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IS MODULATION OF GLUCOCEREBROSIDASE A VIABLE NEW TREATMENT FOR PARKINSON'S DISEASE?

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THESIS FOR LICENTIATE DEGREE

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Expression of gratitude

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1 ABSTRACT

Background: Parkinson`s disease is the second most prevalent neurodegenerative disorder. There are currently no treatments that cure Parkinson's disease (PD) or slow its progression. There is therefore an urgent need for novel treatments. It was recently discovered that patients with mutations in *GBA1* are more likely to develop PD. *GBA1* encodes the glucocerebrosidase protein (GCase). GCase is a lysosomal enzyme that breaks down glucosylceramide. Several studies suggest that a decrease in GCase activity and the accompanying increase in upstream glycosphingolipids cause an increase in levels of α-synuclein and its propensity for aggregation. We investigate if there is a molecular link between GCase activity and α-synuclein. Furthermore, we study the endogenous GCase activator saposin C. Saposin C is a sphingolipid activator protein that acts as a co-factor for glucosylceramide degradation by enabling GCase access to the lipid membrane.

Aims: We aim to investigate how *GBA1* mutations predispose to PD. Additionally we attempt to increase GCase activity using saposin C, and whether activation of GCase with saposin C is protective in PD.

Methods and results: We studied the effect of GCase inhibition or genetic manipulation in SH-SY5Ycells. We establish that knockdown using RNA interference (RNAi) of GCase increases levels of α-synuclein. However, using a potent chemical inhibitor of GCase does not alter levels of α-synuclein. This finding suggests that GCase has an activity independent function that relies on the protein but not its enzymatic action to influence levels of α-synuclein. We are the first group to show that genetic and chemical inhibition produce different results. The increase in α-synuclein can be returned to physiological levels by addition of active recombinant GCase. Our data suggests that using GCase chaperones to aid folding and trafficking could be protective in PD patients with *GBA1* mutations.

To assess whether saposin C has a protective role in PD we generated short peptide fragments of different regions of saposin C. We confirmed that the GCase activating sequence is encoded in amino acids 41-68. We synthesized a cell-permeable peptide but were unable to achieve increase in GCase activity. We therefore, created a cell line overexpressing full length prosaposin. We could show that this leads to a natural increase in GCase activity and decrease in levels of α-synuclein. We show that the decrease in α-synuclein is independent of GCase activity as it

persists in CBE inhibited cells. We also provide preliminary evidence that saposin C can detach α-synuclein from artificial lipid membranes that act as a primary site for α-synuclein aggregation. Saposin C is therefore an interesting target for further studies.

LIST OF SCIENTIFIC PAPERS

I. Zurbruegg, M., Chan, M. Y., & Svenningsson, P. (2019). GBA RNAi but not catalytic inhibition of glucocerebrosidase with Conduritol-β-epoxide increases levels of total α-synuclein in SH-SY5Y cells. Neuroscience Letters. https://doi.org/10.1016/j.neulet.2019.05.027

II. Mark Zurbruegg, Wojciech Paslawski, Per Svenningsson (2019) Saposin C an endogenous activator of glucocerebrosidase reduces levels of α-synuclein (In preparation).

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LIST OF ABBREVIATIONS

AD	Alzheimer's Disease
BCA	Bichronic acid assay
CBE	Conduritol-β-epoxide
CMV	Cytomegalovirus
DLB	Dementia with Lewy bodies
DOPA	Dihydroxyphenylalanine
ELISA	Enzyme-linked immunosorbent assay
GBA 1	Glucocerebrosidase gene
GBA 2	Glucosylceramidase beta 2 gene
GCase	Glucocerebrosidase
GD	Gaucher's disease
GFP	Green fluorescent protein
HBSS	Hank's buffered saline solution
iPSC	Induced pluripotent stem cells
KO	Knock out
LSD	Lysosomal storage disorders
MAO B	Monoamine Oxidase B
MPTP	1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine
NAC	Non-amyloid-β component
OE	Over expressing
PD	Parkinson's Disease
PSAP	Prosaposin gene
RNAi	RNA interference
shRNA	Short hairpin RNA
siRNA	Small interefering RNA
VA	Veraglucerase Alfa

2 INTRODUCTION

2.1 PARKINSON'S DISEASE PREVALENCE

Parkinson's disease (PD) is the second most prevalent neurodegenerative disorder after Alzheimer's disease. The clinical hallmarks of Parkinson's are bradykinesia, resting tremor, rigidity, and postural instability. It has an estimated prevalence of 1-2% of the population aged 60 and above rising up to 3-4% at age 80 or above resulting in a 1.5% lifetime risk of developing PD (Bower, Maraganore, McDonnell, & Rocca, 1999; Nussbaum & Ellis, 2009; Tanner & Goldman, 1996). As prevalence of PD and other neurodegenerative diseases increases there is an equal increase in the care costs of these patients. Not only the cost of care has to be considered but also the great personal tragedy that unfolds in every single case of PD. It is important to remember that every statistic represents actual people that slowly become unable to live their normal lives, losing control of their own body. It is therefore not only an economic but also a moral imperative to further our understanding of this disease, with the eventual hope of a cure.

2.2 HISTORY OF PD

There are reports of Parkinson like illnesses dating back to 600 BC in the ancient Indian Ayurveda texts. The main pathological symptoms were identified as bradykinesia, rigidity of skeletal and facial muscles, resting tremor, as well as postural instability. Surprisingly the treatment developed in ancient India, as early as 300 BC, were seeds from Mucuna pruriens which contain up to 6% levodopa (Ovallath & Deepa, 2013). Further descriptions of patients now thought to have suffered from PD were made by Galen in 175 AD (Lanska, 2009). The first description of PD in modern medicine was made by the eponymous James Parkinson. He reported on six patients. He described them to be afflicted with a shaking palsy that was distinct from other known illnesses. He described Shaking Palsy (Paralysis Agitans) as an "involuntary tremulous motion, with lessened muscular power, in parts not in action and even when supported; with a propensity to bend the trunk forwards, and to pass from a walking to a running pace: the senses and intellects being uninjured" (Parkinson, 1817). While he did not have sufficient means to study this disease fully himself, he encouraged others to do so. Charcot lauded Parkinson for his accurate description of the disease. It was Charcot who

formally characterized the cardinal feature of PD and described all signs and symptoms, but he named it after Parkinson to acknowledge his astute observations (Lanska, 2009). While Parkinson described originally that the intellect remained unaffected it is now widely accepted that nonmotor symptoms such as cognitive decline, autonomic failure and psychiatric problems accompany PD (Corti, Lesage, & Brice, 2011).

Clues to the etiology of PD arose early in the 20th Century when Blocq and Marinescu reported on a patient who had her left substantia nigra destroyed by a tuberculoma. She displayed hemiparetic parkinsonism (Lanska, 2009). The typical Lewy bodies found in the classical PD cases were described by Friedrich Lewy (Lewy, 1912). However, it was not until 1919, when Trétiakoff a physician working with PD patients, that Lewy bodies in the substantia nigra were linked to the pathogenesis of PD (Lanska, 2009). It took nearly 40 years to confirm the importance of Lewy body pathology and loss of striatonigral neurons in PD (Greenfield & Bosanquet, 1953).

