# ABERRANT SYNUCLEIN EXPRESSION AND ITS ROLE IN PATHOGENESIS IN DOPAMINERGIC NEURONS

Bachelor Thesis Life Science & Technology

**B.S.W. Berbers s1859994**
**Supervised by: J. C. V. M. Copray**
**Department of Neuroscience, University Medical Center Groningen**

**5-3-2014**

## Abstract

The most occurring age-related movement disorder is Parkinson's disease, which has proteineous inclusions called Lewy bodies as its main cytopathological hallmark. These inclusions consist mostly of α-synuclein. This knowledge combined with genetic evidence implicates α-synuclein in Parkinson's disease. Non-pathologically, α-synuclein is a chaperone protein to help SNARE-complex formation during vesicle exocytosis in the synapse. However, aberrant synuclein can set multiple pathological pathways in motion leading to cytotoxicity and neurodegeneration. Aggregation of α-synuclein proteins results in soluble oligomers. This form of oligomeric, not fully fibrillar, α-synuclein is thought to exert detrimental effects on cytoplasmic and intra cellular membranes by forming pore-like structures, which can change calcium and dopamine homeostasis. Changes in homeostasis can increase oxidative stress and cause both lysosomal and ubiquitin dysfunction, additionally increasing intracellular α-synuclein concentration. Finally, malfunctioning of the dopamine release can cause spines of connecting neurons to decrease, leading to further neurodegeneration. Therefore, feed-forward amplification loops are now commonly thought to induce apoptosis in neurons, each aberrant α-synuclein pathway enhancing the toxicity of others.

## Abbreviations

| | |
|---|---|
| α-MT | A-methyl-p-tyrosine |
| A30P | Alanine 30 proline |
| A53T | Alanine 53 tyrosine |
| $Ca^{2+}$ | Calcium |
| CSPα | Cysteine string protein-α |
| DAN | Dopaminergic neuron |
| DLB | Lewy body dementia |
| GFP | Green fluorescent protein |
| Hsc70 | Heat-shock protein cognate 70 |
| LB | Lewy body |
| MPTP | 1-methyl-4-phenyl-1,2,3,6-terahydropryridine |
| Munc18 | Mammalian uncoordinated-18 |
| NT | Neurotransmitter |
| NSF | N-ethylmaleimide sensitive factor |
| PBN | Phenyl-N-butyl-N-nitrone |
| PD | Parkinson's disease |
| PSD95 | Postsynaptic density protein 95 |
| ROS | Reactive oxygen species |
| SGT | Small glutamine-rich tetratricopeptide repeat-containing protein |
| SNAP | Soluble NSF-attachment protein |
| t-SNARE | Target-SNARE |
| UPS | Ubiquitin proteasome system |
| v-SNARE | Vesicle-SNARE |
| VAMP | Vesicle associated membrane protein |
| Wt | Wild-type |

## Index

Introduction……………………………………………………………………………………………………………………5

Vesicle Exocytosis in the Synapse……………………………………………………………………………………6

Co-chaperones and α-Synuclein in Exocytosis in the Synapse…………………………………………7

Lewy Bodies Formation in the Synapse…………………………………………………………………………9

Oligomers and Protofibrils of α-Synuclein in the Synapse ……………………………………………10

Synaptic Pathology by α-Synuclein Aberrant Behavior…………………………………………………11

*Protein degradation*……………………………………………………………………………………12

*Dopamine interactions*………………………………………………………………………………12

*Mitochondrial function*………………………………………………………………………………13

*Synpatic function*………………………………………………………………………………………14

Conclusion………………………………………………………………………………………………………………16

References ……………………………………………………………………………………………………………17

## Introduction

Parkinson's disease (PD) is the most occurring age-related movement disorder with a prevalence close to 2 percent of the population older than 65 (Rijk et al. 1999). Symptoms of PD are caused by the loss of dopaminergic neurons in the substantia nigra of the brain. The main cytopathological hallmark of the dopaminergic neurons in PD brains are proteineous inclusions called Lewy bodies. They are also found in other regions of the brain where they are associated with neurological disorders called Lewy body dementias. Spillantini et al. (1997) published a paper clarifying the molecular composition of these Lewy bodies, revealing that they mainly consist of alpha-synuclein. This along with the discovery that a missense mutation in the alpha-synuclein gene can cause heritable PD (Polymeropoulos et al. 1997), pointed toward alpha-synuclein as the new center of attention for PD and other parkinsonian disorders.

The synuclein protein family was discovered years earlier, associated with the cholinergic vesicles of the Pacific electric ray (Maroteaux et al. 1988); later a related sequence was found in rat DNA. Due to the location of the proteins, i.e. at the synapses in the ray and the nucleus in the rat, the name "synuclein" was composed. After several discoveries of this protein in the brains of other animals, it was demonstrated that synucleins are also present in the human brain. A 140 and a 134 amino acid protein were found, respectively referred to as α-synuclein and β-synuclein (Jakes et al. 1994).

Synucleins are found abundantly in neural tissue. A- and β -synuclein are found throughout the whole brain, but concentrations are enriched in the presynaptic terminals (Jakes et al. 1994). The question which role α-synuclein plays in the synaptic terminals was answered much later. The first clues came from an experiment wherein α-synuclein knockout mice turned out to be completely resistant to the dopaminergic neurotoxin 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine (MPTP). MPTP mimics the core neurological symptoms of neurodegeneration that occur in PD and is transported via the dopaminergic vesicle transport pathway. This suggests that α-synuclein has a role in the dopamine transporter mechanism (Dauer et al. 2002). More evidence to support this came forth in a study that used cysteine-string protein-α (CSPα). CSPα is a co-chaperone involved in binding neurotransmitter vesicles to the presynaptic nerve terminals (Chamberlain, Burgoyne 2000). It was shown that transgenic overexpression of α-synuclein can abolish the lethal effect of a CSPα knockout in mice (Chandra et al. 2005). Furthermore α-synuclein was shown to promote neurotransmitter vesicle exocytosis by chaperoning the SNARE-complex formation (Burré et al. 2010).

This all makes a convincing statement that synucleins have a function in signal transduction, but still little is known about the consequence of erroneous folding of α-synuclein at presynaptic nerve terminals. Therefore the goal of this paper is to acquire more information about the presynaptic functions of α-synuclein. There is evidence suggesting that α-synuclein plays a role in binding neurotransmitter vesicles to the membrane of the synaptic cleft. With a better understanding of the non-pathological function of α-synuclein it might be possible to uncover the mechanisms involved in the pathological folding of this protein eventually leading to synapse malfunction and retrogradely to neurodegeneration.

## Vesicle Exocytosis in the Synapse

Synaptic vesicles are crucial organelles in neurons and nerve terminals contain hundreds of these vesicles. The vesicles are filled with neurotransmitters (NT), which are released via exocytosis into the synaptic cleft, when the presynaptic membrane is depolarized by an action potential. After releasing NT the vesicles are recycled. This is the essence of neuronal signal transduction, but the detailed molecular processes are a bit more complicated. To understand the role of α-synuclein in vesicle exocytosis this paper will first look more closely at the mechanisms in the nerve terminals.

Synaptic vesicles can come from either the vesicle pool, recycling, or from an endosome. To use the vesicles for signal transduction they are filled with NTs. This happens via active transport fueled by an electrochemical gradient which is created by a proton pump that acidifies the inside of the vesicle. Before the vesicles can disperse the NTs in the synaptic cleft, they are docked by specific proteins at the active zone of the synapse and primed to become sensitive to a $Ca^{2+}$-signal. When an action potential reaches the presynaptic membrane, $Ca^{2+}$-channels open leading to rising $Ca^{2+}$ levels in the intracellular fluid. This completes the fusion of the synaptic membrane with the vesicle membrane and NTs are released into the synaptic cleft to bind to the receptors in the postsynaptic density. After release of the NTs vesicles can be recycled and used again (figure 1) (Südhof et al. 2011).

