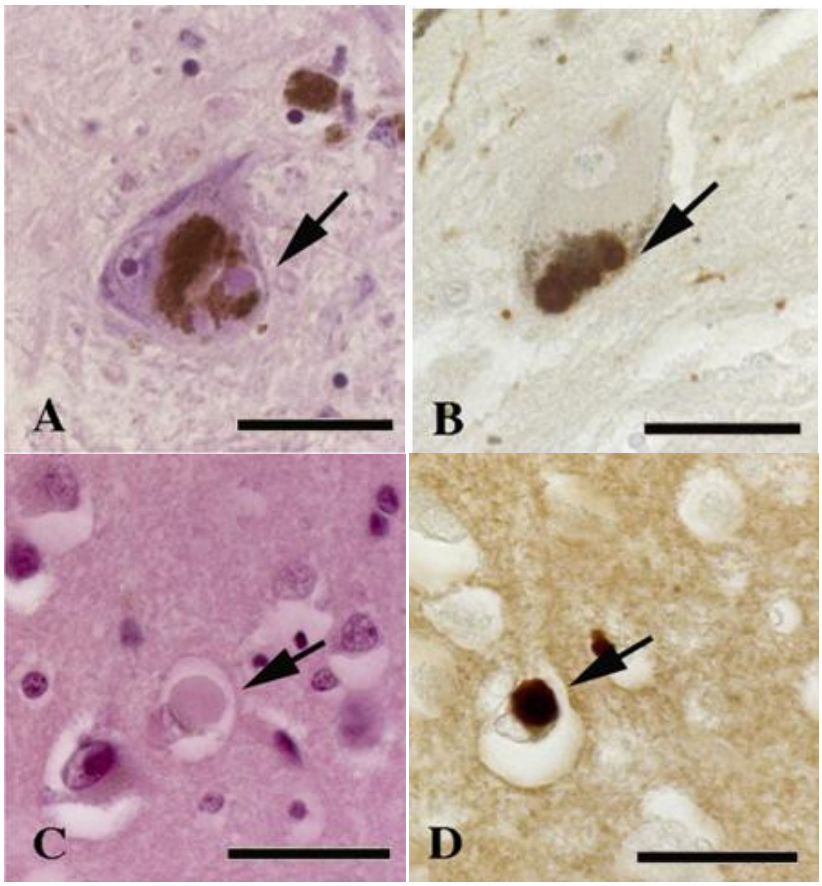

Figure 3. Classic brainstem LBs and cortical LBs in different brain regions. *A and B. Neuron in the substantia nigra (indicated by the arrows) with multiple classic LBs. H&E staining and anti α-synuclein immunostaining, respectively. C and D. Cortical LBs in the cingulated cortex (indicated by the arrows). H&E staining and anti α-synuclein immunostaining, respectively. Scale bar: 50 µm [adapted from 49].*

1.4 Aetiology of Parkinson's disease

Up to now, the exact pathogenesis and etiology of PD are largely elusive. Although genetic factors may be of great importance [30, 40, 41, 55], recent research suggests that mutations are responsible for only a small part of all PD cases [42]. Several genetic mutations have been identified in PD patients, including mutations in the following genes: *SNCA* (α-synuclein), *PINK1* (PTEN-induced putative kinase 1), *Parkin*, *UCH-L1* (ubiquitin C-terminal hydrolase L1)*, DJ-1* (PARK7)*, NR4A2* (nuclear receptor subfamily 4, group A, member 2) and *LRRK2* (leucine-rich repeat kinase 2) [6, 7, 40, 42]. Furthermore, several studies have identified loci across the genome (PARK3, PARK4, PARK6, PARK8, PARK9 and PARK10) containing PD-associated genes that nowadays are still unknown [6]. Gene mutations in *Parkin*, *PINK1* and *DJ-1* are linked to autosomal recessive, early onset familial PD [30, 41, 56], while mutations in *SNCA* and *LRRK2* are associated with autosomal dominant PD [41].

One of the most promising indications in the pathogenesis of PD is the potential role of the α-synuclein gene (*SNCA;* PARK1) [55]. In this gene, three PD associated point-mutations (A30P, E476K and A53T) have been detected, as well as gene duplications and triplications [6, 42, 55]. Although *SNCA* mutations account for less than 1% of PD cases, aggregation of the *SNCA* protein α-synuclein is identified in all patients suffering from PD. Furthermore, α-synuclein is the main protein present in LBs, one of the pathological characteristics of PD [55].

Mitochondrial dysfunction plays an important role in neuronal cell loss and the development of PD. At least two genes are associated with mitochondrial dysfunction in PD: *PINK1* and *Parkin* (PARK2) [30, 42]. PINK1 protein is a serine/threonine kinase, which is located in the mitochondrial

inner membrane and is important for maintaining mitochondrial homeostasis within dopaminergic neurons. PINK1 is able to directly phosphorylate parkin, an ubiquitin protein ligase that is related to the proteasome. After phosphorylation, parkin regulates mitochondrial function [42, 57]. It is not entirely understood how PINK1 can phosphorylate parkin, which is located in the cytoplasm. Probably, PINK1 uses a signal transduction pathway to stimulate parkin to ubiquitinate its targets, which in their turn affect mitochondrial function [57]. *Parkin* is a large gene in which over 70 PD associated mutations have been shown. Familial PD associated mutations in parkin disturb its ubiquitin ligase activity [58]. Symptoms of *parkin*-linked PD vary from classical parkinsonism to severe dystonia [6]. Since parkin and α-synuclein associate, and are both found in LBs, no LBs are present in most cases of *Parkin*-linked PD [6, 58].

The UCH-L1 protein (ubiquitin carboxyl-terminal esterase L1; PARK5) accomodates a connection between the ubiquitin-proteasome system (UPS), the endosomal-lysosomal pathway and LBs in late-onset PD [59]. UCH-L1 is extensively present in the brain and manages a stock of mono-ubiquitin for the UPS [59, 60]. Furthermore, UCHL1 stimulates the preservation of free ubiquitin in the endosomal-lysosomal pathway. Mutations in *UCH-L1* therefore result in impaired functioning of the endosome-lysosome pathway and the UPS system, resulting in LB formation [59]. An I93M mutation in *UCH-L1* is associated with dominantly inherited PD. Besides, *UCH-L1*^{I93M}-transgenic mice show progressive dopaminergic cell loss in the substantia nigra. This implicates that an I93M mutation in *UCH-L1* is one of the factors causing PD [60].

Although *DJ-1 (*PARK7*)* was originally identified as an oncogene, it has also been shown to be important in the development of PD [56]. Furthermore, *DJ-1* appears to be important in other neuropathological diseases, including stroke [6, 56]. *DJ-1* gene mutations result in the development of early onset PD that is autosomal recessive [61]. PD caused by *DJ-1* mutation is characterized by alternating severity and slow disease progression [6]. Nowadays, the exact role and functioning of DJ-1 protein in PD remains unclear, as well as the mechanism of DJ-1 causing dopaminergic cell death [6, 56, 61]. However, this gene seems to respond to initiators of oxidative stress, among which dopaminergic toxins rotenone (a chemical used as pesticide that interferes with the mitochondrial electron transport) and MPTP (1-methyl-4-phenyl-1,2,3,6 tetrahydropyridine; a toxin that affects the substantia nigra and causes neuronal cell death) [6, 56]. This suggests that *DJ-1* mutations play a role in the development of PD.