While the pathophysiological understanding of PD only progressed slowly various treatments were found to be effective based on empirical evidence. The first treatment consisted of anticholinergic alkaloids such as extracts from belladonna. Hyoscyamine was found to be modestly effective in treatment of PD by Charcot's student. After world war II several drugs were synthesized to replace the most common PD therapy which was still treatment with belladonna, these consisted of centrally acting anticholinergic drugs as well as synthetic anti-histamines such as diphenhydramine (Foley, 2003).

A real breakthrough in pharmacotherapy did not occur again until the mid-sixties. It was becoming clear that dopamine was playing an important role in the brain (Montagu, 1957). Analysis of catecholamine distribution revealed it being highly localized within the neostriatum (Bertler & Rosengren, 1959). Very astute observations by Carlsson and colleagues led to the hypothesis that dopamine depletion was the cause of PD. Supporting evidence included the fact that reserpine, a vesicular monoamine transport blocker, could induce PD. Treatment with the precursor DOPA led to reversal of reserpine induced PD (Carlsson, 2001). This effect was then confirmed in idiopathic and influenza induced PD patients, in which DOPA led to a remarkable decrease in bradykinesia (Birkmayer & Hornykiewicz, 1962). It was shown that with a slowly increasing dose of L-dopa and a peripheral

DOPA-decarboxylase inhibitor the side effects were quite small (George C Cotzias, Papavasiliou, & Gellene, 1969). Development of a long acting form of levodopa as well as catechol-O-methyltransferase inhibitors made this therapy even more viable (Lanska, 2009).

One of the problems of L-dopa therapy is that as PD progresses there are marked decreases in neurons that are able to convert DOPA to dopamine, leading to reduced signaling efficacy in the basal ganglia. Remarkably, a loss of 70-80% of tyrosine hydroxylase positive neurons is required before motor symptoms appear. Indicating that the basal ganglia system is very robust and good at compensating for a decrease in dopamine (Bernheimer, Birkmayer, Hornykiewicz, Jellinger, & Seitelberger, 1973). Therefore, a direct way to stimulate dopamine receptors that does not necessitate DOPA to dopamine conversion was desirable. Apomorphine a morphine derivative was noted to be highly similar in structure to dopamine, and it was shown that it acts as a dopamine receptor agonist (Ernst, 1965). Treatment of patients with apomorphine leads to significant improvement reducing bradykinesia, rigidity and tremor. However, apomorphine was never used routinely in the clinic due to liver toxicity (G C Cotzias, Papavasiliou, Tolosa, Mendez, & Bell-Midura, 1976). Instead ergot derivatives such as bromocriptine and pergolide were found to have dopaminergic activity and were used in combination with L-dopa (Calne et al., 1978; Galea Debono, Donaldson, Marsden, & Parkes, 1975). While dopaminergic agonists are less effective at alleviating symptoms of PD than levodopa, they also induce fewer side effects such as dyskinesia. A common therapeutic protocol combines low dose L-dopa with a dopaminergic agonist (Ch. Wolters, Tissingh, Bergmans, & Kuiper, 1995).

In addition to Pharmacotherapy surgical approaches were attempted. Lesion of corticospinal pathways reduced the tremor but also paralyzed patients. Approaches with more finesse such as stereotactic surgery, lesioning the thalamus and caudate nucleus, were shown to improve parkinsonian symptoms without impairing normal function. Lesioning of the thalamus became a fairly common treatment (Gildenberg, 1998). However, use of L-dopa largely replaced the use of surgical approaches. As limitations of pharmacotherapy became clear, renewed interest in surgical approaches led to the realization that lesioning of the external Globus pallidus and sub-thalamic nucleus were effective at treating PD (Gildenberg, 1998). Furthermore, deep brain stimulation of the subthalamic nucleus was shown to decrease

parkinsonian symptoms, with fewer side-effects than destructive ablation (Benabid, Pollak, Louveau, Henry, & de Rougemont, 1987; Lanska, 2009).

There have been tremendous advances in understanding PD pathology and etiology since its first description nearly 2500 years ago. Despite these advances we have not yet found a cure. However, the pace of research has rapidly accelerated and with advances in molecular biology and stem cell therapy, hope remains that one day we can cure PD and reverse the damage it does. However, the by far best approach would be to prevent people from developing PD. To achieve this goal, we must understand what causes PD.

2.3 PD PATHOLOGY

2.3.1 α-synuclein

PD was considered to be a sporadic disease with no genetic involvement until the late 20th century. This was due to the observation that external poisons such as MPTP could induce Parkinsonism, and that patients exposed to the 1918 influenza developed PD. Furthermore, twin studies did not reveal a genetic involvement (Lanska, 2009; Poskanzer & Schwab, 1963). However, in 1990 there was a report of a family with autosomal dominant transmission of PD (Golbe, Di Iorio, Bonavita, Miller, & Duvoisin, 1990). Linkage analysis and sequencing of the suspected region revealed a mutation in the α-synuclein gene (Polymeropoulos et al., 1997). α-synuclein is a 140 amino acid long protein that accumulates in the pre-synaptic nerve terminal. α-synuclein is also an integral part of Lewy bodies seen in PD (Spillantini et al., 1997). It turns out α-synuclein also has an important role in Alzheimer's disease constituting the non-amyloid β component of the AD plaques (Uéda et al., 1993). Patients with α-synuclein mutations typically have a late onset, low case of tremor and large Lewy body inclusions. Common α-synuclein mutations such as, A53T, A30P, and E46K, are autosomal dominant (Lashuel, Overk, Oueslati, & Masliah, 2013). Even sporadic PD patients have α-synuclein inclusions in Lewy bodies and Lewy neurites (Irizarry et al., 1998). While the importance of α-synuclein in PD is widely recognized, the molecular mechanisms by which these mutations mediate neurotoxicity are still not clear. A large number of studies point to importance of oligomerization and fibril formation. (Lashuel et al., 2013; Prusiner, 2012; Volpicelli-Daley, Luk, & Lee, 2014). However, we do not understand the role of α-synuclein in the body making it difficult to pinpoint any specific mechanism.

α-synuclein most likely performs an important physiological role as it is conserved across many vertebrate species (George, 2002). Strong evidence exists that α-synuclein is involved in neurotransmitter re-uptake, as well as moving vesicles from the reserve pool to the readily-releasable pool (Lashuel et al., 2013). α-synuclein knockout (KO) mice show deficits in hippocampal signaling that corresponds to the inability to replenish the readily releasable pool of synaptic vesicles (Cabin et al., 2002). Overexpression of α-synuclein results in reduced neurotransmitter release, and a decrease in readily releasable vesicles (Lashuel et al., 2013). α-synuclein KO mice are viable indicating that this protein is not essential for signaling but rather has a protective function, that becomes important under neural stress (Lashuel et al., 2013). Looking at the structure unfortunately does not reveal much about the proteins function.

α-synuclein is approximately 14.4 kDa and consists of, a lysine rich n-terminal that regulates interactions with the membrane, and an unstructured carboxyl tail that mediates protein and metal binding interactions (Lashuel et al., 2013). Amino acids 65-90 are known as the non-amyloid-β component (NAC) of AD plaque. The NAC region is critical for oligomerization and fibril formation (Lashuel et al., 2013; Luk et al., 2009). Its ability to oligomerize is most likely proportional to the concentration of it.

