From Department of Clinical Neuroscience
Karolinska Institutet, Stockholm, Sweden

TRANSLATIONAL STUDIES OF GLUCOCEREBROSIDASE IN PARKINSON'S DISEASE

Rika Kojima

Karolinska Institutet

Stockholm 2024

Published by Karolinska Institutet.
Printed by Universitetsservice US-AB, 2024

ISBN 978-91-8017-301-8
Cover illustration: Immunostaining of iPSC-derived midbrain dopaminergic neurons expressing tyrosine hydroxylase (purple) and microtubule-associated protein 2 (green).

TRANSLATIONAL STUDIES OF GLUCOCEREBROSIDASE IN PARKINSON'S DISEASE

Thesis for Doctoral Degree (Ph.D.)

By

Rika Kojima

The thesis will be defended in public at J3:14 Kerstin Hagenfeldt, Bioclinicum, Solna, Friday 19th of April at 9:00 a.m.

Principal Supervisor:
Xiaoqun Zhang
Karolinska Institutet
Department of Clinical Neuroscience

Co-supervisor(s):
Per Svenningsson
Karolinska Institutet
Department of Clinical Neuroscience

Ernest Arenas
Karolinska Institutet
Department of Medical Biochemistry and Biophysics

Opponent:
Agnete Kirkeby
University of Copenhagen
Department of Neuroscience

Examination Board:
Per Nilsson
Karolinska Institutet
Department of Neurobiology, Care Sciences and Society

Sophia Schedin Weiss
Karolinska Institutet
Department of Neurobiology, Care Sciences and Society

John Löfblom
KTH Royal Institute of Technology
Department of Protein Science
Division of Protein Engineering

POPULAR SCIENCE SUMMARY OF THE THESIS

Parkinson's disease is a movement disorder common in elderly people, typically over 65 years old. Patients often experience uncontrollable shaking and difficulty in moving their bodies as they intend. In addition to movement inabilities, depression, anxiety, and sleeping problems are also common. Parkinson's disease is caused by a loss of special brain cells. However, the reason why the brain cells die is unknown. A clue for understanding this mystery cell death could be a protein called α-synuclein. α-synuclein is a small protein piling up in the brain of Parkinson's disease patients. The function of α-synuclein is unclear, but it becomes toxic when many α-synuclein gather and form clumps. Therefore, accumulation of α-synuclein is considered associated with the cause of Parkinson's disease. Unfortunately, there is still no cure for Parkinson's disease so far, and only treatments relieving symptoms are available.

Most Parkinson's diseases occur for no specific reason. However, some patients develop Parkinson's disease because they have specific mutations in their genes. There are a lot of gene mutations known to increase the risk of getting Parkinson's disease. One of these risk genes is GBA1. GBA1 gene makes a protein called glucocerebrosidase (GCase). GCase is a protein that breaks lipids, essential components of cells acting as building blocks. A mutation in the GBA1 gene increases the risk of Parkinson's disease by 5-10 folds, but the mechanisms behind this are still unknown.

These days, many researchers take advantage of Induced pluripotent stem cells (iPSCs) to study Parkinson's disease. iPSCs are a sort of cells that have the ability to become any cell type. iPSCs are useful for studying diseases because we can make specific types of cells from them that are difficult to get. For example, brain cells can be generated from iPSCs to study the cause of cell death in Parkinson's disease. iPSCs are also beneficial as they may offer an alternative treatment for many diseases. The brain cells made from iPSCs have the potential to replace the lost cells in the brain of Parkinson's disease patients, so they are being tested in clinical trials with actual patients.

In an aging society, the number of Parkinson's disease patients is growing. Therefore, there has been an urgent need for finding a cure or better treatment options for Parkinson's disease. This thesis aims to promote Parkinson's disease research focusing on the link between GCase and Parkinson's disease.

In Paper I, we examine the liquid surrounding the brain of Parkinson's disease patients who have a GBA1 mutation. The brain liquid contains various proteins, and some contents may reflect the state of the brain. We find some of the proteins in the liquid are distinct to Parkinson's disease with GBA1 mutation. Then, we use iPSCs made from a Parkinson's disease patient to make brain cells. We analyze the brain cells and compare the results with the brain liquid analysis to find the proteins possibly related to the death of brain cells. Finally, we discover five distinct proteins common in the brain liquid and the

brain cells made from iPSCs. These proteins may play a key role in the brain cell death of Parkinson's disease.