NR4A2 (nuclear receptor subfamily 4, group A, member 2) encodes a transcription factor regulating, among others tyrosine hydroxylase, which is necessary for the differentiation and maintenance of dopaminergic neurons [6, 62]. Therefore, a decrease in *NR4A2* in the brain increases the vulnerability of dopaminergic neurons to stress and might thus play a role in the pathogenesis of PD [62]. The phenotype of *NR4A2*-linked parkinsonism is identical to late-onset PD [6].

LRRK2 mutations account for 2 to 6.4 percent of familial PD cases and 0.6 to 3.4 percent for idiopathic PD cases. The protein LRRK2, that is encoded by the *LRRK2* gene, is located in LBs and potentially regulates both α-synuclein and parkin by functioning as a kinase. Therefore, LRRK2 plays a pivotal role in LB formation and PD pathogenesis [63]. Figure 4 provides an overview of the causes of neuronal death in PD.

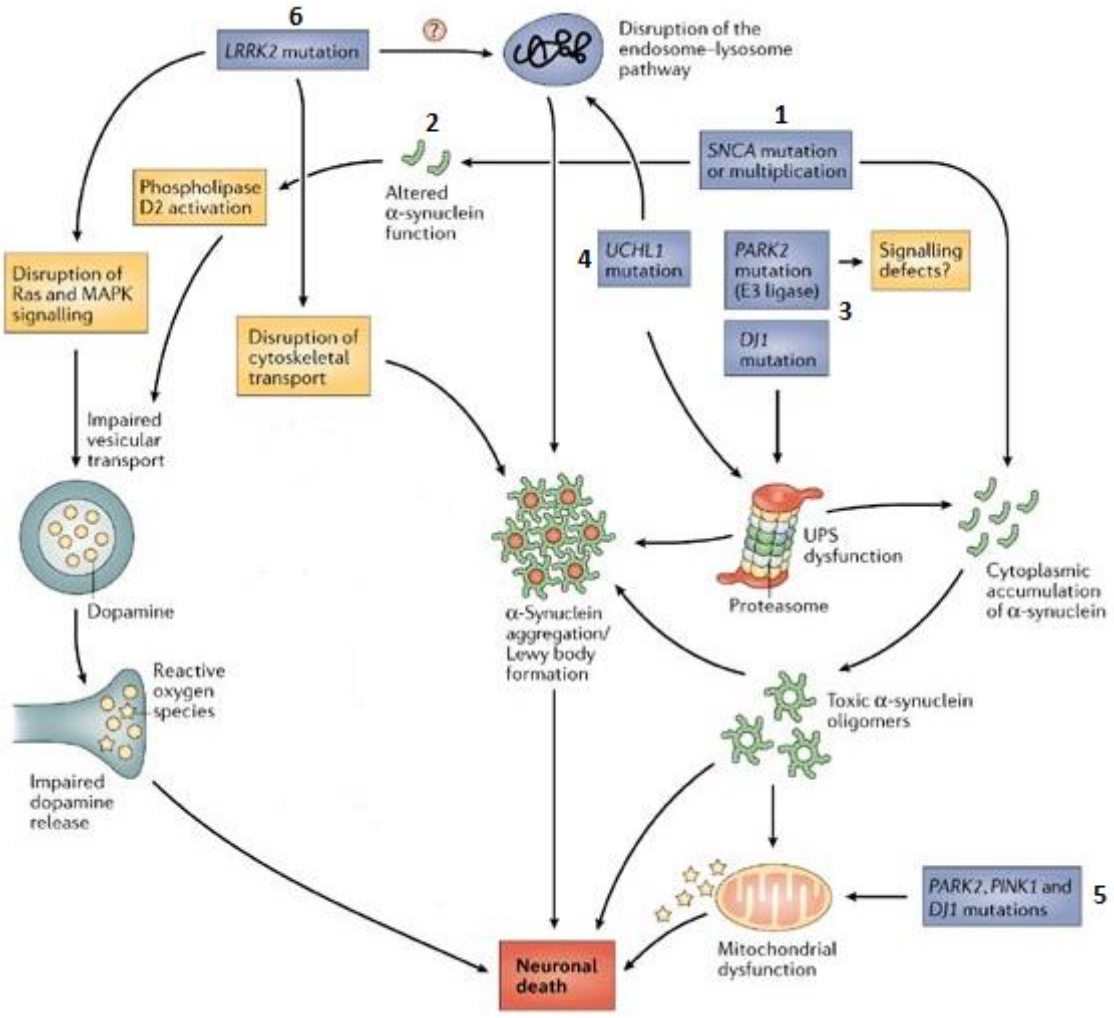

Figure 4. Model for neuronal death in PD. *1. Mutations or multiplications in SNCA result in an increased α-synuclein aggregation, which is toxic to cells and leads to neuronal death. 2. Mutations can alter the function of α-synuclein. This results in activation of phospholipase D2. Phospholipase D2 is an enzyme that is involved in, among others, vesicular transport. Mutations in α-synuclein result in a decreased vesicular transport that, in its turn, results in an impaired dopamine release. As a result of this, dopamine accumulates in the cytosol, leading to the generation of reactive oxygen species and neuronal death. 3. Genes parkin and DJ1 are required for proper functioning of the ubiquitin-proteasome system (UPS). Therefore, mutations in these genes may influence the proteasomal α-synuclein degradation and stimulate aggregation of α-synuclein. 4. UCH-L1 connects the UPS, endosomal-lysosomal pathways and LBs in late-onset PD. UCH-L1 manages a stock of mono-ubiquitin for the UPS and stimulates the preservation of free ubiquitin in the endosomal-lysosomal pathway. Mutations in UCH-L1 therefore result in impaired functioning of the endosome-lysosome pathway and UPS system, resulting in LB formation. 5. Mutations in PARK2, PINK1 and DJ1 all result in mitochondrial dysfunction. Since UPS functioning is dependent on mitochondrial ATP synthesis, mitochondrial dysfunction results in accumulation of α-synuclein and eventually, neuronal death. 6. LRRK2 plays a role in cellular trafficking, phosphorylation and intracellular signaling. Therefore, mutations in LRRK2 lead to impaired signaling pathways, resulting in impaired dopamine release. Furthermore, mutations in LRRK2 lead to destroyed cytoskeletal structure and microtubule instability [adapted from 59].*

1.5 Current therapies

The first symptoms of PD arise after approximately 60 percent of the dopaminergic neurons are damaged and lost. One of the consequences of the neuronal cell loss is a decreased dopamine concentration. This reduction in dopamine concentration in affected brain regions causes the majority of the PD symptoms, including bradykinesia, muscular rigidity and tremor [48]. Until now, it is not possible to prevent or to block the progressive neuronal cell loss in the substantia nigra [48, 64]. Furthermore, none of the therapies used nowadays are able to significantly decrease the disease progression. Treatments only act to reduce symptoms [48].