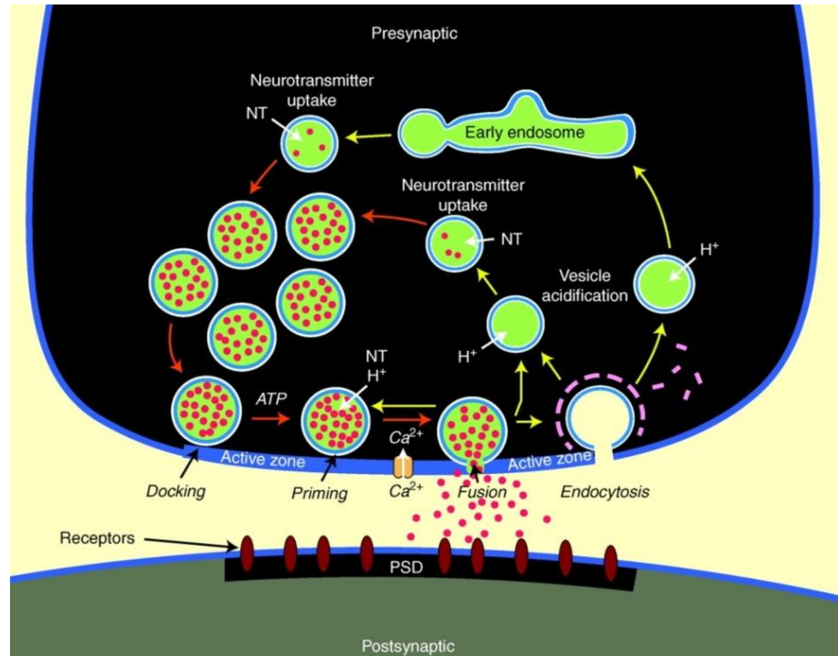


**Figure 1.** (Südhof et al. 2011) Schematic representation of the synaptic vesicle cycle in the presynaptic nerve terminal as it contacts the postsynaptic neuron. Exocytosis is represented by the red arrows, endocytosis and recycling are indicated by yellow arrows. Neurotransmitters (NT; red dots) are actively transported into the synaptic vesicles (green circles) stimulated by an electrochemical gradient created by a proton pump in the vesicle wall. Before synaptic exocystosis the vesicles are docked in the active zone and primed to respond to a $Ca^{2+}$-signal by an ATP dependent process, which will be discussed more thoroughly later on. $Ca^{2+}$-channels open when an action potential depolarizes the nerve terminal. The increased levels of intracellular $Ca^{2+}$ triggers fusion between vesicle and membrane and the NTs flow into the synaptic cleft. After fusion synaptic vesicles can be recycled or new vesicles can be endocytised.

Vesicle membrane fusion with the synaptic membrane and the steps, docking and priming, leading to this are of course a crucial part of the vesicle exocytosis. A series of findings between 1989 and 1993 led to the discovery of the SNARE protein complex and thereby a better understanding of these processes.

In 1989, Wilson et al. were able to isolate N-ethylmaleimide sensitive factor (NSF) in vitro and demonstrated that it is essential for catalyzing the fusion of transport vesicles in co-operation with soluble NSF-attachment proteins (SNAPs). Then in 1992, it was discovered that the neurotoxins tetanus and botulinum target SNARE proteins. It was demonstrated that these toxins enter the nerve terminals and block presynaptic membrane fusion by selective proteolytic cleavage of the vesicle membrane protein synaptobrevin (Schiavo et al 1992). Consequently synaptobrevin was identified as the first essential fusion protein. Shortly thereafter, it was shown that synaptic membrane proteins SNAP-25 (not

to be confused with SNAPs) and syntaxin-1 were also substrates for different types of botulinum toxins, exposing two more essential fusion complexes mediating synaptic vesicle exocytosis (Blasi et al. 1993(2x)). Shortly after the identification of these three essential fusion proteins: synaptobrevin, SNAP-25 and syntaxin-1, it was discovered that they form a complex with each other (Söllner et al. 1993). This complex is dissolvable by NSF leading to the name SNARE complex: soluble NFS-attachment protein receptor.

This all led to the "SNARE hypothesis" stating that SNARE complexes bridge the vesicle and plasma membrane and mediate docking and targeting specificity. The assembly and disassembly of SNARE proteins is a cycle in which the assembly of the components provides energy for fusion by pulling the membranes together, this process is also referred to as priming. Disassembly of the SNARE complex is done by NSF and SNAPs, making all components available for fusion of another vesicle. The cycle starts when the synaptobrevin α-helix on the vesicle membrane, also called v-SNARE (vesicle-SNARE), begins amino- to carboxy-terminal zippering into the three α-helixes of SNAP-25 and syntaxin, commonly referred to as the t-SNARE (target-SNARE). This forms a complex called the trans-SNARE which bridges the gap between the two membranes destined to fuse (Söllner et al. 1993). When the membranes merge, the fusion pore opens and the trans-SNARE is converted to a cis-SNARE. Now the v- and t-SNARE are on the same membrane and the four α-helixes are parallel in a coiled coil structure (figure 2) (Poirier et al. 1998). Cis-SNARE complexes are separated into the original monomer by NSF and SNAPs, thereby loading the SNARE proteins with energy via ATP-dependent dissociation.

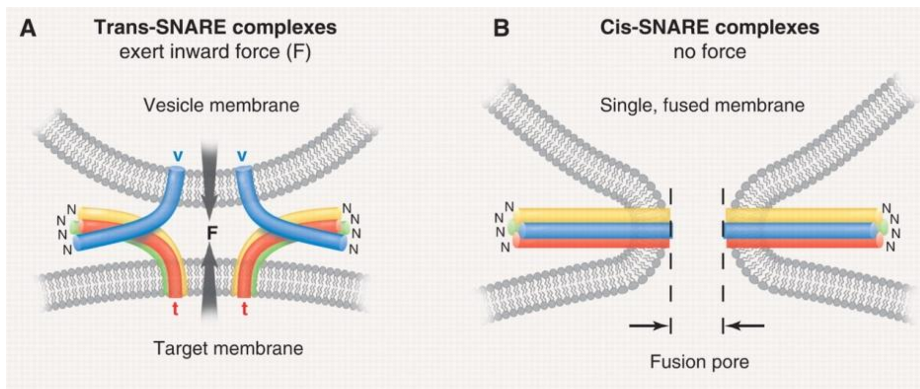


**Figure 2.** (Südhof et al. 2009). Simplified model of membrane fusion catalyzed by zippering of SNARE proteins **A.** Three helices anchored in the cytomembrane (the t-SNARE) assemble with the fourth helix anchored in the vesicle membrane (v-SNARE) to form trans-SNARE complexes. Assembly starts from the N termini towards the C termini at the membrane-side of the proteins. This generates a force (F) that pulls the membranes together, forcing them to fuse. **B.** The zippering can only be completed when the membranes fuse to form a pore and the trans-SNARE is converted to cis-SNARE complex.

## Co-chaperones and α-Synuclein in Exocytosis in the Synapse

Nerve terminals release NT's thousands of time per minute. For every release in the synapse, SNARE complexes are assembled and disassembled by NSF and SNAPs. At any given moment there are hundreds of SNARE proteins folded, unfolded or in a highly reactive intermediate state. All these proteins are susceptible to misfolding and nonspecific interactions, potentially causing problems with interneuronal communication and neuronal survival. Therefore, chaperone systems are needed in neurons to maintain SNARE protein function and promote complex assembly. For vesicle exocytosis there are two chaperone systems, one of these has CSPα as key protein, the other has synucleins.

CSPα is a vesicle protein that works by forming a complex with heat-shock protein cognate (Hsc70) and small glutamine-rich tetratricopeptide repeat-containing protein (SGT). This complex promotes SNARE complex assembly by preventing SNAP-25 aggregation (figure 4). It was demonstrated that

neurodegeneration in mice with a CSPα knockout was aggravated when SNAP-25 levels were decreased by both knockout and knockdown. On the other hand, an overexpression of SNAP-25 can minimize the degenerative effect of the CSPα knockout (Sharma et al 2012).