In Paper II, we use the brain cells made from the patient's iPSCs to study α-synuclein and lipids in the cells. We find that the brain cells with GBA1 mutation release more α-synuclein to the outside of the cells. GBA1 mutation does not change most lipids in the brain cells, but we identified that one lipid called α-2,3SpG is decreased in the brain cells with GBA1 mutation. The connection between the increased release of α-synuclein and the decreased lipid may be clarified in future studies.

In Paper III, we study a protein called prosaposin to explore its function on α-synuclein accumulation. Prosaposin consists of four small proteins, called saposins A, B, C, and D. Among them, saposin C helps GCase to break lipids down. Also, prosaposin is shown to protect brain cells from damage and stress. To examine if prosaposin is helpful for Parkinson's disease, we increase the amount of prosaposin produced in the cells. We find that α-synuclein is decreased in the cells with more prosaposin. On the contrary, when we reduce the amount of prosaposin, α-synuclein accumulates. Finally, we find that saposin C can remove α-synuclein from lipids. We believe the function of saposin C to separate α-synuclein from lipids is important to prevent α-synuclein from accumulation.

In conclusion, this thesis provides new insights about GCase and Parkinson's disease, which will help understand the cause of Parkinson's disease and how the GBA1 gene is involved. We believe our studies contribute to developing new treatments for Parkinson's disease in the future.

ABSTRACT

A number of genetic variants have been linked to Parkinson's disease (PD). Among these, mutations in the *GBA1* gene are identified as one of the most common risk factors for developing PD. The connection between increased PD risk and individuals with *GBA1* mutations was first recognized in the 1990s, but even today, the mechanism remains unclear. *GBA1* gene encodes glucocerebrosidase (GCase), a lysosomal hydrolase degrading glucosylceramide (GlcCer). The enzyme activity of GCase is reduced in PD patients, particularly among *GBA1* mutation carriers. However, compromised GCase activity alone does not lead to disease development, adding complexity to its contribution to the greater risk of PD. Therefore, this thesis aims to untangle the intricate connection between *GBA1* variants and PD pathogenesis.

In this thesis, we take advantage of patient-derived induced pluripotent stem cells (iPSCs) as a tool for exploring the role of the *GBA1* mutations in PD pathogenesis. Firstly, we develop an optimized protocol for efficiently generating midbrain dopaminergic (mDA) neurons from iPSCs. The established protocol is validated to produce mDA neurons with high reproducibility across several iPS cell lines.

Using GBA1-PD patient-derived iPSCs as a reference for human samples, we investigate GBA1-specific secretome alterations in the cerebrospinal fluid (CSF) of GBA1-PD patients. The comparison of CSF and iPSC-derived mDA neurons allows us to excerpt the CSF proteins attributed to mDA neuronal populations. Notably, the most significantly altered protein in the CSF of GBA1-PD patients, FKBP4, is upregulated in the GBA1-PD mDA neurons. Our identification of FKBP4, a member of the immunophilin protein family, suggests an involvement of immune systems in GBA1-associated PD.

Glycosphingolipids (GSLs) are brain-enriched lipids metabolized by lysosomal glycosidases, including GCase. Herein, we report that GBA1-PD mDA neurons exhibit decreased levels of α-2,3SpG, a neolacto-series GSL, compared with its isogenic control neurons. Also, we show a 1.39-fold elevation of α-synuclein release in GBA1-PD mDA neurons. These results highlight the alterations in GSL and α-synuclein secretion specific in GBA1-PD mDA neurons.

Prosaposin (PSAP) is a precursor protein of saposin C, an essential activator of GCase. To investigate the potential of PSAP/saposin C as a therapeutic target for PD, we employ gene overexpression systems and evaluate the role of PSAP in α-synuclein pathology. Human neuroblastoma SH-SY5Y cells stably overexpressing PSAP display enhanced GCase activities with a concomitant decrease in intracellular/extracellular α-synuclein levels. small interfering RNA-mediated knockdown of endogenous PSAP shows the opposite effect on α-synuclein levels, indicating the involvement of PSAP in α-synuclein regulation. Furthermore, we demonstrate that saposin C detaches α-synuclein from an artificial lipid bilayer membrane containing GlcCer, leading us to postulate that

observed α-synuclein regulation of PSAP might be attributed to saposin C's ability to interfere with α-synuclein-to-lipid membrane interaction.

To conclude, this thesis contributes to elucidating the missing link in our understanding of the molecular mechanisms underlying the elevated risk of PD among *GBA1* mutation carriers, providing valuable insights into the pathogenesis of GBA1-associated PD.