Dopamine replacement therapy with its precursor levodopa (L-3,4-dihydroxyphenylalanine; L-dopa), is considered to be the most effective treatment of PD, since its introduction in the 1960's [48, 64]. However, long- term treatment with L-dopa results in a diminished efficacy and the development of side effects, including hypokinesia, hyperkinesia or akinesia [48, 64]. Also dopaminergic receptor agonists and dopamine agonists play a major role in PD treatment nowadays [48, 64, 65]. In early stages of PD, treatment with dopamine agonists is often used. Therapy with dopamine agonists can reduce dyskinesia. However, treatment with dopamine-agonists is less effective than treatment with L-dopa. The use of dopamine agonists from the start of the disease can postpone the use of L-dopa or can reduce its dose for a longer period of time. As the disease progresses, increasing concentrations of dopamine agonists are needed, as a result of which side effects develop [64]. Although symptomatic therapy by increasing dopamine levels improves the quality of life, long term treatment is unwanted due to the side-effects, lowering of the drug efficacy, and ongoing progression of the disease [48, 66].

Other drugs currently used are COMT (Catechol-*o*-methyltransferase) inhibitors, MAO-B (monoamine oxidase B) inhibitors and apomorphine [48, 64, 65]. Next to L-dopa therapy to treat PD, COMT inhibitors and MAO-B inhibitors are the drugs of second choice [68]. COMT inhibitors inhibit the metabolism of L-dopa to 3-*o*-methyldopa (3-OMD). As a result of this, the half-life of L-dopa extends, causing a prolonged effect of this drug [65]. MAO-B inhibitors inhibit the intracellular enzyme monoamine oxidase and consequently decline the elimination of dopamine. This results in an increased dopamine concentration. Since MAO-B is, for the greater part, present in astrocytes, inhibition of MAO-B results in an increase in both endogenous and exogenous dopamine concentrations [65]. In comparison to the administration of L-dopa alone, the combination of L-dopa and either a COMT or MAO-B inhibitor has been shown to be most effective in treatment of PD [68].

Apomorphine is a dopamine receptor agonist that is used already for a long time. After injection, this drug has a quick effect. However, the effect does not last as long as the effect of levodopa. Treatment with apomorphine is mainly used as emergency treatment. The side effects are comparable with other dopamine agonists and include nausea, vomiting, hypersomnia and psychiatric disorders (confusion and hallucination) [64].

In case of advanced PD that does not respond to L-dopa therapy, deep brain stimulation (DBS) can be used as treatment [45, 67]. DBS is used as treatment for PD since 1987 and can only be applied if the patient does not suffer from dementia or other neurological symptoms [45, 67]. DBS is accomplished via implantation of electrodes into the affected brain areas [67]. Several studies have demonstrated an improvement of PD motor symptoms, and a significant decrease in tremor, bradykinesia and rigidity, following electrical stimulation of the brain [45, 67, 69]. Furthermore, DBS can reduce side effects of pharmaceutical treatment [45]. Although worldwide 30,000 patients underwent DBS for PD treatment, extended research is necessary to gain more insight into the functional anatomy and physiology of PD and the precise effects of DBS on PD. Furthermore, more research has to be conducted to confirm the long-term effects and possible side effects of DBS [45, 67].

Therapies including gene therapy, neural tissue transplantation and nanotechnology show great promise for future treatments of PD. However, extended research is needed for the safety and efficacy of these methods [45]. In several ways, nanotechnology could play a role in treatment of PD and other neurodegenerative diseases. For example, nanoparticles could be applied to directly

deliver drugs to their targets. Besides, nanotechnology can be used to repair DNA and to eliminate aggregated proteins. Furthermore, nanotechnology can be used to insert genes or proteins that are necessary for correct functioning of the brain. Presently, it is unknown if these suggestions can ever be used as therapy for PD [70]. Therefore, extended research is of great importance.

Treatment of PD should be multidisciplinary, comprising both pharmacological and non-pharmacological therapy. Non-pharmacological therapy involves, for example, exercise therapy. Exercise therapy could enhance activities of daily living and improve the quality of life in patients with PD. In 2009, Yousefi et al. showed that a 10-week exercise therapy in combination with drug therapy has positive effects on the activities of daily living (ADL) and the quality of life (QOL) in patients suffering from PD [71]. At the same time, other studies show that for PD therapy, a combination of drug therapy and exercise is more effective than only using pharmacological therapy [72-74]. The positive effects of exercise could be explained by the fact that exercise helps break the cycle of immobility that is experienced by many PD patients [71, 72].

2. α-Synuclein

2.1 The characteristics of α-synuclein

α-Synuclein is a small (~14 kD) cytoplasmic protein that belongs to the synuclein family. This family consists of three known proteins, α-, β-, and γ-synuclein [75-78]. The synuclein genes are mainly expressed in brain areas including the thalamus, the substantia nigra, the caudate nucleus and the amygdala. All synuclein proteins are found in neuronal cells present in the brain and the central nervous system (CNS) [77]. α-Synuclein is expressed by neuronal cells and is located in presynaptic nerve terminals [77, 79]. However, it can also be found in blood platelets, haematopoietic cells, neuromuscular connections and cardiac tissue [77].

α-Synuclein comprises 3 domains: an amphipathic N-terminal domain, a hydrophobic centre and a hydrophilic C-terminal domain. This composition results in a α-helical, random loop formation that facilitates α-synuclein interaction with phospholipid-containing membranes [77, 79-81].

Although the exact function of α-synuclein is largely unknown [77], it is highly expressed during neuronal development [75]. α-Synuclein is likely to be involved in neuronal differentiation, regulation of synaptic plasticity and regulation of dopamine release [77]. Furthermore, there seems to be a role for α-synuclein in the aetiology of several human neurodegenerative disorders, including PD, dementia with Lewy bodies (DLB), multiple system atrophy (MSA) and amyotrophic lateral sclerosis (ALS) [75, 77].

2.2 α-Synuclein aggregation

Multiple factors can induce α-synuclein misfolding and the formation of aggregates, including pH, temperature, gene mutations, α-synuclein over expression and oxidative stress [46, 75, 77, 80, 82]. Furthermore, in vitro studies have been shown that the hydrophobic centre of α-synuclein is able to spontaneously form aggregates [83]. In the process of forming aggregates, first misfolded α-synuclein forms relatively soluble oligomers. Second, these oligomers form insoluble fibrillar structures, which are also called fibrils or aggregates (Fig. 5). The aggregates contain β-pleated sheets, which are secondary protein structures, consisting of two or more polypeptide chains, connected by hydrogen bonds. Since the aggregates cannot be degraded by the ubiquitin-proteasome system (UPS), they accumulate in the cytoplasm [80, 81]. Although α-synuclein aggregates are found to be present in LBs in PD, the α-synuclein oligomers seem to be most toxic to cells [81]. The oligomers are likely to form pores in both the plasma membrane and intracellular membranes. Furukawa et al. described an increased cell permeability in cells expressing α-synuclein, supporting this thought [84].