Concentration of α-synuclein depend on rate of synthesis as well as degradation. Imbalance in production of α-synuclein, as well as failure to degrade the protein at a normal rate, may lead to disease (*Figure 1*). Copy number variations resulting in higher levels of α-synuclein greatly increase the risk for PD. Most common mutations do not increase the concentration but increases α-synucleins propensity to oligomerize (Lashuel et al., 2013). Therefore, we need to consider not only the concentration but also the aggregation propensity.

α-synuclein most likely exists as a stable monomer and it is not yet known whether oligomerization or fibrillization have a physiological effect. However, conservation of the NAC region suggests that the ability to aggregate has an unknown use. While, α-synuclein is unstructured in solution, various factors have been shown to stabilize its structure as well as modulate its aggregation propensity. Proteolysis, post-translational modifications, oxidative stress, phospholipids and metal ions modulate aggregation propensity (Lashuel et al., 2013). α-synuclein has been shown to take a variety of conformations including dimers, tetramers, oligomers, proto-fibrils and

fibrils (Deleersnijder, Gerard, Debyser, & Baekelandt, 2013). The importance of these various α-synuclein species in health and disease are still under debate. Monomers can be considered non-toxic as they occur naturally within the body. Tetramers have recently been suggested to be the physiological conformation of α-synuclein, and are resistant to aggregation (Bartels, Choi, & Selkoe, 2011). Furthermore, mutations in α-synuclein have been shown to decrease tetramer levels, increasing aggregation propensity (Dettmer et al., 2015) . However, there is still debate about the existence of tetramers as a large-scale verification study found that monomeric α-synuclein is the dominant conformation in the central nervous system, as well as various other α-synuclein sources. These experiments were conducted using a wide variety of techniques, and by seven independent research groups (Fauvet et al., 2012).

The nature of the toxic α-synuclein species is also contentious. Initially, the toxic species was thought to be the fibrillar aggregates found in Lewy neurites and Lewy bodies. However, the main toxic species is now thought to be oligomeric (Lashuel et al., 2013). Furthermore, it cannot be ruled out that even monomers may contribute to toxicity due to their unstructured nature. Monomers could activate any number of toxic downstream pathways in PD. The majority of in-vitro, in-vivo and post-mortem analysis assume oligomers or fibrils are toxic, finding a good correlation between aggregated oligomeric and fibrillar α-synuclein, and neurotoxicity. Deletions of α-synuclein amino acids 71-82 abolishes its ability to aggregate and with it the typically observed neurotoxicity (Periquet, Fulga, Myllykangas, Schlossmacher, & Feany, 2007). Therefore, the aggregation mechanics of α-synuclein are directly linked to the observed toxicity and PD progression.

To summarize, the level of α-synuclein aggregates depend on both the concentration of monomers, the rate at which these monomers form aggregates and the rate at which these aggregates is cleared. Therefore, strategies that target α-synuclein concentration and aggregation propensity present a good target for novel therapeutic approaches *(Figure 1)*.

Factor	Evidence	Therapeutic approaches	Reference
Rate of transcription	Methylation of α-synuclein is reduced in PD patient brains	transcriptional activators / repressors, epigenetic modulation	(Jowaed, Schmitt, Kaut, & Wüllner, 2010)
Stability of mRNA	Reduced α-synuclein levels observed after siRNA treatment leading to improved disease condition	siRNA, miRNA	(Gorbatyuk et al., 2010; McCormack et al., 2010)
Rate of translation	no evidence	no specific target opportunities	
Propensity to aggregate	Δ71-82 deletion abolishes aggregation, pathogenic mutations increase aggregation propensity	gene editing, stabilization of monomers	(Deleersnijder et al., 2013; Periquet et al., 2007)
Degradation of monomers	inhibition of proteasome leads to inclusion body formation in ventral mesencephalic cultures	Targeting monomers to lysosome or proteasome	(McNaught et al., 2002)
Degradation of toxic aggregates	Catecholamines can stabilize proto-fibrillar aggregates and increase toxicity, defects in protein degradation are seen frequently in PD patients	decreasing aggregate stabilizers that may include catecholamines, targeting aggregates to lysosome or proteasome, immunotherapy targeting aggregates	(Conway et al., 2001; Masliah et al., 2011)

Figure 1. *Factors affecting level of α-synuclein and potential treatment opportunities*

2.3.2 Mitochondrial recycling and oxidative stress

Parkin is another gene important for PD was discovered just a year later. This gene segregated in an autosomal recessive fashion in a Japanese cohort (Kitada et al., 1998). Parkin mutations result in juvenile onset PD, with symmetric onset of symptoms and slow progression. Parkin mutations were not associated with α-synuclein positive Lewy bodies initially, but it is now accepted that some Lewy bodies may form in the substantia nigra and locus coeruleus (Pramstaller et al., 2005). α-synuclein is also a substrate for parkin, and mutations in parkin result in aggregation of α-synuclein (Shimura et al., 2001). However homozygous loss of parkin function leads to pure nigral degeneration without Lewy body inclusions. Therefore, Parkin is able to cause PD independently of α-synuclein (Hardy, Lewis, Revesz, Lees, & Paisan-Ruiz, 2009).

Parkin is also involved in the ubiquitin-proteasome system acting as an E3 ligase responsible for ubiquitinating proteins. Parkin is essential for mitochondrial quality control together with Pink-1. Pink-1 accumulates on the mitochondrial membrane. Through elegant experimentation in drosophila it was shown that Pink-1 is upstream of Parkin (Park et al., 2006). Post translational modification of Parkin such as phosphorylation by Pink-1 results in activation of Parkin leading to ubiquitination of various mitochondrial proteins and eventually autophagy of the mitochondrion (Durcan & Fon, 2015). Both mutations in Parkin and PINK-1 can lead to PD in an autosomal recessive manner. It is unclear how damaged mitochondrial recycling results in PD.

A widely accepted but not conclusively proven theory states, that nigral dopaminergic cells are very sensitive to oxidative stress (M. P. Murphy, 2009). This is due to the production of catecholamines already resulting in large quantities of reactive oxygen species (ROS). Mitochondria are another major source of ROS in the cell. Decreased functioning of complex I leading to a high protonmotive is one of the major drivers behind ROS production (M. P. Murphy, 2009). MPTP a known PD inducing neurotoxin blocks complex I. Inability to clear damaged mitochondria results in a marked increase of reactive oxygen species (ROS) and may cause the toxicity specifically affecting midbrain dopaminergic neurons. DJ-1 is another gene linked to PD and functions as a sensor for oxidative stress and protects neurons from the stress. Incorrect mitochondrial recycling leading to oxidative toxicity may therefore be one of the pathways leading to PD.

John Hardy suggested that there are multiple pathways leading to PD, based on the known PD genes (Hardy et al., 2009). Mitochondrial dysregulation as well as aggregation of α-synuclein can lead to PD. These pathways have complex interactions but are able to trigger PD independently (Hardy et al., 2009; Henchcliffe & Beal, 2008). In addition to these two well established canonical PD pathways there has been the proposition of a third pathway that induces PD by affecting lysosomal processing (Hardy et al., 2009).

LRRK-2 is another gene which if mutated can cause PD and while penetrance of PD varies with mutation, the most common G2019S has nearly complete penetrance at the age of 80. Again, very little is known about why LRRK-2 causes PD. Lewy bodies are not present in all cases meaning that while this mutation seems to promote formation of Lewy bodies, it has secondary toxicity independent of α-synuclein aggregation (Hardy et al., 2009).