The synuclein protein family consists of three types: α-, $\beta$- and γ-synuclein. Attention started to focus on synucleins when it was discovered that a missense mutation in the α-synuclein gene can cause heritable PD (Polymeropoulos et al. 1997). Moreover, it appeared that the major component of the Lewy body proteinaceous inclusions, the main pathological hallmark of PD, was α-synuclein (Spillantini et al 1997). Further evidence supporting the link between α-synuclein and PD came from a study in which neurodegeneration was mimicked in mice using MPTP, a dopaminergic neurotoxin which causes parkinsonism in humans and mice. All α-synuclein knock-out mice prove to be completely resistant to MPTP (Dauer et al 2002).

These are all indications that α-synuclein has a pathological role in PD. But, nonpathologically, it appears that all types of synuclein facilitate SNARE complex assembly. The N-terminal repeats of synucleins bind to the negatively charged phospholipids in vesicle membranes, shaping the proteins into α-helical structures with a hydrophilic C-terminal tail (figure 3) (Jao et al. 2004). Clues to what role synucleins fulfill in the vesicle membranes came when it was discovered that overexpression of α-synuclein can counter the detrimental effects of an earlier mentioned CSPα knockout in mice (Chandra et al. 2005). Previous research had shown that an α- and $\beta$-synuclein double knockout, although not causing a major neurodegenerative phenotype, led to a decrease in dopamine levels in mouse brains (Chandra 2004). And more recently a study using mice with an α$\beta$γ-synuclein knockout reveal age-dependent neuronal dysfunction and diminished survival. This was especially attributed to the dramatic effect deletion of synucleins has on synaptic structure and size (Greten-Harrison et al. 2010). Also in the triple knockout group a notable age-dependent decrease in synaptobrevin and large increase of CSPα were observed. Although other protein quantities remained more or less unchanged, this alteration in synaptic protein composition was accompanied by a decrease in SNARE-complex assembly (Burré et al. 2010).

Thus, we can conclude that the

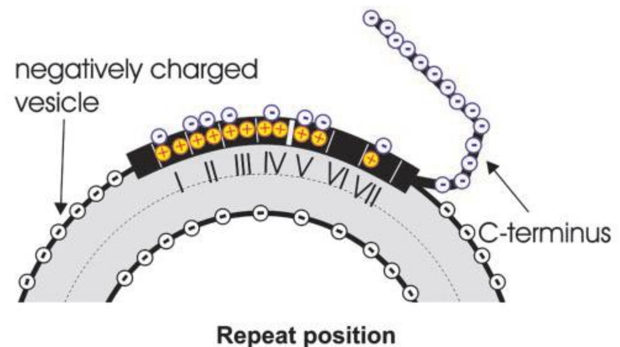


**Figure 3.** (Jao et al 2004). Synuclein folding on vesicle membrane. The yellow circles represent the positively charged N-terminal repeats. The white circle represents negatively charged parts of the synuclein, which are solvent. The C-terminus is the hydrophilic tail of the synuclein, which does not bind to the membrane due to its charge.

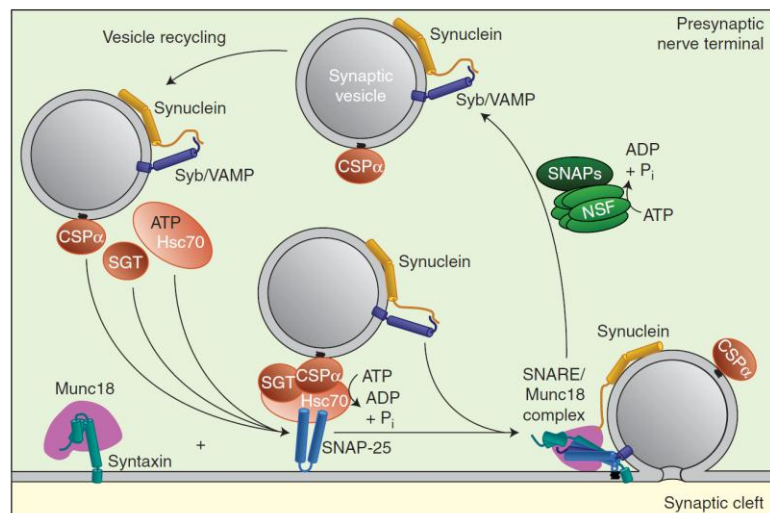


**Figure 4.** (Südhof et al 2011). Synaptic vesicle exocytosis by SNARE-complex assembly and disassembly with chaperones. At the bottom of the vesicle CSPα forms a complex with SGT and Hsc70 (orange circles), which binds SNAP-25 to stabilize it. On the side of the vesicle synuclein (yellow shape) binds synaptobrevin (Syb) or VAMP in order to stabilize it as it zippers into SNAP-25 and the Syntaxin Munc18 complex. After exocytosis, the SNARE-complex is disassembled by SNAPs and NSF, while the chaperone proteins remain secured to the vesicle membrane.

combination of synaptobrevin and synucleins is required for maintaining normal SNARE-complex assembly during aging in mice. This was emphasized by the finding that, when co-expressing each SNARE protein separately with α-synuclein, only synaptobrevin formed a complex with α-synuclein (Burré et al. 2010). When the C-terminal of α-synuclein was removed, this complex cannot be formed, demonstrating that the hydrophilic C-terminal tail of α-synuclein is crucial for promoting SNARE-complex formation.

Consequently, we can conclude that both absence and presence of synucleins can cause neuropathies. Absence of α-synuclein impedes SNARE-complex assembly. But increased levels of α-synuclein are thought to cause PD either by, misfolding to produce toxic conformations, or by additional alterations upsetting the physiological balance, or both. The malfunctioning of this protein will be discussed more thoroughly in this paper.

## Lewy Bodies Formation in the Synapse

A mutation in the α-synuclein gene was linked to heritable PD (Polymeropoulos et al. 1997). And α-synuclein was found to be the main component of the insoluble proteinaceous aggregates called Lewy bodies (LB) found in the substantia nigra of patients with sporadic PD (figure 5) (Spillantini et al 1997). This led to the believe that LB's are somehow involved in the nerve cell loss. And till this day the neuropathological diagnosis of PD is based on the detection and quantification of Lewy bodies.

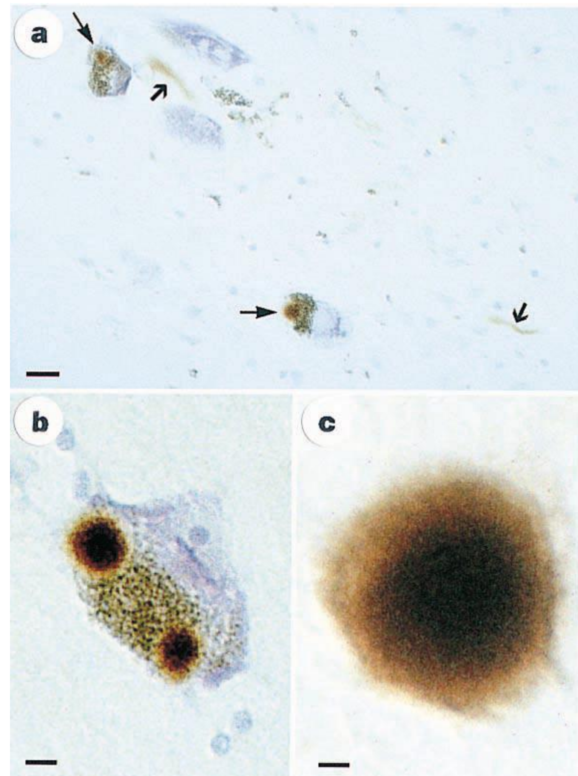


**Figure 5.** (Spillantini et al. 1997). Substantia nigra from patients with PD immunostained for α-synuclein. A: two pigmented nerve cells containing α-synuclein-positive LB's (thin arrows). Also in this picture two α-synuclein-positive Lewy neuritis (thick arrows) B: one pigmented nerve cell with two α-synuclein-positive LB's. C: extracellular α-synuclein-positive LB. Scale bar: A = 20µm, B = 8 µm, C = 4 µm.

However, it has been demonstrated that patients with mild to moderate loss of neurons in the substantia nigra have a higher LB count than patient suffering severe neuronal depletion. This suggests that neurons containing LB's can be considered dying neurons. But it does not imply that LB's inclusions are the cause of neuronal cell death (Wakabayashi et al. 2007). Moreover, it seems unlikely that every lost nerve cell has gone through a stage of LB formation. This is supported by the notion that LB's do not predispose neurons to undergo apoptotic-like cell death and that the majority of the neurons lost in the substantia nigra die without- or before LB's can occur (Tompkins et al. 1997).