Since PD is accompanied by loss of dopaminergic neurons, there most likely is a link between dopamine metabolism and the aggregation of α-synuclein [85, 86]. In vitro studies have shown that α-synuclein forms oligomers in the presence of dopamine or its oxidative intermediates. However, the exact mechanism of interaction between dopamine and α-synuclein is nowadays not completely understood [86].

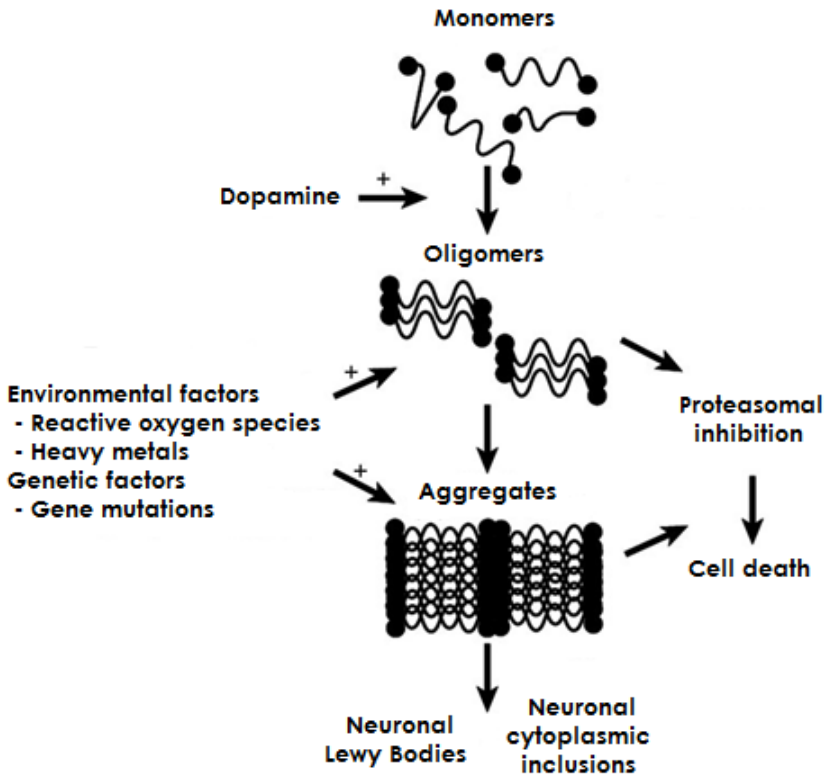

Figure 5. Model of α-synuclein aggregation. *In its native state, α-synuclein is a monomer and has a random coil conformation. Several factors can induce misfolding of the protein, resulting in the formation of oligomers. Eventually, the oligomers form β-pleated sheets, and aggregates are formed. Factors that enhance the formation of these aggregates are depicted by the arrows [adapted from 87].*

2.3 Interaction between α-synuclein and phoshpolipid membranes

α-Synuclein oligomers seem to be most toxic to cells, as a result of their ability to penetrate membranes [81, 88]. Ultrastructural and biochemical studies imply that α-synuclein oligomers are able to form pores in the membrane that will eventually result in cell death. In 2008, Tsigelny et al demonstrated that pentameric α-synuclein binds to and penetrates a phospholipid (1-palmitoyl-2-eleoyl-sn-glycero-3-phosphocholine) bilayer (Fig. 6) [89].

When α-synuclein oligomerisation occurs, first stable homodimers are formed. The dimers are prone to bind to phospholipid bilayers, after which they stabilise and form an oligomere [88, 90]. The formation of a pentamer, as shown in Figure 6A, is just one possible manner in which oligomerisation can occur. Further penetration of the α-synuclein pentamer can lead to pore formation in the membrane [90]. In contrast, α-synuclein monomers may bind to the membrane and do not cause pore formation [88].

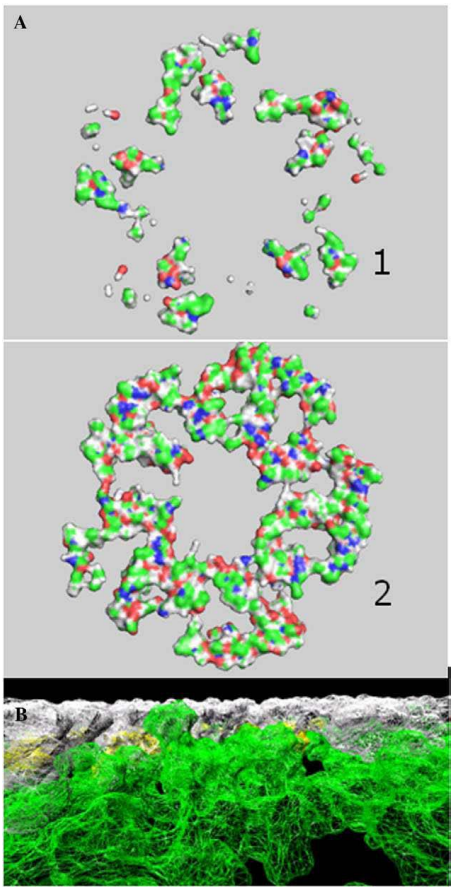

Figure 6. Binding of pentameric α-synuclein to a membrane. *A. Pentameric α-synuclein is shown behind the surface of a POPC (1-palmitoyl-2-eleoyl-sn-glycero-3-phosphocholine) membrane, before MD (molecular dynamics) simulation (1), and 0.5 nanoseconds after starting the MD simulation (2). B. The pentameric α-synuclein (white) is bound to the POPC membrane (green) [88].*

2.4 The role of α-synuclein in the pathogenesis of Parkinson's disease

Since α-synuclein is a major component of LBs (Fig. 7) [75, 76, 91, 92], α-synuclein aggregation seems to be an important factor for neuronal degeneration in PD patients [46, 91]. Although the reason for aggregation is still unknown, it has been shown that α-synuclein also forms aggregates in patients suffering from DLB and MSA [46].

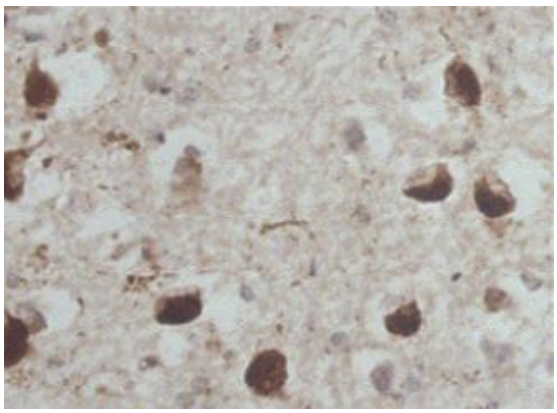

Figure 7. The localization of α-synuclein in Lewy bodies. *α-Synuclein aggregates are present in Lewy bodies. Magnification ×400 [adapted from 93].*

In 1997, Polymeropoulos et al were the first to show that an A53T mutation in the α-synuclein gene was genetically associated with early-onset, familial PD (Fig. 8). As a result of the substitution of alanine with threonine, the normal α-helix structure of this part of the protein is disorganized, and the β-pleated sheets extend. Consequently, the protein is more prone to aggregation [94]. Furthermore, in 1998 Kruger et al identified a second mutation in the α-synuclein gene, A30P, that is also associated with PD [95]. The role of these mutations in the pathogenesis of PD was supported by the finding that α-synuclein is the major component of LBs [83, 91]. Subsequently, linkage studies have shown that PD can also be caused by several other mutations in the α-synuclein gene (*SNCA*). In addition to these findings, recently it has been shown that *SNCA* gene duplications and triplications can be a cause of PD. This implies that also α-synuclein over expression can result in PD. However, *SNCA* mutations are infrequent and only occur in 1 percent of all PD cases [96].