Despite a variety of PD genes being identified and characterized we still know surprisingly little about the cause of the majority of PD cases. This is because most PD patients do not have a mutation in disease causing gene. PD is most likely polygenic and shows strong environmental influence, this makes the causes leading to PD extremely difficult to detect.

2.3.3 Environmental causes of PD

PD in most cases is sporadic meaning that the disease cause is not known or not determinable. Studies have mainly focused on genetic factors to help us understand molecular pathways that are obfuscated in sporadic disease. However, a few environmental factors have extremely clear link to being causative of PD. The earliest environmental link was found in a subset of patients suffering from the Spanish flu. These patients developed post-encephalitic Parkinsonism. This originally lead to the false conclusion that PD is entirely determined by environmental factors, but after the flu of 1918 no other influenza virus has been linked to causing PD (Poskanzer & Schwab, 1963). Another argument for the environmental hypothesis was the famous neurotoxin 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine (MPTP). The faulty synthesis of meperidine an opioid analgesic resulted in MPTP being a major impurity that a variety of people ended up self-injecting. People who injected this mixture rapidly presented with aggressive parkinsonian symptoms (Davis et al., 1979; Langston, Ballard, Tetrud, & Irwin, 1983). MPTP acts as a

prodrug and is converted to MPP+ by monoamine oxidase B (MAO-B). MPP+ can then act as a complex I inhibitor in the mitochondria resulting in severe loss of dopaminergic neurons in the substantia nigra (Richardson et al., 2007).

Rotenone is a commonly used insecticide and piscicide that works very similar to MPTP. Rotenone blocks electron transfer in complex I, resulting in ROS creation. While highly toxic to fish and insect's, rotenone is only mildly toxic in humans and other mammals. At commonly used concentrations it is not taken up through the skin. However, exposure to rotenone even at very low concentration has been linked to increased risk of PD (Tanner et al., 2011). Paraquat a herbicide that works by creation of ROS has also been linked to PD (Tanner et al., 2011). Traumatic brain injuries have also been shown to have a small but significant effect in increasing the risk of developing PD (Gardner et al., 2015). It is not clear why sustaining brain injury would lead to PD, but it may be by potentially inducing oxidative stress in response to the injury. Studying both environmental and genetic pathways shows that there are clear overlapping molecular pathways. Especially oxidative stress and damage to mitochondria seem to cause PD.

Another interesting environmental interaction is that of smoking which reduces the risk of PD. This link was noticed as early as 1959, when there was a large scale effort to link health concerns to smoking (Dorn, 1959). It is commonly believed to be the nicotine in cigarettes that reduces the risk of developing PD, but no nicotine receptor targeting therapeutics are currently licensed for PD. It has been suggested that decreased activity of MAO-B in smokers reduces the risk of PD as it can reduce the impact of environmental toxins, as well as decrease breakdown of dopamine to toxic intermediates (Fowler et al., 1998). However, the molecular pathway underlying protection from PD in smokers is still unclear.

2.3.4 Parkinsonism plus syndrome

Additionally, various diseases are now classified as Parkinson's disease plus syndrome and fall into the category of synucleinopathies (dementia with Lewy bodies and multiple systems atrophy) or Tauopathies (Corticobasal degeneration, frontotemporal dementia and Parkinsonism linked to chromosome 17, Pick's disease, progressive supra-nuclear palsy). Given that PD can have such varied etiology it is likely that the nigral dopaminergic neurons are especially susceptible to damage explaining why several independent pathways converge on a set of clinical

symptoms defined as PD. PD is therefore not a single disease but rather appears to be an endpoint resulting from failure of a variety of different pathways. Great variety can be seen in the presentation of the disease which appears to be related to the heterogeneous molecular causes of the disease. By studying pathways that lead to PD we may gain a better understanding of how to treat it. Interestingly, patients with Gaucher's disease and their relatives are particularly susceptible to PD. It is therefore warranted that the link between the two diseases is investigated in greater detail.

2.4 GAUCHER'S DISEASE

Gaucher's disease (GD) is one of the 41 known lysosomal storage disorders (LSD). It was first described by Gaucher in 1882 (Kahn, 2007). This fairly rare disorder has an incidence of 1 in 100,00 of the general population, but is far more common in Ashkenazi Jews reaching a prevalence of 1 in 1,000 (Stirnemann & Belmatoug, 2012). GD is autosomal recessive and characterized by deficiency in glucocerebrosidase activity. Glucocerebrosidase is a lysosomal enzyme that is responsible for cleaving the β-glucosidic linkage in glucosylceramide (Mehta, 2006). Decrease in glucocerebrosidase activity can result in three distinct forms of GD. The most common form is type 1, which is also described as non-neuronopathic. The most common symptoms are enlarged liver and spleen, anemia, thrombocytopenia and bone disease. Type II and type III GD are neuronopathic with variable pathology. Type II presents early in life and shows rapid progressive neurological deterioration, leading to death. Type III GD also presents variable pathology but can include myoclonic epilepsy, horizontal saccadic eye movements and hydrocephalus. All types of GD are caused by mutations in the *GBA 1* gene (Ellen Sidransky & Lopez, 2012). Two therapies are currently used to treat GD. Enzyme replacement therapy relies on injections of recombinant GCase and is an effective treatment for type I GD. Substrate reduction therapy is based on applying a glucosylceramide synthase inhibitor, which reduces the amount of glucosylceramide produced, and is used for mild forms of GD. Treatment of the neurological symptoms however has always been poor, as the recombinant enzyme does not pass through the blood brain barrier.

2.5 THE LINK BETWEEN GAUCHER'S DISEASE AND PARKINSON'S DISEASE

GBA 1 mutations were only identified as a risk factor for PD thanks to astute clinical observation. Epidemiologic analysis, linkage studies and genome wide analysis all failed to pick up the correlation (Rogaeva & Hardy, 2008). Early evidence came from a study in which six patients with GD type 1 developed PD (Neudorfer et al., 1996). It was later confirmed that patients and relatives of patients with Gaucher's disease developed PD at a significantly higher rate (O Goker-Alpan et al., 2004). This sparked interest in finding out whether these two diseases were related. Large multi-center trials were carried out and the rate of *GBA1* mutations was a striking 18% in PD patients, compared to 4% in age matched controls (Gan-Or et al., 2008). This finding was replicated in other patient cohorts such as British patients, where *GBA1* mutations were found in 4.2% of PD patients versus 1.17% of control (Neumann et al., 2009). Similar results were obtained from a European screening study of 1130 PD patients of which 6.7% had *GBA1* mutations versus 1.0% in 391 controls (Lesage et al., 2011). Even by conservative estimates *GBA1* is the most common genetic risk factor for PD. Most physicians interpret *GBA1* mutations to be autosomal dominant with reduced penetrance. However, the penetrance of *GBA1* mutations is most likely multigenic, making genetic counseling difficult without knowing more about the molecular pathways resulting in penetrance (Ellen Sidransky & Lopez, 2012).