A study of fibrillization speed of wild type α-synuclein in comparison to A53T and A30P α-synuclein, two different types of mutations linked to early onset of PD, added to the notion that LB's may not be the major culprit in PD. For this study found that the A53T mutation accelerated fibrillization, while the A30P slows down the formation of fibrils. This effect was also registered to lesser extent in the heterozygotes (as is present in patients with

familial PD) (Conway et al. 2000). But, the lack of correlation in fibrillization speed between the PD-causing mutations suggests the fibril might not be the most significant pathogen.
Therefore, no strong correlation between the LB density and the severity of the clinical PD symptoms can be found. As demonstrated in a study wherein 904 autopsies were examined, 106 α-synuclein–positive individuals were found. 32 of these 106 individuals had been diagnosed with a neurodegenerative disorder, suggesting that LB inclusions are not definite markers for neuronal dysfunction and that LB's can be present without clinical symptoms (Parkkinen et al. 2005).
Hence, LB's themselves may not cause the process of neurodegeneration. This is also supported by series of studies comparing neurons with and without LB's from the substantia nigra. These studies found no significant difference between LB affected and unaffected cells, in the size of the perikarya and nucleolar size(Gertz et al. 1994), in the protein levels of the dopamine converting enzyme tyrosine hydroxylase (Kastner et al. 1993), and in apoptotic-like changes (Tompkins et al. 1997). Furthermore, a form of early-onset PD does not typically exhibit LB's (Kitada et al. 1998). This all makes a compelling argument that the presence of LB's is rather a symptom of PD, than that it causes the neurodegeneration in the substantia nigra.

## Oligomers and Protofibrils of α-Synuclein in the Synapse

So, it has been determined that LB's consist mainly of α-synuclein, but essentially in aqueous solution α-synuclein is natively an unfolded protein. As discussed before, upon contact with a water-lipid interface the hydrophobic repeats in the N-terminal of α-synuclein will coil up against the membrane surface, while the C-terminal remains unfolded. However, α-synuclein can occur in a number of quaternary structures which can be divided into three categories: the natively unfolded monomer, transient oligomeric fibril precursors (protofibrils) and the amyloid fibril (figure 6). The amyloid fibrils have cross-β-structures, making them stable insoluble protein aggregates, which fuse to form LB's (Beyer et al. 2007).

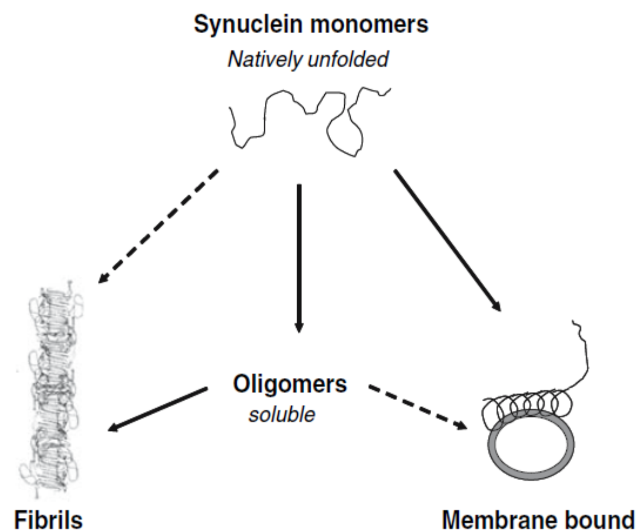


**Figure 6.** (Beyer et al. 2007). In this simplified representation of organizational variability of α-synuclein the transitions that are supported by experimental evidence are designated by solid arrows

Because of increasing doubt to whether LB's cause the pathology in PD, another explanation for neurotoxicity was sought. And a new consensus was reached, when more and more research indicated that the transient oligomeric state of α-synuclein en route from monomeric proteins to fibrillar aggregates were cytotoxic. Experiments using in vitro fibrillogenesis found oligomerization of α-synuclein, creating β-sheet enriched protofibrils. These protofibrils form spherical structures, which can either form linear chains, or form circular ring structures (Conway et al. 2000). Experiments demonstrated that when incubating these spherical protofibrils with brain-derived membranes in vitro, they bind much more tightly than monomeric or fibrillar α-synuclein. Especially interesting is that atomic force microscopy revealed that membrane-bound protofibrils form porelike annular structures. These annular protofibrils strongly resemble a class of pore-forming bacterial toxins (Lashuel et al. 2002) and have an inner diameter between 2 nm and 2,5 nm, making it possible for molecules less than 2,5nm in

diameter to pass through membranes (figure 7) (Ding et al. 2002). Consequently, annular protofibrils cause vesicles as well as cytoplasma membranes to leak (Kayed et al. 2003), without disrupting their spatial integrity. Due to the size specificity of the pores, channels $Ca^{2+}$ and dopamine can be released, while larger molecules remain within the vesicles (Volles et al. 2001).

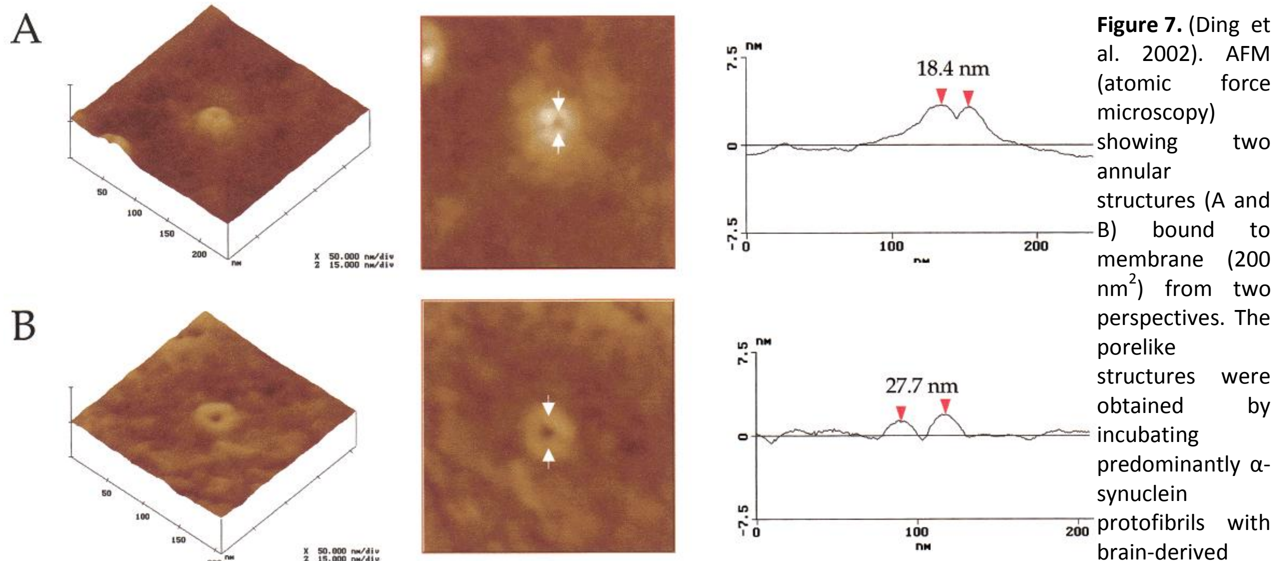


**Figure 7.** (Ding et al. 2002). AFM (atomic force microscopy) showing two annular structures (A and B) bound to membrane (200 $nm^2$) from two perspectives. The porelike structures were obtained by incubating predominantly α-synuclein protofibrils with brain-derived

The amount of protofibrils present in neurons is dependent on the levels of α-synuclein present in the cell, which is regulated by a balance of synthesis, degradation, and secretion. Studies have shown that the intracellular α-synuclein concentration increases, when lysosome or proteasome activity is inhibited. This implies that α-synuclein is degraded by ubiquitin proteasome system as well as the autophagy-lysosome pathway (McNaught et al. 2004, Webb et al. 2003). In the proteasome system aggregated proteins can impede the degradative capacity of the proteasome (Bence et al. 2001), which means elevated quantities of aggregated α-synuclein can lead to further increase of these levels. Furthermore, when α-synuclein is modified by dopamine, it becomes difficult to be degraded by autophagy and can even block degradation of other related substrates. This blockage can also possibly activate a vicious cycle increasing α-synuclein levels and stress in neurons (Martinez-Vicente et al. 2008).
This interaction between α-synuclein by dopamine could also explain why the depletion of neurons is mostly restricted to of dopaminergic neurons, for it has been demonstrated that a high cytosolic dopamine concentration promote protofibril formation in dopaminergic neurons (Lee et al. 2011). Furthermore, dopamine has also proved to stabilize protofibrils, consequently inhibiting fibril formation and accumulating protofibrils (Conway et al. 2001).