The most common alteration found in α-synuclein present in LBs, is a phosphorylation at Ser-129 (P-S129) [79, 97]. Since P-S129 α-synuclein is also present in the normal brain, the phosphorylation is most likely associated with normal α-synuclein metabolism [98]. However, ubiquitinated P-S129 α-synuclein is only found in LBs, indicating that ubiquitination occurs after P-S129 α-synuclein translocated into LBs [98].

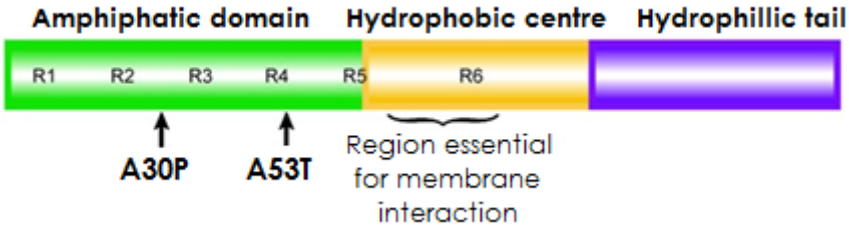

Figure 8. Schematic structure of α-synuclein. *The arrows indicate the location of mutations associated with early-onset familial PD [adapted from 87].*

Normally, α-synuclein monomers are degraded by the ubiquitin-proteasome pathway [99]. After poly-ubiquitination of α-synuclein, the protein is degraded by the proteasome, a proteolytic complex for protein degradation that is located in the cytoplasm [91]. In PD, mutated α-synuclein is degraded slower, as a result of which aggregates are formed [99]. This is confirmed by the presence of α-synuclein, as well as ubiquitin and proteasome subunits in LBs [91]. Since aggregated α-synuclein cannot be labeled by ubiquitin for proteasomal degradation, this results in a build-up of intracellular α-synuclein oligomers, which are toxic to cells. In addition, the toxic α-synuclein oligomers that are formed after accumulation of α-synuclein inhibit the UPS functioning [46, 72, 77, 100-102].

Another mechanism for degradation of α-synuclein is via lysosomal autophagy. HSC70 (heat shock protein cognate 70) and HSP90 (heat shock protein 90) are both lysosomal chaperone proteins that enable the relocation of α-synuclein to lysosomes and therefore prevent α-synuclein to accumulate and form aggregates. Since PD is associated with dysfunction of the lysosomal autophagy pathway, this contributes to the accumulation and fibril formation of α-synuclein in the cytoplasm [102].

Both in vitro and in vivo experiments have shown that there are links between α-synuclein, oxidative stress and the development of PD. Oxidative stress seems to result in an altered α-synuclein conformation, causing the formation of aggregates. Furthermore, oxidative stress causes dopaminergic dysfunction, which leads to a decreased dopamine release and neuronal death [46, 80].

3. Resemblance between Parkinson's disease and other diseases caused by pathological aggregation of proteins

3.1 Introduction to amyloidoses

Amyloidoses are a group of protein misfolding diseases, which are accompanied by failure of the organs that are involved [103, 104]. Specific proteins or peptides fold incorrectly, as a result of which they aggregate and form extracellular amyloid deposits in various tissues. Misfolding and aggregation of proteins can be induced by several factors, among which specific gene mutations and interactions with metal ions. It can also be caused by changes in the environment, including changes in pH or temperature [103]. Furthermore, the formation of amyloid deposits is dependent on the concentrations of the aggregating proteins, the interactions between the individual molecules of these proteins and the interactions between proteins and cells [105].

Amyloid deposits are characterized by the presence of β-sheets [103] and can be systemic, but they can also affect a single-organ [104, 105]. In the case of neurodegenerative disorders, disease is likely to be caused by the association of aggregated proteins with cellular components. In the case of systemic amyloidosis, disease seems to be caused by the aggregation of proteins and their accumulation in vital organs. Nowadays, the presence of amyloid deposits is identified in more than 20 degenerative diseases that affect various organs or the central nervous system (CNS) [103]. Examples of amyloidoses that affect the brain and the central nervous system, are Alzheimer's disease (AD) and Creutzfeldt-Jakob disease [103, 106]. Other amyloidoses involve organs including the liver, heart and pancreas (for example systemic amyloidoses and type II diabetes mellitus (DM2) [7, 40, 103, 105]. Table 3 provides an overview of peptides and proteins involved in amyloid diseases [103].

***Table 3**. Summary of the main amyloidoses and the proteins or peptides involved [103].*

Disease	Main aggregate component
Alzheimer's disease	Aβ peptides (plaques); tau protein (tangles)
Spongiform encephalopathies	Prion (whole or fragments)
Parkinson's disease	α-synuclein (wt or mutant)
Primary systemic amyloidosis	Ig light chains (whole or fragments)
Secondary systemic amyloidosis	Serum amyloid A (whole or 76-residue fragment)
Fronto-temporal dementias	Tau (wt or mutant)
Senile systemic amyloidosis	Transthyretin (whole or fragments)
Familial amyloid polyneuropathy I	Transthyretin (over 45 mutants)
Hereditary cerebral amyloid angiopathy	Cystatin C (minus a 10-residue fragment)
Haemodialysis-related amyloidosis	β_2-microglobulin
Familial amyloid polyneuropathy III	Apolipoprotein AI (fragments)
Finnish hereditary systemic amyloidosis	Gelsolin (71 amino acid fragment)
Type II diabetes	Amylin (fragment)
Medullary carcinoma of the thyroid	Calcitonin (fragment)
Atrial amyloidosis	Atrial natriuretic factor
Hereditary non-neuropathic systemic amyloidosis	Lysozyme (whole or fragments)
Injection-localised amyloidosis	Insulin
Hereditary renal amyloidosis	Fibrinogen α-A chain, transthyretin, apolipoprotein AI, apolipoprotein AII, lysozyme, gelsolin, cystatin C
Amyotrophic lateral sclerosis	Superoxide dismutase 1 (wt or mutant)
Huntington's disease	Huntingtin
Spinal and bulbar muscular atrophy	Androgen receptor [whole or poly(Q) fragments]
Spinocerebellar ataxias	Ataxins [whole or poly(Q) fragments]
Spinocerebellar ataxia 17	TATA box-binding protein [whole or poly(Q) fragments]

3.2 Resemblance between Parkinson's disease and amyloidoses

In PD, similar deposits to those in amyloidoses are found. In contrast to other amyloid diseases, these deposits are located intracellularly, in inclusion bodies that are present in dopaminergic neurons [103]. As described in chapter 1 and 2, the protein deposits in PD comprise α-synuclein aggregates that are located in LBs in the cytoplasm of dopaminergic neurons [42, 48, 103]. Although several factors are described that can induce α-synuclein misfolding and aggregation, the cause for aggregation is still unknown [46]. Since the α-synuclein aggregates cannot be degraded by the ubiquitin-proteasome system (UPS), they accumulate in the cytoplasm [80, 81].