A prospective study in Ashkenazi Jews found that carrying mutated alleles of *GBA1* decreased the age at which you develop PD. Homozygotes developed PD at a younger age (54.2 years) versus heterozygotes (65.2 years) and non-carriers (70.2). Furthermore, a significantly larger percentage of subjects developed PD if they were homo/heterozygotic for *GBA1* (Alcalay et al., 2014; Bultron et al., 2010; Nalls et al., 2013; E Sidransky et al., 2009). It was therefore clear that *GBA1* was a strong risk factor for PD. While the association between *GBA1* mutations and PD has been clearly demonstrated, the molecular mechanisms is still unknown. Both toxic gain of function and loss of function were proposed as potential explanations (Ellen Sidransky & Lopez, 2012). Mutated GCase could promote α-synuclein aggregation or be toxic to nigrostriatal dopamine neurons. Alternatively, loss of GCase function could promote substrate accumulation which may be toxic or loss of function results in reduced α-synuclein clearance.

Initial observations supported the gain of function hypothesis. *GBA1* heterozygotes with one non-functioning allele still develop PD at a much higher rate than normal. However, *GBA1* does not demonstrate haploinsufficiency and patients with one mutated allele have nearly normal GCase functioning (Alcalay et al., 2014; Bultron et al., 2010; Nalls et al., 2013; E Sidransky et al., 2009). Furthermore, the E326K *GBA1* mutation does not cause a large reduction in GCase function. Homozygotic carriers of this mutation had GCase function at the lower end of the normal spectrum and did not have GD. Despite having little impact on GCase activity the E326K mutation increased the risk of developing PD (Duran et al., 2013).

Furthermore, misfolded GCase is found in Lewy bodies suggesting that it may potentially aid α-synuclein oligomerization. It has been suggested that GCase and α-synuclein interact in the lysosome (Yap et al., 2011). Lastly most GD patients do not develop PD indicating that it is not the absence of function that leads to development of the disease (Mazzulli et al., 2011). Therefore, a large body of evidence indicates that *GBA1* mutations cause a toxic gain of function.

However, loss of GCase function may also contribute to PD. *GBA1* KO mice have increased levels of α-synuclein and a single injection of CBE raised the Striato-nigral levels of α-synuclein. Furthermore, decrease in GCase function may put stress onto the mitochondria leading to reactive oxygen species production, a process indicated in PD(Manning-Boğ, Schüle, & Langston, 2009; Osellame et al., 2013).

Additional evidence for loss of function come from molecular studies. It has become clear that a decrease in GCase activity leads to increased α-synuclein both in vitro and in vivo (Manning-Boğ et al., 2009; Mazzulli et al., 2011). Increased levels of α-synuclein increase the likelihood and speed at which α-synuclein oligomers form. PD patient brain analysis shows that even in sporadic PD patients there is a decrease in both the level of GCase protein and its activity level (K. E. Murphy et al., 2014). Therefore, both gain of function and loss of function seem important.

2.6 THE RELATIONSHIP BETWEEN GCASE AND A-SYNUCLEIN

Both gain of function and loss of function theories seem to implicate α-synuclein as the link between *GBA1* mutations and PD. A variety of crucial questions still remain unanswered. Does α-synuclein cause a decrease in GCase protein level and activity? Does decreased GCase activity increase levels of α-synuclein? Does one cause precede the other?

Let us first look at the question of timing. In patients with homozygous or heterozygous *GBA1* mutations it is obviously a decrease in GCase activity that precedes increased levels of α-synuclein. Conversely, in PD patients that have a SNCA triplication, the increased level of α-synuclein precedes decreased GCase activity. If we look at sporadic cases of PD there is no clear answer but interestingly PD patients with no mutations in *GBA1* still show reduced GCase activity (K. E. Murphy et al., 2014). One of the most popular current theories is that there is no clear cause and effect but rather a cycle.

The theory proposes a bi-directional feedback loop between GCase activity and level of α-synuclein (Mazzulli et al., 2011). Decrease in GCase activity leads to an increase in glucosylceramide which may stabilize small soluble α-synuclein oligomers. These oligomers then further impede the trafficking of GCase from the endoplasmic reticulum to the Golgi. This leads to depletion of lysosomal GCase and eventual dysfunction of the lysosome in general. This increases α-synuclein levels, due to a decrease in α-synuclein degradation in the lysosome (K. E. Murphy et al., 2014; Sevlever, Jiang, & Yen, 2008; Vogiatzi, Xilouri, Vekrellis, & Stefanis, 2008). It therefore seems likely that both an increased level of α-synuclein and a decreased GCase activity can start a cycle resulting in the final observed pathogenic levels of α-synuclein. Disrupting this cycle by increasing the functionality of GCase may be a viable way to interfere with PD pathogenesis or impede progression of PD.

This feedback theory is supported by a study using cells that both overexpress α-synuclein and are *GBA1* -/-. In these cells there is profound lysosomal dysfunction is mimicked and there is an accumulation of insoluble α-synuclein oligomers. These data indicate that loss of *GBA1* contributes to developing PD (Bae et al., 2015).

However, other studies indicate that there is no correlation between GCase activity and α-synuclein levels (Cullen et al., 2011; Dermentzaki, Dimitriou, Xilouri, Michelakakis, & Stefanis, 2013). In these papers, relationship of α-synuclein and GCase is assessed by inhibiting GCase with the specific inhibitor conduritol-β-epoxide (CBE), rather than using genetic knock-out or knock-down strategies. After CBE treatment, which reduced GCase activity 95%, there was no significant increase in α-synuclein. However transfecting cells with AAVs expressing mutant isoforms of GCase results in marked increase of α-synuclein supporting the idea that there is a toxic gain of function mutation responsible for predisposing to PD rather than a loss of function.

The idea of a toxic gain of function is also better supported by clinical evidence, as patients with GCase mutations have an increased incorporation of mutant GCase into Lewy bodies with about 90% of Lewy bodies being GCase positive in mutant carriers compared to 10% in age-matched controls (Ellen Sidransky & Lopez, 2012). GCase mutations are also present in diffuse Lewy body dementia but not in multiple system atrophy (Ellen Sidransky & Lopez, 2012). This indicates that GCase and α-synuclein interact directly in some way. In-vitro studies do show that GCase and α-synuclein can interact on artificial lipid membranes (Yap, Gruschus, Velayati, Sidransky, & Lee, 2013; Yap et al., 2011). Therefore, it is still plausible that interactions between lipids, mutated GCase and α-synuclein is the cause for increased risk of developing PD.

To summarize, the molecular mechanism by which *GBA1* mutations act is still unknown. Many studies show that decrease in GCase activity increases levels of α-synuclein and that α-synuclein can in turn reduce GCase activity (Manning-Boğ et al., 2009; Mazzulli et al., 2011; Yap et al., 2013, 2011). However, opposing studies did not find a correlation between GCase activity and α-synuclein. Instead they implicate misfolding of GCase as the primary cause showing that a variety of misfolded mutants are responsible for increased levels of α-synuclein (Cullen et al., 2011; Dermentzaki et al., 2013). However, most studies agree that the mutations have an effect on α-synuclein levels, which increases likelihood of α-synuclein aggregation.