## Synaptic Pathology by α-Synuclein Aberrant Behavior

It is, therefore, commonly accepted that the oligomerization of α-synuclein to small protofibrils is the main cause of neurodegeneration in PD. But the mechanisms as to how these protofibrils induce cytotoxicity in neurons are understood to lesser extent. A couple of possible pathways are described with the information available.

### *Protein degradation*

As most PD pathologies are ascribed to α-synuclein aggregation, malfunctioning of the balance between synthesis, degradation and secretion of this protein seems a good place to start. As mentioned before, α-synuclein is degraded by the ubiquitin proteasome system (UPS) and the autophagy-lysosome pathway. Both of these pathways are inhibited by excess amounts of aberrant α-synuclein, slowing down degradation and further increasing α-synuclein levels. α-Synuclein modified by noncovalent interaction with oxidized dopamine not only blocks its own autophagy, but also the degradation of other substrates in this pathway, increasing dopaminergic neurons vulnerability to stressors, which can eventually lead to apoptosis (Martinez-Vicente et al. 2008).

Protein aggregates also affect proteasome-mediated protein turnover. α-Synuclein aggregates can directly bind to certain proteasomal subunits impairing the proteolytic activity (Lindersson et al. 2004), and are therefore simultaneously inhibitors and the products that result from inhibition of that pathway (Bence et al. 2001). This effect is demonstrated, in a study wherein rats that were systematically exposed to proteasome inhibitors, exhibited many key features of PD, including selective nigrostriatal neurodegeneration, and the formation of LB's (McNaugt et al. 2004).

### *Dopamine interactions*

Another important subject for PD research is the effect dopamine can have on α-synuclein, for this could explain why the neurodegeneration is relatively restricted to dopaminergic neurons. It has been demonstrated that catecholamines, particularly dopamine, form covalent bonds with α-synuclein inhibiting fibrillization by stabilizing the oligomers (Conway et al. 2001). This results in an accumulation of protofibrils.

In cultured cells α-synuclein toxicity appears to be dependent on the presence of dopamine. α-Synuclein was not toxic, and even had a neuroprotective effect in non-dopaminergic human cortical neurons, but in cells with an endogenous dopamine production degeneration was registered. When dopamine production was inhibited by a highly specific tyrosine hydroxylase inhibitor α-methyl-p-tyrosine (α-MT) in dopaminergic neurons, the α-synuclein induced apoptosis was also completely inhibited, suggesting the toxicity is dopamine dependent (figure 8A) (Xu et al. 2002).

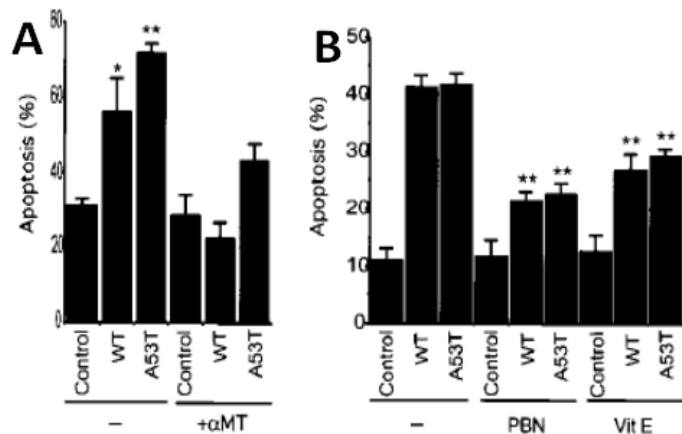


**Figure 8.** (Xu et al. 2002). The effect of dopamine on α-synuclein induced apoptosis. **A.** Cultures of human fetal dopaminergic neuron (DAN cells) transfected with wild-type (WT) or A53T mutant α-synuclein constructs, in absence (-) or presence (+α-MT) of 1 mM α-MT, an inhibitor of dopamine biosynthesis and incubated with $H_2O_2$ and $FeCl_2$. α-MT does not significantly affect apoptosis induced by oxidative stress in the control cultures, but completely inhibits wild-type α-synuclein-induced apoptosis, and decreases mutant α-synuclein induced apoptosis significantly. **B.** α-Synuclein induced neurotoxicity is inhibited by antioxidants. The same transfected DAN cell cultures in absence (-) and presence of antioxidant 100 μM phenyl-N-butyl-nitrone (PBN) or 250 μM vitamin E (Vit E). presence of both antioxidants inhibits α-synuclein-induced apoptosis. Values represent the mean ± s.e.m.; n = 4, **, $P < 0.01$; *, $P < 0.05$ relative to control, by analysis of variance (ANOVA) with post-hoc Student-Neumann-Kiels test.

Excess amounts of dopamine are associated with oxidative stress, because increased dopamine turnover can result in excess hydrogen peroxide and other reactive oxygen species (ROS) formation (Olanow 1990). Antioxidants (e.g. vitamin E and phenyl-N-butyl-nitrone PBN) decrease the apoptosis induced by α-synuclein, suggesting α-synuclein increases the dopamine-dependent generation of ROS, causing apoptosis (figure 8B) (Xu et al. 2002).

Thus, it appears α-synuclein increases cytosolic dopamine levels, which, due to the high oxidative potential of dopamine metabolism, promotes oxidative stress and apoptosis.

### *Mitochondrial function*

In PD mitochondrial alterations are well established, proposing α-synuclein has effect on this organelle. This notion is seconded by the detection of α-synuclein inside the mitochondrial membrane of the brain of rodents, and acceleration of α-synuclein aggregation induced by mitochondrial complex-I inhibitors such as rotenone and MPTP (Betarbet et al. 2000). Inhibition of the complex-I even replicates many of the key features of PD including selective dopaminergic neuronal degeneration, but α-synuclein knockout mice do not exhibit any of the pathologies caused by mitochondrial inhibition (Dauer et al. 2002). Thus, α-synuclein and mitochondrial function are inextricably linked.

Not only does inhibition of the mitochondrial complex-I increase α-synuclein aggregation, overexpression of α-synuclein also inhibits this complex' activity and is thought to colocalize with mitochondria binding its membrane. Especially small α-synuclein oligomers bind membrane strongly with the α-helix of the N-terminal, changing the membrane curvature, which leads to swelling and fragmentation of mitochondria in vitro and in vivo (figure 9). This fragmentation can result in loss of the loss of mitochondrial transmembrane potential and elevated release of ROS increasing cytotoxicity (Nakamura et al. 2011).

Most likely this process also leads to autophagy of mitochondria, or mitophagy. In this case mitochondria are degraded leading to depletion of chemical energy, inducing apoptosis. Inhibition of mitophagy partially counters deterioration of neurons, demonstrating the effect of this degenerative pathway (Choubey et al. 2011).