As depicted in Figure 4, the formation of α-synuclein oligomers and aggregates results in neuronal death [59]. Most likely, cell death is caused by the association of the α-synuclein aggregates with cellular components [103]. As described in chapter 2.3, α-synuclein oligomers are able to insert in both cell membranes, and intracellular membranes, resulting in an increased cell permeability, and eventually cell death [81, 88].

3.3 Alzheimer's disease

Alzheimer's disease (AD) is the most common neurodegenerative disease in human and the most common cause of dementia [1, 36, 107, 108]. AD is characterized by the presence of intracellular neurofibrillary tangles (NFTs) that arise after twisting of the neuronal cytoskeleton [210], and the formation of extracellular fibrillary amyloid plaques in the brain (senile plaques). Amyloid plaques (Fig. 9) mainly consist of aggregates of the amyloid-β (Aβ) peptide. Aβ peptides in the AD brain aggregate into oligomers, which then accumulate and form amyloid plaques [105, 107, 108].

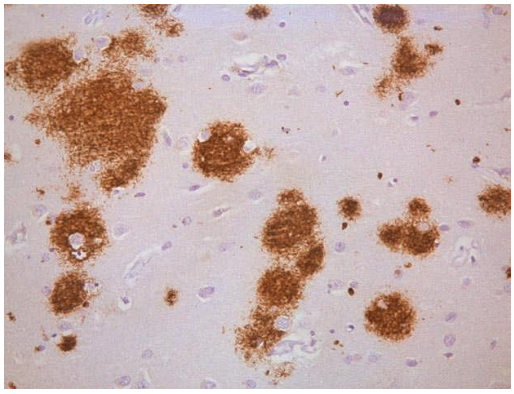

Figure 9. Presence of amyloid plaques in the brain of a patient affected by Alzheimer's disease*. Cortical section from a patient affected by AD, stained with amyloid-β specific antibodies. The dark colouring represents the amyloid plaques [105].*

Figure 10 shows the formation of Aβ from amyloid-β precursor protein (APP). APP belongs to a family of transmembrane proteins and is located in the cell membrane. In order to form Aβ, first β-secretase cleaves transmembrane APP at the N-terminus of Aβ. As a result of this, both soluble APP ($sAPP_\beta$) and a membrane bound APP fragment appear (CTF_β). Next, γ-secretase cleaves CTF_β, leading to the generation of the Aβ protein [108]. Monomeric Aβ is not toxic, however, oligomerisation of Aβ leads to accumulation and eventually the formation of senile plaques [108, 109]. Furthermore, the oligomeric Aβ may enhance, among others, tau hyperphosphorylation, proteasomal dysfunction and mitochondrial dysfunction [109].

Tau is a soluble protein that has six isoforms, and is associated with microtubules. By interacting with tubulin, tau stabilizes microtubule assembly. Hyperphosphorylation of tau results in the transformation of normal tau into PHF-tau (paired helical filament) and NFTs, leading to microtubule destabilization and the formation of PHF-tau tangles. The PHF-tau tangles are insoluble

aggregates that are toxic to cells, causing a disrupted axonal transport, which can result in cell death [109]. Figure 11 describes the role of APP and of tau in inducing neuronal death.

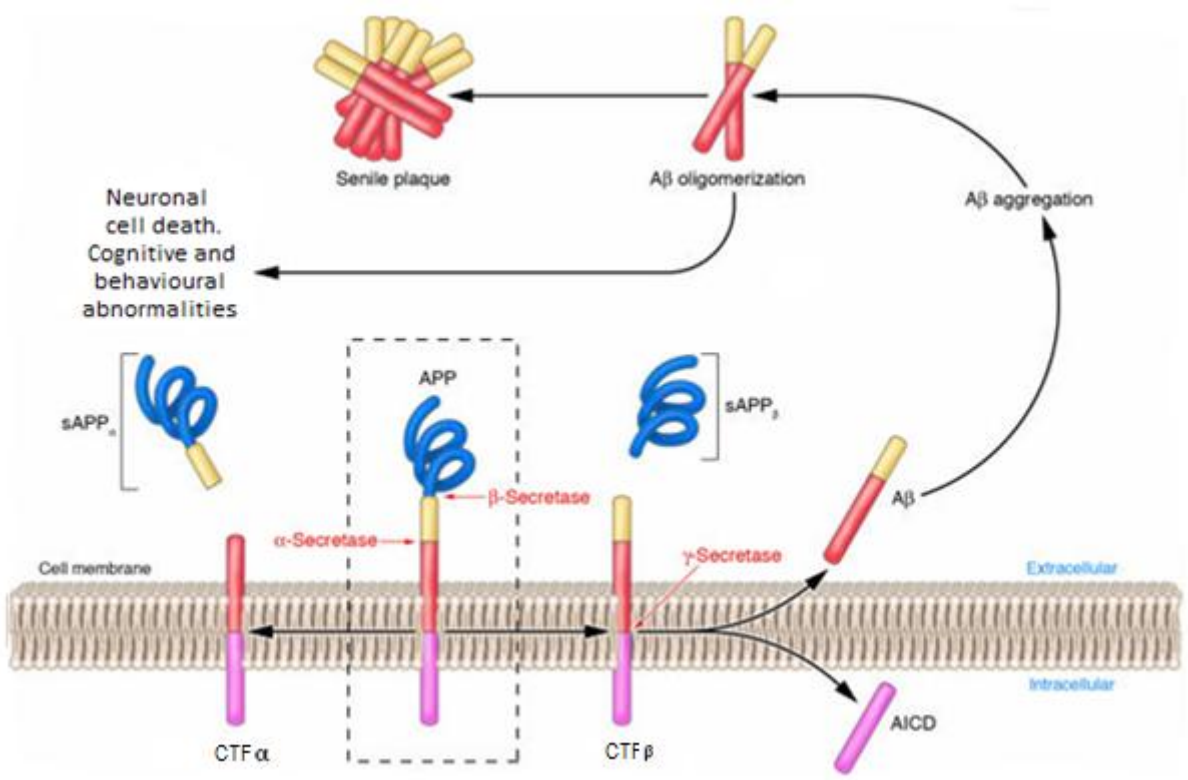

Figure 10. Overview of the formation of senile plaques in AD. *Membrane bound APP (shown in the dashed box) can be metabolized in two ways. In the first manner, α-secretase cleaves membrane bound APP, resulting in the formation of* CTF_{α}*. No amyloid (senile plaque) is formed in this case. In the second manner, β-secretase cleaves membrane bound APP, after which* CTF_{β} *is formed. After cleavage of* CTF_{β} *by γ-secretase, the Aβ protein is generated. This protein aggregates and forms oligomers, after which the oligomers accumulate and form senile plaques [adapted from 108].*