LIST OF SCIENTIFIC PAPERS

I. **Kojima R**, Paslawski W, Lyu G, Arenas E, Zhang X, Svenningsson P. Secretome Analyses Identify FKBP4 as a GBA1-Associated Protein in CSF and iPS Cells from Parkinson's Disease Patients with GBA1 Mutations. *Int J Mol Sci.* 2024 Jan 4;25(1):683.

II. **Kojima R**, Wallom KL, Lyu G, Paslawski W, Zhang X, Arenas E, Platt F, Svenningsson P.
Altered sialyl(α2-3)paragloboside levels in *GBA1* N409S (N370S) Parkinson's disease iPSC-derived midbrain dopaminergic neurons. Manuscript.

III. **Kojima R**, Zurbruegg M, Li T, Paslawski W, Zhang X, Svenningsson P. Prosaposin Reduces α-Synuclein in Cells and Saposin C Dislodges it from Glucosylceramide-enriched Lipid Membranes. *J Mol Neurosci.* 2022 Nov;72(11):2313-2325.

CONTENTS

LIST OF ABBREVIATIONS

AA	Ascorbic acid
AADC	Aromatic amino acid decarboxylase
AD	Alzheimer's disease
AJ	Ashkenazi Jewish
ALDH1A1	Aldehyde dehydrogenase 1 family A1
AMP-dNM	N-(5-adamantane-1-yl-methoxy-pentyl)-Deoxynojirimycin
ANOVA	Analysis of variance
AP	Anterior-posterior
BBB	Blood-brain barrier
BDNF	Brain-derived neurotrophic factor
BiP/GRP78	Binding immunoglobulin protein/Glucose-regulated protein 78
BMP	Bone morphogenetic protein
CALB	Calbindin
CBD	Corticobasal degeneration
CBE	Conduritol B epoxide
COMT	Catechol-O-methyltransferase
CSF	Cerebrospinal fluid
DA	Dopamine
dbcAMP	Dibutyryl cyclic adenosine monophosphate
DBS	Deep-brain stimulation
DDC	Dopa decarboxylase
DMSO	Dimethyl sulfoxide
DOPA	L-3,4-dihydroxyphenylalanine
DOPAL	3,4-dihydroxyphenylacetaldehyde
DV	Dorsal-ventral
ECM	Extracellular matrix
EN1	Engrailed 1
ER	Endoplasmic reticulum
ERT	Enzyme replacement therapy

FDR	False discovery rate
FGFs	Fibroblast growth factors
FP	Floor plate
GCase	Glucocerebrosidase
GD	Gaucher disease
GDNF	Grial cell line-derived neurotrophic factor
GIRK2	G-protein-regulated inward-rectifier potassium channel 2
GlcCer	Glucosylceramide
GlcSph	Glucosylsphingosine
GPR37	G protein-coupled receptor 37
GPR37L1	G protein-coupled receptor 37 like 1
GSLs	Glycosphingolipids
GWAS	Genome-wide association study
hESCs	Human embryonic stem cells
hiPSCs	Human induced pluripotent stem cells
HY	Hoehn and Yahr scale
iPD	Idiopathic Parkinson's disease
LEDD	L-dopa equivalent doses
LXR	Liver X receptor
MAOA/B	Monoamine oxidase type A/B
MAPS	Misfolding-associated protein secretion
mDA	Midbrain dopaminergic
mFP	Midbrain floor plate
MHB	Midbrain-hindbrain boundary
MoCA	Montreal cognitive assessment
MPTP	1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine
mRNA	Messenger ribonucleic acid
MSA	Multiple system atrophy
MVB	Multivesicular body
NAC	Non-amyloid-component

NM	Neuromelanin
NMDA	N-methyl-D-aspartate
NP-HPLC	Normal-phase high-performance liquid chromatography
PAGE	Polyacrylamide gel electrophoresis
PD	Parkinson's disease
PEA	Proximity extension assay
PFA	Paraformaldehyde
PFB-FDGlu	5-(Pentafluorobenzoylamino)Fluorescein Di-β-D-glucopyranoside
PGRN	Progranulin
PLO	Poly-L-ornithine
PSAP	Prosaposin
PSP	Progressive supranuclear palsy
pS129	Phosphorylation at Serine 129
PTMs	Post-translational modifications
PVDF	Polyvinylidene difluoride
qPCR	Quantitative polymerase chain reaction
ROS	Reactive oxygen species
ROUT	Robust regression and outlier removal
RP	Roof plate
RRF	Retrorubal field
SAA	Seed amplification assay
SDI	Socio-demographic Index
SDS	Sodium dodecyl sulfate
SEM	Standard error of the mean
SHH	Sonic hedgehog
siRNA	Small interfering ribonucleic acid
SNARE	Soluble N-ethylmaleimide attachment protein receptor
SRT	Substrate reduction therapy
STN	Subthalamic nucleus
SV	Synaptic vesicle