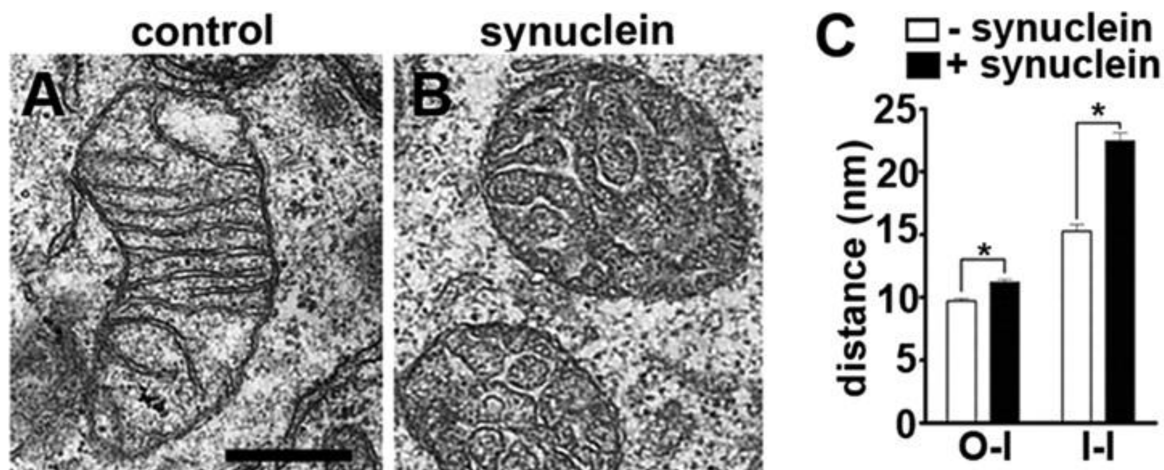


**Figure 9.** (Nakamura et al. 2011). COS cells were transfected with mitoGFP and a vector with either control (white bars) or α-synuclein (black bars). The cells were sorted for GFP fluorescence, and the quartile of top expression was plated onto culture discs. After fixing the cells, they were examined by electron microscopy **A, B**. The mitochondria of COS cells are shown for control (**A**), and the disrupted ultrastructure of the α-synuclein transfected cells(**B**). Scale bar indicates 500 nm. **C** represents the analyzed distance between the outer and inner mitochondrial membrane (O-I) and between adjacent inner mitochondrial membranes (I-I). The mitochondria were randomly selected and presented as the mean of n: 138-176 for O-I and 222-335 for I-I. The electron microscopy indicated mitochondrial swelling in the α-synuclein positive cells, which is confirmed by increase in distance between both the membrane measurements. *, $p < 0.01$ by two one-way analysis of variance and Newman-Keuls post hoc test. con, control.

### *Synpatic function*

As mentioned, in healthy neurons α-synuclein is involved in the process of vesicle exocytosis. The protein chaperones synaptobrevin in forming a SNARE-complex with the SNARE proteins on the cytomembrane (Südhof et al. 2011). Therefore, α-synuclein is mainly located in the presynaptic terminals. In a study using mice with an αβγ-synuclein an age-dependent neuronal dysfunction and diminished survival was revealed (Greten-Harrison et al. 2010), emphasizing the importance of properly functioning synucleins.

In contrast, aberrant α-synuclein behavior specifically in the synapse is associated with detrimental pathways leading to neuronal apoptosis. Even modest increase in α-synuclein can lead to reduction in NT release in dopaminergic neurons (Nemani et al. 2011). The mechanism responsible for this is most likely twofold, and has to do with impaired NT release and dopamine displacement.

As discussed, NTs are released from nerve terminals by vesicles exocytosis. This process requires SNARE-complex assembly and disassembly. If for any reason the important chaperone for this process, α-synuclein, is malfunctioning, vesicles cannot be exocytized. NT release is also dependent on the availability of vesicles in the synaptic recycling or reserve pool. A study with slight α-synuclein overexpression uncovered that there is a problem with getting the recycled vesicles to the active zone of the synapse. This results in a reduced number of synaptic vesicles that can be used for signal transduction (Nemani et al. 2011).

The second problem is caused by the high aggregation potential of α-synuclein. α-Synuclein oligomers bind membrane and are able to form annular protofibrils with porelike structures. The pore is estimated to have an inner diameter of 2,5 nm, which is larger than dopamine molecules. Therefore, when protofibrils bind to vesicle membranes, they can have a permeabilizing effect. Through these pores dopamine can leak from the vesicles into the cytosol. Excess amounts of cytosolic dopamine increase oxidative stress in the neuron. Simultaneously, due to the

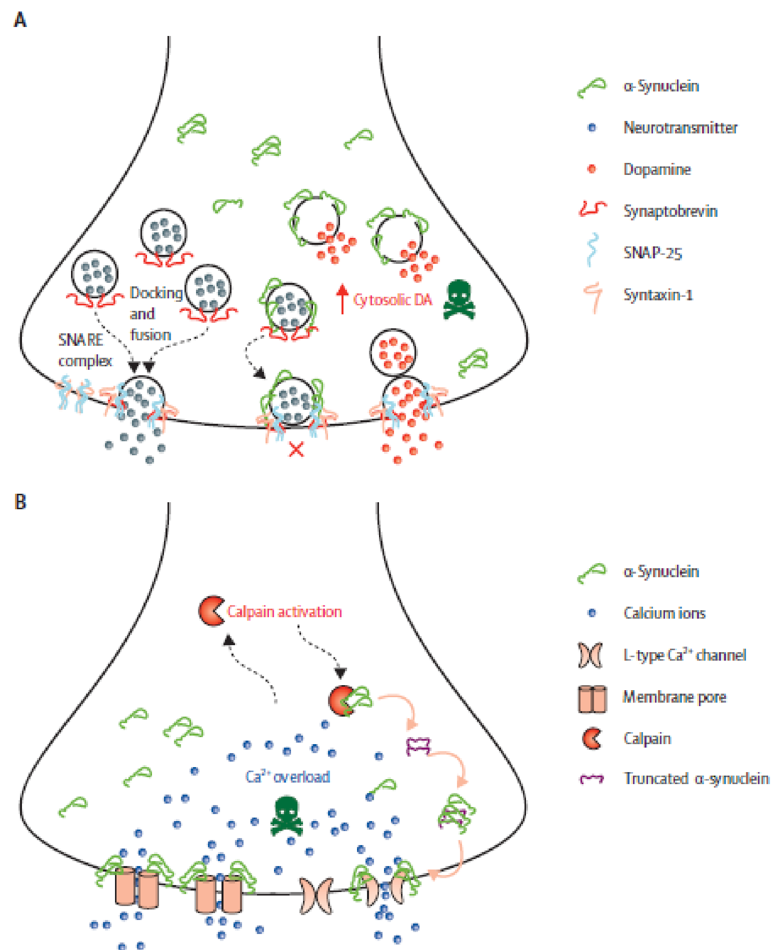


**Figure 10.** (Vekrellis et al. 2011). **A.** Elevated α–synuclein levels decrease dopamine release and increase cytotoxicity. The left side shows normal vesicle exocytosis with docking, fusion, and NT release. In the middle vesicle exocytosis is inhibited by malfunctioning of the SNARE-complex chaperone α–synuclein. The right side shows the porelike structures through which dopamine can leak from the vesicle into the cytosol. This can increase cytotoxicity, due to the high potential of the dopamine metabolism. **B.** α–synuclein aggregation can increase $Ca^{2+}$ concentration. Protofibrils can also form pores in the cytomembrane. This increases the intracellular $Ca^{2+}$ concentration, which can open the $Ca^{2+}$ channel, this can cause a $Ca^{2+}$ overload. $Ca^{2+}$ can induce apoptosis through different pathways. One of these is calpain activation. Activated calpain can cleave α–synuclein making it even more prone to aggregation.

dopamine leaking from the vesicle, there are less NT's available for signal transduction (figure 10A) (Mosharov et al. 2006).
The annular protofribrilar pores can also increase toxicity via another pathway, because α-synuclein misfolding and aggregation may not solely affect intracellular organelles, but also the outer leaflet of the cytoplasma membrane permeability. This can alter the calcium ($Ca^{2+}$) homeostasis (Danzer et al. 2007), which can result in an intracellular $Ca^{2+}$ overload. $Ca^{2+}$ overload can trigger apoptosis through a number of pathways, one of these could be apoptosis through calcium-dependent calpain (figure 10B) (Squier et al. 1994).