3 GBA RNAI BUT NOT CATALYTIC INHIBITION OF GLUCOCEREBROSIDASE WITH CONDURITOL-β-EPOXIDE INCREASES LEVELS OF TOTAL α-SYNUCLEIN IN SH-SY5Y CELLS

3.1 BACKGROUND FOR STUDY 1

There is copious evidence that PD and GD are linked (Ellen Sidransky & Lopez, 2012). The results of all currently published studies have been compiled in *figure 2*. However, the underlying molecular mechanism is still under debate.

one of the first molecular links showed that administration of the GCase inhibitor conduritol-β-epoxide (CBE) led to an increase in levels of α-synuclein (Manning-Boğ et al., 2009). The results of this study showed a clear elevation of α-synuclein in SH-SY5Ycells after treatment with 50 μM of CBE. However, escalating the dose to 100

or 200 μM showed a lower level of α-synuclein despite increased GCase inhibition. Furthermore, a thorough replication study of these original experiments was unable to confirm an increase in α-synuclein (Dermentzaki et al., 2013). Another study found that mutant *GBA1* forms can increase levels of α-synuclein. Administration of CBE did not lead to an increase in α-synuclein but overexpression of wildtype GCase decreased levels of α-synuclein (Cullen et al., 2011). However, Knockdown of GCase reliably increases levels of α-synuclein (Mazzulli et al., 2011; Schöndorf et al., 2014). One plausible mechanism by which alterations in GCase activity could affect α-synuclein is through increase in GCase substrate.

Loss of GCase function leads to an increase in glucosylceramide and glucosylsphingosine (Dekker et al., 2011; Hamler et al., 2017). Incubation of α-synuclein with these two lipids increased β-sheet confirmation (Taguchi et al., 2017). Furthermore, α-synuclein could be turned into pre-formed fibrils by incubation with glucosylceramide and glucosylsphingosine but not other common lipids. When lipid formed α-synuclein fibrils were added to cells, there was clear aggregation of α-synuclein inside those cells (Taguchi et al., 2017). Therefore, loss of GCase function results in increased levels of glucosylceramide and glucosylsphingosine leading to α-synuclein oligomerization.

However, the loss of function hypothesis ignores some critical evidence. Mutant GCase is often found in Lewy bodies but normal GCase is not (Ozlem Goker-Alpan, Stubblefield, Giasson, & Sidransky, 2010). Furthermore, Cullen and colleagues transfected various cell lines with either mutant or wild-type GCase plasmids. Over-expression of mutated GCase alongside WT GCase led to a significant increase in levels of α-synuclein independent of GCase activity (Cullen et al., 2011). Additionally, mutant GCase can be retained in the endoplasmic reticulum triggering the unfolded protein response and stressing the cell, possibly causing the cell death (Ron & Horowitz, 2005). Furthermore, patients with mutations that have little effect on GCase activity and heterozygous mutants still show an increased risk for PD even though they are sub-clinical for GD (Ellen Sidransky & Lopez, 2012).

It is therefore, not clear yet whether a decrease in GCase activity, a decrease in GCase protein level or an increase in toxic mutant GCase function is the cause of increased α-synuclein and therefore the increased PD risk. We aimed to disentangle the complex cause of increased α-synuclein by directly comparing genetic knock-down strategies and chemical inhibition strategies. We could show that RNA

interference, but not chemical inhibition leads to an increase in α-synuclein. We suggest that chemical inhibition and genetic ablation strategies produce two distinct effects. Our results suggest caution in choosing which cellular *GBA1* model is used to best mimic PD with *GBA1* mutations. Based on the existing literature and our results we hypothesize that reduction in GCase levels decreases α-synuclein degradation via a direct interaction. (Aflaki, Westbroek, & Sidransky, 2017). This direct interaction would explain why mutated GCase accumulates with α-synuclein in Lewy bodies. Our data shows an increase in α-synuclein levels only when using RNAi and not by chemical inhibition. This hypothesis is consistent with the variety of different results observed (*Figure 2*).

Chemical inhibition of Glucocerebrosidase	Genetic Manipulation of GBA 1
Treatment of differentiated SH-SY5Y cells with 50-200 μM CBE for 48 hours increased levels of α-synuclein. Animals treated with CBE showed increased α-synuclein immunofluorescence (Manning-Boğ et al., 2009).	Lentiviral shRNA targeting GBA 1 resulted in 50% Glucocerebrosidase KD and a 60% increase of α-synuclein (Mazzulli et al., 2011)
Treatment of differentiated SH-SY5Ycells and rat primary cortical neurons with 50-200 μM CBE from one day to one week has no effect on α-synuclein (Dermentzaki et al., 2013).	IPSC derived from GBA 1 mutation carriers (RecNil, L444P, N370S). Even RecNil iPSC had higher levels of α-synuclein (Schöndorf et al., 2014)
Treatment of SH-SY5Ywith 50 μM CBE for one month showed a significant increase in α-synuclein. Knockdown of *GBA1* with siRNA did not yield significant increase in α-synuclein.(Cleeter et al., 2013).	Treating iPSC that have *GBA1* mutations with small-molecule chaperone restores lysosomal glucocerebrosidase localization and activity and reduces α-synuclein levels to that comparable of controls. (Mazzulli et al., 2016)
Treatment with CBE (10 - 100 μM) had no effect on α-synuclein levels in MES-SNCA cells. Overexpression of glucocerebrosidase mutants especially D409V caused remarkable increase in α-synuclein levels and this increase was unrelated to GCase activity. (Cullen et al., 2011).	Overexpression of mutated glucocerebrosidase (D409H, L444P, N370S, E235A, E340A) but not WT GBA 1 led to a great increase in α-synuclein levels. (Cullen et al., 2011)
Treatment with 25 μM CBE for 48 hours caused visual increase in α-synuclein immunolabeling in differentiated control iPSC (Schöndorf et al., 2014).	Knockout of GBA 1 in SH-SY5Ycells results in double the level of α-synuclein. (Bae et al., 2015)
Treatment of primary cultures with 10 μM CBE replacing media every 3 days for 10 days. There was approximately 50% increase in WB and a 10% increase using ELISA to quantify α-synuclein (Magalhaes et al., 2016).	
Treatment of iPSC with 50μM CBE for 7 days did not increase levels of soluble α-synuclein but did increase levels of insoluble α-synuclein. (Zunke et al., 2018)	

Figure 2. *Overview of cellular experiments manipulating GCase activity and measuring* α-synuclein.

4 SUMMARY PAPER I

GBA RNAi but not catalytic inhibition of glucocerebrosidase with Conduritol-β-epoxide increases levels of total α-synuclein in SH-SY5Y cells

4.1 ARTICLE SUMMARY

Our aim was to investigate the correlation between α-synuclein and GCase activity in greater detail. We focused on this specific problem because of conflicting reports in the literature and our own experimental data (Dermentzaki et al., 2013; Mazzulli et al., 2011). We used four different cell models: 1) undifferentiated SH-SY5Y cells, 2) differentiated SH-SY5Y cells, 3) N2a cells over-expressing α-synuclein, 4) rat midbrain primary cultures.

Our primary goal was to confirm or refute that CBE increases levels of α-synuclein. First, we investigated the effect of CBE treatment in primary cultures and differentiated SH-SY5Y cells over a 10 day timespan. CBE treatment reduced GCase activity by over 95%, however we did not see any significant increase in α-synuclein (Art 1, Figure 1). We could show that even when treated over a long-time span of 30 days, there is no noticeable increase in soluble α-synuclein as measured by western blot (Paper 1, Figure 2). This confirmed the data published by Dermentzaki and colleagues (Dermentzaki et al., 2013).

We then decided to go ahead and still attempt a genetic manipulation of GCase levels by use of siRNAs. Our theory was that while inhibition of GCase with CBE does not increase levels of α-synuclein, genetic manipulation of *GBA1* does increase α-synuclein. This was based on multiple publications showing a significant increase in α-synuclein after genetic manipulation (Figure 2). We could confirm that treatment for 10 days with *GBA1* siRNA significantly increased levels of α-synuclein. We carefully controlled this experiment by also using *GBA2* siRNA and CBE. We therefore show that knockdown of GCase but not chemical inhibition, causes the increase in α-synuclein.