TBS-T	Tris-buffered saline with 0.1% Tween 20
TGFβ	Transforming growth factor β
UPDRS	Unified Parkinson's disease rating scale
UPR	Unfolded protein response
VM	Ventral midbrain
VTA	Ventral tegmental area
α-2,3SpG	Sialyl(α2-3)paragloboside
2-AA	Anthranilic acid
4-MU	4-methylumbelliferone
4-MUG	4-methylumbelliferyl β-D-glucopyranoside
6-OHDA	6-hydroxydopamine

INTRODUCTION

In an aging society, the increasing prevalence of age-related disorders, including Parkinson's disease (PD), has been a global issue to be addressed. The urgent need for establishing preventive measures and cures for those diseases has emerged. PD is a neurodegenerative disorder characterized by motor dysfunctions accompanied by a variety of non-motor symptoms, which result from the progressive loss of dopaminergic neurons. Several pathophysiological features, such as the accumulation of aggregated α-synuclein, have been associated with the cause of neuronal death. Yet, the definite molecular mechanisms of PD pathogenesis are undisclosed.

Mutations in the *GBA1* gene are one of the most common risk factors for PD. However, the vast majority of *GBA1* mutation carriers do not develop PD throughout their life, indicating the mutation alone is not sufficient to induce PD. The *GBA1* gene encodes a lysosomal enzyme, glucocerebrosidase (GCase), and compromised GCase function is considered to aggravate pre-existing PD-related risk factors, predisposing to disease development. GCase requires a co-factor, saposin C, for its activation. Saposin C enhances GCase activity by facilitating GCase-substrate binding. Saposin C and its precursor protein, prosaposin (PSAP), have been deemed a candidate disease modifier for PD, while their therapeutic potential has not been fully explored yet.

GCase is responsible for the degradation of glycosphingolipids (GSLs), typically glucosylceramide (GlcCer). GSLs, particularly gangliosides, are enriched in the brain, playing a vital role in neuronal function and development. Recently, lipid alteration and its association with GCase deficiency have been arising as one of the mechanisms underlying PD pathogenesis. Several GSLs are known to interact with α-synuclein and are considered to modify their aggregation tendency. The association between GlcCer accumulation caused by impaired GCase and augmented α-synuclein pathology has been suggested as a mechanism underlying GBA1-PD. However, its comprehensive molecular machinery has not been established yet.

Induced pluripotent stem cells (iPSCs) have been employed in PD research. iPSCs not limited to serving as in vitro cellular models alone but providing alternative therapeutic potentials. Clinical trials for transplantation of iPSC-derived midbrain dopaminergic (mDA) neurons are underway. With the growing demand for obtaining pure mDA neurons for transplantation, the technologies to make mDA neurons from stem cells have progressed dramatically. A better understanding of human brain development allows further protocol refinement, facilitating PD research as well as cell therapy for PD.

Hence, to elucidate the molecular pathogenesis of PD, a profound understanding of the pathophysiological role of GBA1 in PD is crucial. Untangling the complex GBA1-centered interplay will contribute to shedding light on the molecular mechanisms of PD, providing new therapeutic prospects for PD.

1 LITERATURE REVIEW

1.1. Parkinson's disease

Parkinson's disease (PD) is the second most common neurodegenerative disorder after Alzheimer's disease (AD), affecting 6.1 million people over the world in 2016[1]. PD is a progressive disease characterized by motor symptoms, including resting tremors, rigidity (muscle stiffness), bradykinesia (slowed movements), and postural instability caused by the loss of dopaminergic neurons in the substantia nigra[2]. Currently, the standard therapeutic options for PD are limited to symptomatic treatments that alleviate motor symptoms by modifying deteriorated dopaminergic functions. The increasing trends in the incidence and prevalence of PD have been observed globally due to the growing aged population[3]. The economic burden of PD is estimated to surge concomitantly, emphasizing the urgent need to develop treatments that prevent or cure PD[4].