All the mentioned ways aberrant α-synuclein can cause neurodegeneration, are mostly through presynaptic toxicity. However, these forms of presynaptic malfunctioning have carryover effects postsynaptically. Formation of postsynaptic dendrites and their size is dependent on presynaptic activity. Long-term potentiation induces formation of new dendrites. On the other hand, deprivation causes reduction in connected surface (Yuste et al. 2001).
As stated previously, even a slight increase in α-synuclein levels can considerably diminish the NT release in neurons. This is most probably due to vesicle misplacement and dopamine leaking from the vesicles into the cytosol (Nemani et al. 2011). A decrease in NT release is accompanied by a decrease in dendritic spine size and number.
The gravity of this side effect can be demonstrated by measuring the levels of postsynaptic marker proteins. Drebrin is an actin-binding protein involved in dendritic spine formation and maintenance. Kramer and Schulz-Schaeffer 2007 observed such a vast reduction in drebrin that it was beyond the limit of detection (figure 11). This severe decline in drebrin is also displayed by the stained neurons exhibiting a dramatic spine reduction (figure 12) (Kramer et al. 2007).

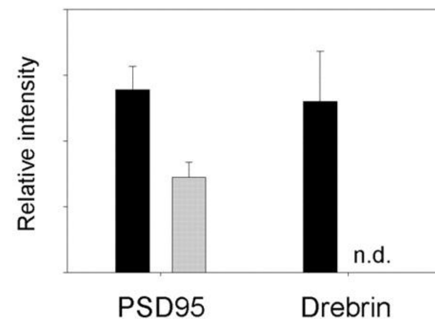


**Figure 11.** (Kramer et al. 2007). Brain homogenates of six confirmed lewy body dementia (DLB) cases (light grey bars) compared to age-matched controls (black bars). The relative densities of the stains found by western blot analysis of postsynaptic density protein (PSD95) and drebrin. (N.d. = nondetectable)

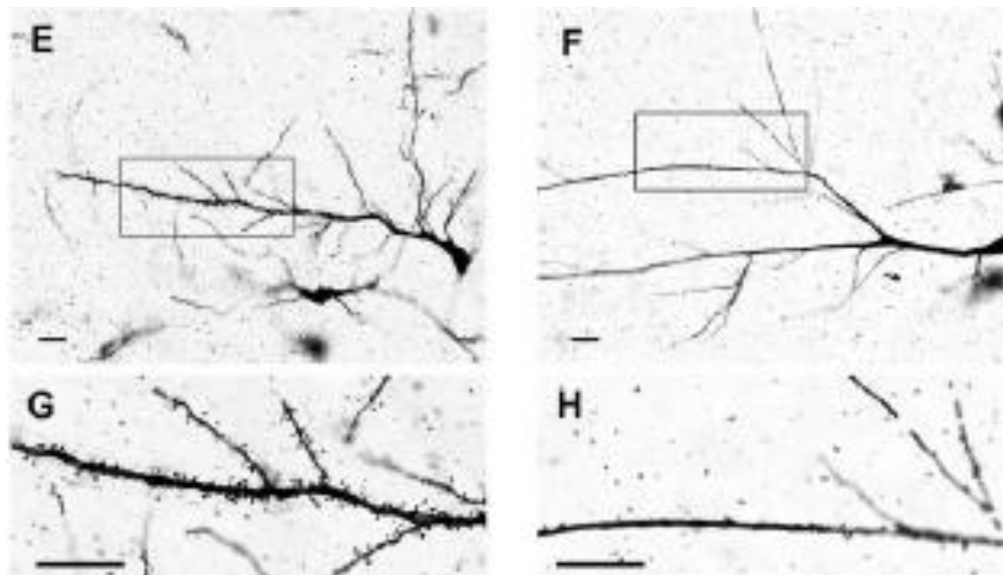


**Figure 12.** (Kramer et al. 2007). Golgi-Cox-Davenport staining of brain slices for the visualization of dendrites and their spines with DLB (**F,H**) compared to age-matched controls (**E,G**). **G** and **H** show the magnification of the boxes in **E** and **F**. Scale bars: 50 µm.

## Conclusion

There is wide consensus that the main cause of the neurodegenerative process in Parkinson's disease (PD) is triggered by dopaminergic synapse dysfunction. Disentangling the underlying mechanisms involved in synapse malfunction seems crucial to finding an effective treatment. The discovery of a link between familial PD and mutations in the α-synuclein gene and α-synuclein dose dependent effects emphasizes the involvement of α-synuclein as culprit in neurodegeneration. Furthermore, the main pathological hallmark of PD, Lewy bodies (LBs), consists almost entirely of α-synuclein. It therefore makes sense to look further into the mechanisms of aberrant α-synuclein.

The physiological role of α-synuclein is to chaperone the synaptobrevin and thereby help form the SNARE-complex during vesicle exocytosis into the synaptic cleft. It, therefore, seems logical that, when α-synuclein malfunctions, problematic signal transduction will develop in the synapse.
Although α-synuclein is inextricably linked to PD, it seems, based on fibrillation studies and autopsies, that LBs are not responsible for neuropathy. Through which mechanism aberrant α-synuclein induces neurotoxicity remains to be uncovered precisely, but there are a few pathways that have been demonstrated to disrupt neuronal physiology. Research supports a theory that accumulation of small soluble α-synuclein oligomers are the main cause for neurodegeneration. The neurotoxicity is mostly limited to dopaminergic neurons, because dopamine has a stabilizing effect on protofibrils, inhibiting fibrillation, and inducing oligomerization.

Protofibrils have the ability to bind membrane very tightly and assume an annular conformation. Due to this property, oligomers form pore-like structures, which can change membrane permeability. This can especially upset $Ca^{2+}$ homeostasis and the dopamine metabolism. Through the permeabilized cytomembrane, extracellular $Ca^{2+}$ can leak into the cytosol causing a $Ca^{2+}$ overload, which can trigger multiple apoptotic pathways. Through the pore-like structures dopamine can leak from synaptic vesicles into the cytosol, displacing dopamine for exocytosis and further increasing the intracellular dopamine concentration.
Elevated dopamine metabolism, as well as the fragmentation of mitochondria by α-synuclein oligomers, can drastically increase ROS levels. In an environment with high ROS and dopamine levels, α-synuclein has a higher tendency to aggregate, further deteriorating the cellular condition. This problem is a feed-forward amplification loop, wherein different pathogenic pathways amplify one another. Not only this pathway has this problem, all the mentioned malfunctioning pathways can amplify the toxicity of others. UPS inhibition by protofibrils results in less α-synuclein degradation, and therefore more aggregation. And dopamine interaction with α-synuclein increases aggregation, subsequently, more oligomers raise the dopamine concentration.
All these effects of defect α-synuclein diminish synapse function and reduce dopamine release through, either toxicity, or declined availability of dopamine-filled vesicles. When the synapse is not working properly, the dendritic spines are deprived of activity, and will reduce. Spine imbalance therefore further impedes signal transduction. In this stage, when neurons lose connection, retrograde signals lead to cell degeneration and symptoms of PD start to become noticeable.

In the end, the pathway to neuronal apoptosis is not singular nor is it simplistic, making it difficult to determine a target for an effective therapy. Each malfunctioning pathway disrupts others ultimately resulting in cell death. I can, however, conclude that the onset of neurodegeneration starts with aberrant α-synuclein folding and aggregation, and that the major culprit in PD appears to be the oligomeric form of α-synuclein, in contrast to LBs which seem to be quite harmless in the pathogenesis of PD.

## References


Bence, N. F., et al. (2001). "Impairment of the ubiquitin-proteasome system by protein aggregation." Science **292**(5521): 1552-1555.

Betarbet, R., et al. (2000). "Chronic systemic pesticide exposure reproduces features of Parkinson's disease." Nat Neurosci **3**(12): 1301-1306.

Beyer, K. (2007). "Mechanistic aspects of Parkinson's disease: alpha-synuclein and the biomembrane" Cell Biochem Biophys **47**(2):285-299.

Blasi, J., et al. (1993). "Botulinum neurotoxin A selectively cleaves the synaptic protein SNAP-25." Nature **365**(6442): 160-163.

Blasi, J., et al. (1993). "Botulinum neurotoxin C1 blocks neurotransmitter release by means of cleaving HPC-1/syntaxin." EMBO J **12**(12): 4821-4828.

Burre, J., et al. (2010). "Alpha-synuclein promotes SNARE-complex assembly in vivo and in vitro." Science **329**(5999): 1663-1667.