This result led us to speculate that GCase influenced α-synuclein in an activity independent way. We based this on *in-vitro* studies, that demonstrated α-synuclein binding to and inhibiting GCase (Yap et al., 2013). We therefore designed an experiment in which we would add recombinant GCase to our cell model. We again demonstrated that siRNA treatment but not addition of CBE increased α-synuclein.

Furthermore, we show that dual addition of CBE and *GBA1* siRNA do not have a greater effect than just adding the siRNA. We could also show that addition of recombinant GCase restores GCase activity and reduces α-synuclein. However, we were not able to confirm that this is done by an activity independent mechanism. Treatment of differentiated SH-SY5Y cells with *GBA1* siRNA followed by addition of recombinant GCase that is preincubated with CBE, did not restore physiological levels of α-synuclein (Paper 1, Figure 4).

In conclusion, using careful controls, we could show that knockdown of GCase increases levels of α-synuclein, while chemical inhibition with CBE, did not increase levels of α-synuclein. This fact is of major importance to the literature as it explains the variety of results that were obtained by different experimental approaches (figure 2). This distinction is a subtle one but has important physiological implications for potential therapeutic applications. When designing new treatments, priority should be given to approaches that stabilize GCase rather than focus solely on the increase in GCase activity. It also suggests that treatments aimed at increasing levels of GCase in PD related areas may be a viable strategy to reduce levels of α-synuclein. Already a variety of potential treatments are adopting this strategy by using small molecular chaperones such as Ambroxol (Sanchez-Martinez et al., 2016).

5 SAPOSIN C REDUCES LEVELS OF A-SYNUCLEIN AND DISLODGES IT FROM GLUCOSYLCERAMIDE-ENRICHED LIPID MEMBRANES

5.1 BACKGROUND TO STUDY 2

Various studies have shown that restoring normal GCase functioning in cells with *GBA1* mutations results in a decrease in levels of α-synuclein (*figure 2)*. iPSC's obtained from patients with *GBA1* mutations have higher levels of α-synuclein than control. Using Zinc-finger nucleases to insert a non-mutated *GBA1* alleles results in a decrease in levels of α-synuclein (Schöndorf et al., 2014). Overexpression of WT GCase in HEK293-SNCA cells led to a significant decrease in α-synuclein (Cullen et al., 2011). Use of a small molecular chaperone to aid folding and processing of mutant GCase through the endoplasmic reticulum into the lysosome was able to revert the increase in α-synuclein observed in cell lines with *GBA1* mutations (Mazzulli et al., 2016; Sanchez-Martinez et al., 2016). This led us to hypothesize that activating GCase could be used as a potential treatment for PD. We decided to use saposin C, an endogenous activator of GCase, to investigate potential beneficial effects of saposin C in PD.

Prosaposin is the propeptide of saposin C and is cleaved into saposin A-D. Saposin's A-D all assist in lysosomal degradation of lipids by acting as sphingolipid activator proteins (Kishimoto, Hiraiwa, & O'Brien, 1992; Solly Weiler, Tomich, Kishimoto, O'Brien, & Barranger, 1995). Saposin C assists GCase by modifying the lipid membrane in a detergent like manner and exposing the headgroup of the lipids (Lieberman, 2011).

There are many reasons why saposin C could be beneficial in PD.(i) Saposin C can increase GCase activity by acting as a chaperone, preventing degradation of GCase and extending its half-life (Sun, Qi, & Grabowski, 2003). (ii) Saposin C contains a neurotrophic sequence close to its n-terminal (O'Brien, Carson, Seo, Hiraiwa, & Kishimoto, 1994; O'Brien et al., 1995). (iii) Saposin C is a potential ligand for the GPR37 orphan receptor (Meyer, Giddens, Coleman, & Hall, 2014). GPR37 is found in Lewy bodies and may provide a druggable target for treatment of PD, because it can interact with the D2 receptor and DAT, to alter dopaminergic signaling. (iv) GPR 37 can be neuroprotective when activated. (v) GPR37 forms part of the core

structure in idiopathic PD Lewy bodies. (Leinartaité & Svenningsson, 2017). (vi) Furthermore, ex-vivo experiments show that α-synuclein can inhibit GCase activity at lysosomal pH. This inhibition is reversed by addition of Saposin C (Yap et al., 2013).

Therefore, Saposin C is an interesting peptide that interacts with multiple PD pathways. To investigate whether saposin C had neuroprotective properties in PD, we generated short saposin C peptides. We could confirm that saposin C activates GCase and that this depends on amino acids 41-68 (S Weiler, Kishimoto, O'Brien, Barranger, & Tomich, 1995). However, attempting to attach a cell penetrating motive and applying the peptide to SH-SY5Ycells was not sufficient to increase GCase activity. Instead we created a stable cell line overexpressing prosaposin under the CMV promoter. This leads to a significant increase in GCase activity and a subsequent decrease in α-synuclein. The decrease in α-synuclein persisted even after inhibition of GCase with CBE indicating another mechanism of action. We showed that saposin C can effectively detach α-synuclein from artificial membranes, which are a primary site of α-synuclein aggregation (Kalia & Lang, 2015). Our data indicates that saposin C is important in PD and may be a potential treatment target.

6 SUMMARY PAPER II

Saposin C reduces levels of α-synuclein and dislodges it from glucosylceramide-enriched lipid membranes

6.1 ARTICLE SUMMARY

In paper II we investigate saposin C as a potential treatment for PD. Our initial idea was to produce small peptide analogues of saposin C, containing the activating sequence for GCase. We thought that these small peptides could increase GCase activity and therefore be protective in PD. We designed a variety of sequences and were able to confirm that amino acids 41-68 of the saposin C peptide activate GCase (S Weiler et al., 1995). We attached a cell penetrating peptide sequence to the activating region of saposin C (Lindgren, Hällbrink, Prochiantz, & Langel, 2000). However, we quickly noticed that while activating GCase in extracted lysosomes was easy, getting the peptide into the cell in sufficient quantities was very difficult. We decided that changing our approach and using an overexpressing cell line would be more feasible for our studies.

We designed a vector containing prosaposin, the propeptide of saposin C. It expresses prosaposin linked to GFP under a CMV promoter. We showed that PSAP-GFP was successfully expressed in our cell line by western blot. We also confirmed that PSAP is trafficked to the lysosome as there was correlation of GFP fluorescence and lysotracker deep red, a chemical dye that stains lysosomes. We could also show that there is an increase in GCase activity and a decrease in levels of α-synuclein (Paper 2, figure 2).

This result was very interesting, and we wanted to investigate it further. We used siRNA targeting prosaposin to evaluate whether knockdown of prosaposin causes increase in α-synuclein levels. We treated cells over a period of 10 days and found that reducing prosaposin levels has the inverse effect of prosaposin overexpression. We saw a significant increase in α-synuclein (Paper 2, figure 3). However, knockdown of prosaposin was also slightly toxic to the cells as it is an important lysosomal protein. The increase in α-synuclein may therefore be a general sign of lysosomal dysfunction (Ebrahimi-Fakhari, Wahlster, & McLean, 2012). Nonetheless, we provide further evidence that prosaposin influences α-synuclein metabolism and is therefore an interesting target in PD.