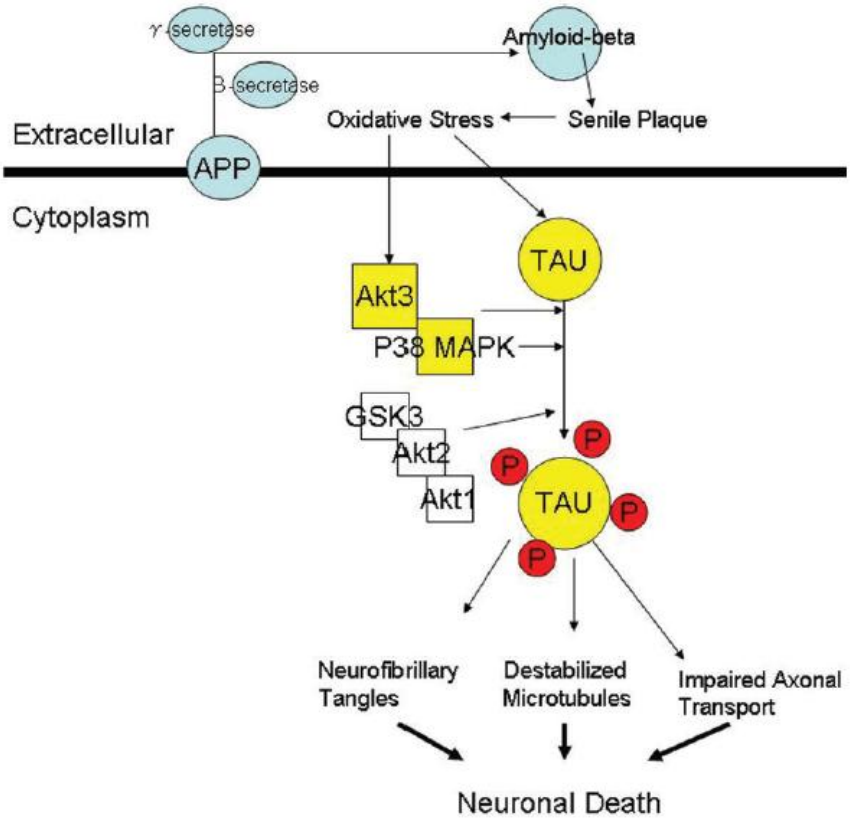

Figure 11. Model of the role of tau in inducing neuronal death. *Transmembrane APP is processed by β- and γ-secretase, resulting in Aβ aggregation and the formation of senile plaques. Via oxidative stress, Aβ oligomers and senile plaques cause hyperphosphorylation of the tau protein. This results in the generation of NFTs, destabilized microtubules and an impaired axonal transport, ultimately leading to neuronal death [110].*

3.4 Diabetes mellitus type II

Worldwide, type 2 diabetes mellitus (DM2) is a prevalent metabolic disease that is characterized by impaired insulin secretion, insulin resistance of target tissues, β-cell failure, increased β-cell death and the presence of islet amyloid [104, 111-113]. In approximately 90 percent of DM2 patients, extracellular accumulation of islet amyloid polypeptide (IAPP, amylin) is present in the pancreatic islets of Langerhans (Fig. 12) [115]. IAPP is a 37 amino acid long peptide that is mainly expressed by β-cells in the pancreas [113]. The precursor protein of IAPP, pro-IAPP, is cleaved at both the N and C terminus of IAPP, resulting in the origination of the mature IAPP protein [114]. Since pro-insulin is processed by the same enzyme as IAPP, they are stored together in secretory granules of the β-cells [112-114]. In the case of insulin resistance, where there is an increased need for insulin, and therefore an increased insulin production, the production of IAPP also seems to be elevated [114].

The amyloid protein can be subjected to conformational changes, resulting in oligomers with an increased number of β-sheets (Fig. 13) [104, 114, 115]. Similar to other amyloidogenic proteins, these oligomers are toxic and are able to insert into membranes after which they form pores [111-116]. Furthermore, the oligomers form fibrils that are deposited in the insulin-producing islet β-cells in the pancreas [111, 113, 114, 116]. The presence of IAPP deposits in DM2 is associated with a diminished β-cell volume [115].

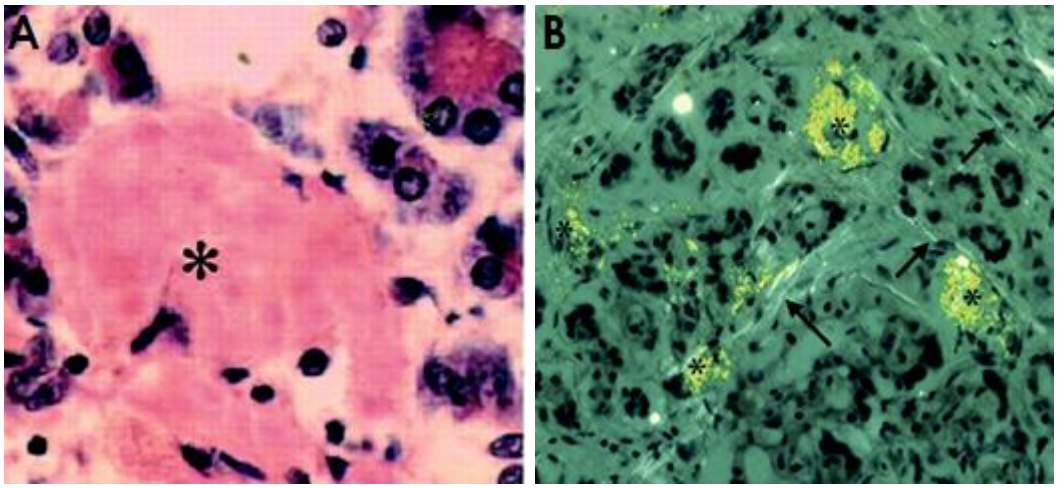

Figure 12. Characteristics of DM2 in the human pancreas. *Islet amyloid deposits are depicted by the asterisks (A and B) and in pink (A) and yellow (B). (A. Congo red staining, magnification 400x, white light) (B. Congo red staining, magnification 200x, polarizing light) [117].*