Chandra, S., et al. (2004). "Double-knockout mice for alpha- and beta-synucleins: effect on synaptic functions." Proc Natl Acad Sci U S A **101**(41): 14966-14971.

Chandra, S., et al. (2005). "Alpha-synuclein cooperates with CSPalpha in preventing neurodegeneration." Cell **123**(3): 383-396.

Choubey, V., et al. (2011). "Mutant A53T alpha-synuclein induces neuronal death by increasing mitochondrial autophagy." J Biol Chem **286**(12): 10814-10824.

Conway, K. A., et al. (2000). "Fibrils formed in vitro from alpha-synuclein and two mutant forms linked to Parkinson's disease are typical amyloid." Biochemistry **39**(10): 2552-2563.

Conway, K. A., et al. (2000). "Acceleration of oligomerization, not fibrillization, is a shared property of both alpha-synuclein mutations linked to early-onset Parkinson's disease: implications for pathogenesis and therapy." Proc Natl Acad Sci U S A **97**(2): 571-576.

Conway, K. A., et al. (2001). "Kinetic stabilization of the alpha-synuclein protofibril by a dopamine-alpha-synuclein adduct." Science **294**(5545): 1346-1349.

Danzer, K. M., et al. (2007). "Different species of alpha-synuclein oligomers induce calcium influx and seeding." J Neurosci **27**(34): 9220-9232.

Dauer, W., et al. (2002). "Resistance of alpha -synuclein null mice to the parkinsonian neurotoxin MPTP." Proc Natl Acad Sci U S A **99**(22): 14524-14529.

Ding, T. T., et al. (2002). "Annular alpha-synuclein protofibrils are produced when spherical protofibrils are incubated in solution or bound to brain-derived membranes." Biochemistry **41**(32): 10209-10217.

Gertz, H. J., et al. (1994). "Stability of cell size and nucleolar size in Lewy body containing neurons of substantia nigra in Parkinson's disease." Brain Res **637**(1-2): 339-341.

Greten-Harrison, B., et al. (2010). "alphabetagamma-Synuclein triple knockout mice reveal age-dependent neuronal dysfunction." Proc Natl Acad Sci U S A **107**(45): 19573-19578.

Jakes, R., et al. (1994). "Identification of two distinct synucleins from human brain." FEBS Lett **345**(1): 27-32.

Jao, C. C., et al. (2004). "Structure of membrane-bound alpha-synuclein studied by site-directed spin labeling." Proc Natl Acad Sci U S A **101**(22): 8331-8336.

Kastner, A., et al. (1993). "Immunocytochemical quantification of tyrosine hydroxylase at a cellular level in the mesencephalon of control subjects and patients with Parkinson's and Alzheimer's disease." J Neurochem **61**(3): 1024-1034.

Kayed, R., et al. (2003). "Common structure of soluble amyloid oligomers implies common mechanism of pathogenesis." Science **300**(5618): 486-489.

Kitada, T., et al. (1998). "Mutations in the parkin gene cause autosomal recessive juvenile parkinsonism." Nature **392**(6676): 605-608.

Kramer, M. L. and W. J. Schulz-Schaeffer (2007). "Presynaptic alpha-synuclein aggregates, not Lewy bodies, cause neurodegeneration in dementia with Lewy bodies." J Neurosci **27**(6): 1405-1410.

Lashuel, H. A., et al. (2002). "Neurodegenerative disease: amyloid pores from pathogenic mutations." Nature **418**(6895): 291.

Lee, H. J., et al. (2011). "Dopamine promotes formation and secretion of non-fibrillar alpha-synuclein oligomers." Exp Mol Med **43**(4): 216-222.

Lindersson, E., et al. (2004). "Proteasomal inhibition by alpha-synuclein filaments and oligomers." J Biol Chem **279**(13): 12924-12934.

Maroteaux, L., et al. (1988). "Synuclein: a neuron-specific protein localized to the nucleus and presynaptic nerve terminal." J Neurosci **8**(8): 2804-2815.

Martinez-Vicente, M., et al. (2008). "Dopamine-modified alpha-synuclein blocks chaperone-mediated autophagy." J Clin Invest **118**(2): 777-788.

McNaught, K. S., et al. (2004). "Systemic exposure to proteasome inhibitors causes a progressive model of Parkinson's disease." Ann Neurol **56**(1): 149-162.

Mosharov, E. V., et al. (2006). "Alpha-synuclein overexpression increases cytosolic catecholamine concentration." J Neurosci **26**(36): 9304-9311.

Nakamura, K., et al. (2011). "Direct membrane association drives mitochondrial fission by the Parkinson disease-associated protein alpha-synuclein." J Biol Chem **286**(23): 20710-20726.

Nemani, V. M., et al. (2010). "Increased expression of alpha-synuclein reduces neurotransmitter release by inhibiting synaptic vesicle reclustering after endocytosis." Neuron **65**(1): 66-79.

Olanow, C. W. (1990). "Oxidation reactions in Parkinson's disease." Neurology **40**(10 Suppl 3): suppl 32-37; discussion 37-39.

Parkkinen, L., et al. (2005). "Alpha-synuclein pathology does not predict extrapyramidal symptoms or dementia." Ann Neurol **57**(1): 82-91.

Poirier, M. A., et al. (1998). "The synaptic SNARE complex is a parallel four-stranded helical bundle." Nat Struct Biol **5**(9): 765-769.

Polymeropoulos, M. H., et al. (1997). "Mutation in the alpha-synuclein gene identified in families with Parkinson's disease." Science **276**(5321): 2045-2047.

Rijk de, M. C., et al. (1999). "Prevalence of Parkinson's disease in Europe: A collaborative study of population-based cohorts. Neurologic in the Elderly Research Group." Neurology **54**(11):21-23.

Schiavo, G., et al. (1992). "Tetanus toxin is a zinc protein and its inhibition of neurotransmitter release and protease activity depend on zinc." EMBO J **11**(10): 3577-3583.

Sharma, M., et al. (2012). "CSPalpha knockout causes neurodegeneration by impairing SNAP-25 function." EMBO J **31**(4): 829-841.

Sollner, T., et al. (1993). "A protein assembly-disassembly pathway in vitro that may correspond to sequential steps of synaptic vesicle docking, activation, and fusion." Cell **75**(3): 409-418.

Spillantini, M. G., et al. (1997). "Alpha-synuclein in Lewy bodies." Nature **388**(6645): 839-840.

Squier, M. K., et al. (1994). "Calpain activation in apoptosis." J Cell Physiol **159**(2): 229-237.

Sudhof, T. C. and J. Rizo (2011). "Synaptic vesicle exocytosis." Cold Spring Harb Perspect Biol **3**(12).

Sudhof, T. C. and J. E. Rothman (2009). "Membrane fusion: grappling with SNARE and SM proteins." Science **323**(5913): 474-477.

Tompkins, M. M. and W. D. Hill (1997). "Contribution of somal Lewy bodies to neuronal death." Brain Res **775**(1-2): 24-29.

Vekrellis, K., et al. (2011). "Pathological roles of alpha-synuclein in neurological disorders." Lancet Neurol **10**(11): 1015-1025.

Volles, M. J., et al. (2001). "Vesicle permeabilization by protofibrillar alpha-synuclein: implications for the pathogenesis and treatment of Parkinson's disease." Biochemistry **40**(26): 7812-7819.

Wakabayashi, K., et al. (2007). "The Lewy body in Parkinson's disease: molecules implicated in the formation and degradation of alpha-synuclein aggregates." Neuropathology **27**(5): 494-506.

Webb, J. L., et al. (2003). "Alpha-Synuclein is degraded by both autophagy and the proteasome." J Biol Chem **278**(27): 25009-25013.

Wilson, D. W., et al. (1989). "A fusion protein required for vesicle-mediated transport in both mammalian cells and yeast." Nature **339**(6223): 355-359.

Xu, J., et al. (2002). "Dopamine-dependent neurotoxicity of alpha-synuclein: a mechanism for selective neurodegeneration in Parkinson disease." Nat Med **8**(6): 600-606.

Yuste, R. and T. Bonhoeffer (2001). "Morphological changes in dendritic spines associated with long-term synaptic plasticity." Annu Rev Neurosci **24**: 1071-1089.