It was at this time point, that our data from paper 1 indicated that GCase activity was not related to levels of α-synuclein. We therefore, confirmed that this was also the case in our PSAP-GFP cell line. We could show that inhibition of GCase with CBE in our PSAP overexpressing cell line did not alter levels of α-synuclein. Prosaposin overexpressing cells still had significantly lower levels of α-synuclein, compared to controls.

At this point we began looking for alternative theories to explain the phenomena. One hypothesis we developed is that α-synuclein binds to lipid membranes and that the saposins can dislodge α-synuclein from these membranes making it more accessible to degradation. This was based on data that α-synuclein interacts with lipids and that lipid rafts may act as a source of α-synuclein aggregation (Alattia, Shaw, Yip, & Prive, 2007; Simons & Gruenberg, 2000; Taguchi et al., 2017). To investigate this hypothesis, we generated recombinant lipid vesicles using phosphocholine and glucosylceramide. We also generated recombinant α-synuclein and saposin C.

The artificial vesicles were incubated with α-synuclein. The vesicles were then loaded into a spin filter that would retain the vesicles and bound α-synuclein. We confirmed that α-synuclein readily binds to lipid vesicles. We then added either recombinant saposin C or control peptide to the spin filter. We show that especially saposin C can dislodge α-synuclein from lipid membranes (Article 2, figure 4).

We conclude that saposin C presents an interesting target for PD. It reduces α-synuclein levels and increases GCase activity. Additionally, it helps dislodge α-synuclein from lipid membranes, making it potentially more accessible for degradation and preventing aggregates from forming.

7 CONCLUDING REMARKS AND FUTURE PERSPECTIVES

Our overarching question for this thesis was whether modulation of GCase is a potential treatment for PD?

In study I, we show that using RNAi to decrease levels of GCase leads to an increase in levels of α-synuclein. Using CBE on the other hand does not change levels of α-synuclein. Our study is supported by a recent publication showing that administration of CBE can cause aggregation of α-synuclein without changing the soluble levels (Zunke et al., 2018). We therefore gain valuable mechanistic insight into how *GBA1* mutations predispose to PD. Our data suggests that increasing the amount of GCase trafficked to the lysosome is protective in PD.

Our data supports a variety of treatments such as administration of GCase in the central nervous system. Rocha and colleagues showed that viral injection causing GCase overexpression in rats was protective in PD models (Rocha et al., 2015). Our results especially support the use of GCase chaperones in PD patients with *GBA1* mutations. This is supported by recent animal data showing that ambroxol a molecular chaperone of GCase is protective against 6-Hydroxydopamine treatment in rats (Mishra, Chandravanshi, Trigun, & Krishnamurthy, 2018). Additionally, more and more molecular chaperones are becoming available for GCase. Ambroxol has already shown great promise as a potential treatment for PD (Bouscary et al., 2019; Sanchez-Martinez et al., 2016; Yang, Beavan, Chau, Taanman, & Schapira, 2017). Recently, a group demonstrated a slow release system for ambroxol (Enshaei et al., 2019). Another, interesting recent finding is that Arimoclomol, a heat shock protein amplifier has been shown to increase levels of GCase and GCase activity in GD models (Fog et al., 2018). Therefore, our first study supports the claim that GCase modulation can be a useful treatment for PD and indicates that chaperone therapy enabling correct folding and trafficking of GCase is a promising approach.

In Study II we present evidence that the endogenous GCase activating protein saposin C, has the ability to increase GCase activity and protein levels. Saposin C therefore not only acts as a co-factor for GCase but may also act as a molecular chaperone preventing GCase degradation. Overexpressing prosaposin leads to a decrease in α-synuclein. Therefore, increasing *PSAP* expression is most likely protective in PD for a variety of reasons. I) it increases GCase activity, II) it increases

the protein level of GCase. III) Saposin C can detach α-synuclein from biological membranes containing glucosylceramide. IV) Saposin C contains a neurotrophic and neuroprotective fragment that may be beneficial in PD. V) Saposin C is a suggested ligand for GPR37 and GPR37L1 receptors and activation of these receptors may be beneficial in PD (Meyer et al., 2014). Again, our data supports the claim that GCase modulation may be protective in PD.

One finding that was particularly surprising is that saposin C can detach α-synuclein from artificial glucosylceramide vesicles. Additionally, it can break interaction between GCase and α-synuclein (Yap et al., 2013, 2015). So while Saposin C may act directly on GCase to produce a protective effect, it also acts directly on α-synuclein.

To conclude we present data suggesting that GCase levels rather than GCase activity are important for preventing PD. Furthermore, we investigated saposin C and highlight its potential as a target in PD. We hope that this work can contribute to the ever-growing literature on PD, and that work on saposin C as a potential clinically relevant protein for PD can continue.

While we have provided some promising data. Much work remains to be done. The exact effect of saposin C in PD needs to be characterized with great care. The main aim of future studies should be to prove a protective effect of saposin C overexpression in clinically relevant models such as dopaminergic neurons differentiated from iPSC's of patients with GD and PD. Exploring the effect of saposin C overexpression in animal PD models using viral constructs is also desirable. A particularly relevant models would be that of Luk and co-workers. Aggregation of α-synuclein seems to be a primary mechanism for developing PD. Injecting mice with pre-formed fibrillar α-synuclein can induce a pathology strikingly similar to PD (Luk et al., 2012). A decrease in α-synuclein due to *PSAP* overexpression logically dictates a decrease in aggregation speed of α-synuclein. We therefore hypothesize that over-expression of PSAP in this mouse model should have a protective effect.

Furthermore, we need to unambiguously show that it is saposin C, and not other saposins, or full length prosaposin, which are responsible for the decrease in α-synuclein. As saposin C is cleaved from a precursor peptide in the lysosome, it is

difficult to pinpoint a specific effect to saposin C and exclude the action of the other saposins.

Finally, we would like to investigate approaches that could be used to increase prosaposin, and therefore saposin C levels in PD patients. Prosaposin is trafficked to the lysosome by interaction of its c-terminal region with the sortillin receptor (Carvelli, Libin, & Morales, 2015). However, prosaposin can also be secreted into the extracellular space. It is known that the 65 kDa form of prosaposin is secreted, while the 68-70kDA PSAP is targeted to the lysosome. The mechanism gating this trafficking is unknown (Carvelli et al., 2015). Knowing more about the trafficking of PSAP may aid in developing strategies that increase intralysosomal saposin C levels. Finally, our original idea was to generate small synthetic peptides that could be used for treatment of PD. However, due to the complexity of the targeting involved we were unable to do so. Therefore, the biology of prosaposin is still not understood well enough to effectively use this protein as a potential treatment. We hope by showing that it has a possible protective effects in PD, we will encourage research to further explore this promising pathway.

Finally, we wish to remark that PD is a devastating neurodegenerative disorder. Patients afflicted with this disease gradually lose the ability to perform voluntary movement. Therefore, these patients often lose their independence and the ability to continue living a normal life in the home they have known for decades. Many PD patients also face cognitive decline, and depression is one of the most frequent co-morbidities in PD. As scientists we must not forget our humanity and let it guide our efforts. Hopefully, with the combined skill and patience of thousands of dedicated researchers, we are inching closer towards the truth, allowing us to prevent or at least slow this devastating disease.

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