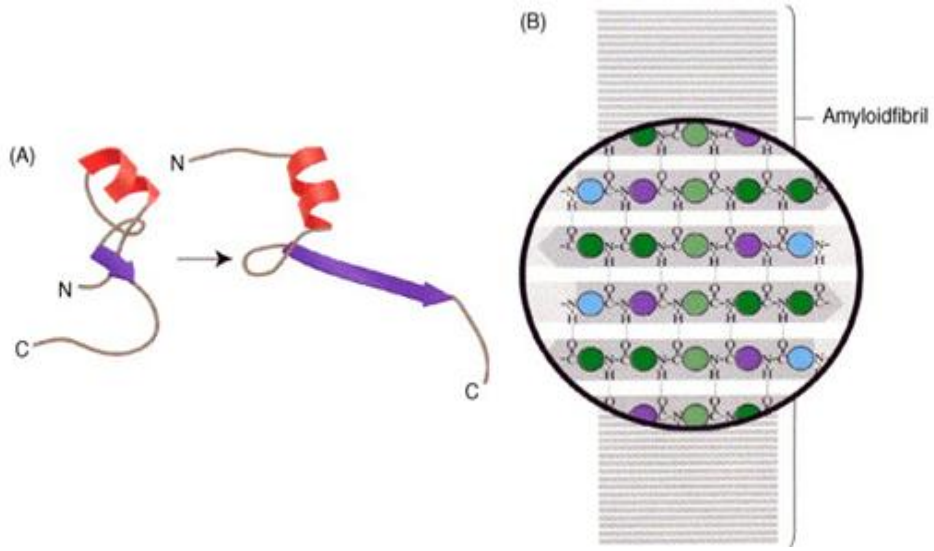

Figure 13. Model of amyloid fibril formation. *A. Amyloid monomers undergo structural changes, resulting in the formation of β-sheets (indicated in purple). B. Aggregation of amyloid molecules is the result of linkage of the molecules via hydrogen bonds between amino acids (coloured circles) in the β-sheets [104].*

3.5 Prion diseases

Transmissible spongiform encephalopathies (TSEs) or prion diseases comprise a group of infectious human and animal diseases that involve misfolding of the prion protein (PrP), resulting in neurodegenerative conditions [118]. In humans, prion diseases include Creutzfeldt-Jacob disease (in cattle known as mad-cow disease; characterized by amnesia, ataxia, unintentional movements and rigidity), kuru (incurable neurodegenerative disease, characterized by body tremors), fatal familial insomnia and Gerstmann-Sträussler-Scheinker syndrome (a rare, fatal neurodegenerative disorder, characterized by difficulty speaking, unsteadiness and progressive dementia) [105, 119].

A characteristic of prion diseases is the extensive extracellular accumulation of atypically folded human prion protein in the brain [105]. In its native conformation, the benign prion protein

(PrPC) is a soluble monomer with mainly an α-helical structure [120]. In all known prion diseases, however, PrPC converts into the malignant PrPSc form, which comprises a higher degree of β-pleated sheets, compared to PrPC (Fig. 14A) [105, 120, 121]. However, the cause of this conversion is still unknown [121]. Following the conversion from PrPc into the pathogenic PrPSc, the protein forms insoluble oligomers and after aggregation, fibrils are formed [120]. This also results in the formation of amyloid deposits in the brain (Fig. 14B) [105]. In vitro experiments have shown that both PrPSc oligomers and fibrils are toxic to neuronal cells [122]. The PrPSc fibrils form intraneuronal amyloidogenic plaques and seem to be the cause for the neurological symptoms in prion diseases [123-125].

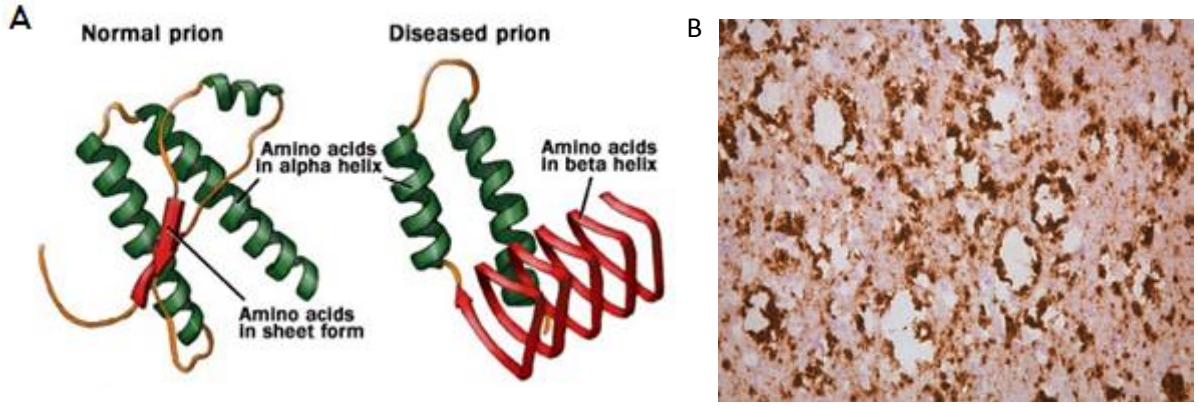

Figure 14. Conformation of a normal prion protein (PrPC) and a pathogenic prion protein (PrPSc) and the presence of protein accumulation in a patient affected by prion disease. *A. Models of the structure of normal and misfolded prion proteins, respectively [126]. B. Cortical section from a patient affected by prion disease. The section is stained with human prion protein-specific antibodies. The dark colour shows the accumulation of human prion proteins [105].*

Since various prion diseases are caused by the same prion proteins, there must be an explanation for the different phenotypes of the disease. The conversion of the normal prion protein into the diseased prion protein results in an alteration in the function of the protein. Since chaperone proteins, among others, enhance this conformation [127], the type of chaperone protein possibly plays a role in the development of a specific disease phenotype. Furthermore, during several phases in the replication and maturation of the prion proteins, alterations can occur [127], that can be involved in determining the type and severity of the disease that develops. During a cell division, the prion proteins are distributed unevenly to the daughter cells. In cells that receive higher amounts of diseased prion proteins, more prion proteins seem to accumulate, as a result of which a stronger disease phenotype develops [127]. Possibly, this process not only determines the severity of the disease, but also its phenotype. Also in the process of protein synthesis, folding, trafficking and degradation alterations can occur that enhance the development of different disease phenotypes. Finally, environmental factors, including stress, could play a role in determining the disease phenotype [127].

3.6 Conclusion

Alzheimer's disease (AD), as well as diabetes mellitus type 2 (DM2) and prion diseases, are all associated with misfolding and aggregation of specific proteins. These diseases belong to the group of amyloidoses and are accompanied by failure of the organs that are involved. In literature, there are contradictory opinions whether PD belongs to the group of amyloid diseases. However, PD shows great resemblance with other amyloidoses, including the diseases described in this chapter. In all

described conditions, extracellular amyloid deposits are found, either in the brain (AD and prion disease) or in the β-cells of the pancreas (DM2). Interestingly, although similar deposits have been found in PD, these are located intracellularly.

The diseases described in this chapter, including PD, are all associated with the formation of protein oligomers, which eventually develop into aggregates. In all four described diseases, oligomers are the toxic components, rather than the protein aggregates. Most likely, the toxic characteristics of the oligomers are determined by their ability to penetrate membranes, leading to pore formation and death of the target cell.

Although several factors have been described that can induce protein misfolding and aggregation, the motive for the formation of oligomers and aggregates is still unknown. Furthermore, the exact structure of these aggregates is not completely clear and it still is undefined to what extent oligomers are involved in cell death of their target cells. Therefore, extended research is necessary to each of the described diseases. However, since the diseases show great resemblance, studies on one disease can also be informative for the other diseases, as well as for other amyloidoses that have not been described in this thesis.

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