1.1.1. Epidemiology

PD is a late-onset disease that has an average onset at 65-70 years old with a peak prevalence between 85-89 years of age[1,5]. The prevalence of PD is higher in males than in females, which might be associated with a protective effect of estrogen and environmental factors such as sex differences in occupations and work-related chronic stress[5,6]. Worldwide studies revealed that there were geological differences in PD incidence, prevalence rates, and trends, possibly due to regional divergence in the Socio-demographic Index (SDI), lifestyles, environmental pollution, and so on[1,3]. Generally, there is a positive association between SDI and the burden of PD, indicating that environmental factors play a significant role in disease development[1].

1.1.2. Diagnosis

Diagnosis of PD is based on clinical criteria assessed by clinical examinations. The clinical criteria of PD consist of three cardinal features: Bradykinesia, rigidity, and rest tremor[7,8]. Various rating scales are used for the evaluation of the motor impairment and disability of PD. The most widely used and established scales are the Hoehn and Yahr scale (HY)[9] and the Unified Parkinson's Disease Rating Scale (UPDRS)[10]. The clinical criteria of PD also incorporate non-motor features of PD, such as sleep disturbances, autonomic dysfunction (constipation, daytime urinary urgency), loss of smell (hyposmia), and psychiatric dysfunction (depression, anxiety, or hallucinations)[7]. Due to the lack of a definitive diagnosis during life, the clinical diagnosis of PD is still challenging, particularly in the early stages of the disease[11,12]. Atypical parkinsonian syndromes, including progressive supranuclear palsy (PSP), multiple system atrophy (MSA), and corticobasal degeneration (CBD), share many key clinical features with PD, which makes it difficult to clinically distinguish them from each other[13]. Particularly, patients misdiagnosed with PD

are often retrospectively diagnosed with PSP or MSA by biopsy[14]. Therefore, to improve the accuracy of diagnosis, reliable biomarkers that substantiate the clinical diagnosis of PD and differentiate from other Parkinsonian disorders need to be established.

1.1.3. Symptoms and Treatments

1.1.3.1. Motor symptoms

In addition to major motor symptoms (bradykinesia, rigidity, rest tremor), several minor motor abnormalities are present among PD patients. Primitive reflexes, difficulties in speech (dysarthria) and swallowing (dysphagia), and abnormality in eye movements (saccades) are commonly observed in PD, which are thought to be derived from impaired dopaminergic function[11]. Motor symptoms are ameliorated by pharmacological treatment restoring dopamine (DA) deficiency including levodopa and DA agonists[15]. Administration of levodopa, the precursor of DA, has been the gold standard for the treatment of motor symptoms. Levodopa is often prescribed combined with other drugs which enhance the effect of levodopa[2,16,17]. Inhibitors of aromatic amino acid decarboxylase (AADC), and catechol-*O*-methyltransferase (COMT) improve the bioavailability of levodopa by preventing peripheral DA metabolisms[18]. Monoamine oxidase type B (MAOB) inhibitors increase the concentration of DA in the synapse by preventing DA degradation[19]. DA agonists, directly stimulating DA receptors, serve as levodopa substitutions[20]. Over time, response towards DA medication decreases, and a higher dosage and frequency are required to achieve a sufficient therapeutic effect. Concomitant levodopa-induced dyskinesia (involuntary movement) and severe wearing-off (motor fluctuations) are the main issues arising from long-term levodopa treatment[21]. To resolve the problem, non-dopaminergic therapeutics have been under development. The only established non-dopaminergic drug is Amantadine, a non-competitive N-methyl-D-aspartate (NMDA) receptor antagonist, which has been clinically used for the treatment of levodopa-induced dyskinesia[22]. For advanced PD patients experiencing levodopa-induced motor complications, which are uncontrollable with oral pharmacological treatment, deep-brain stimulation (DBS) is an option to manage the symptoms. DBS is a neurosurgical therapy that implants electrodes in the brain region responsible for body movements, such as the subthalamic nucleus and globus pallidus internal segment[23]. To conclude, motor symptoms of PD are managed by a combination of treatment options although motor complications in advanced PD are still challenging[24]. A better understanding of dopaminergic systems is crucial to overcoming the drawbacks of current levodopa-centered therapy.

1.1.3.2. Non-motor symptoms

Non-motor symptoms often appear preceding motor symptoms[25,25]. Some PD patients experience problems with sleep[26], constipation[27], depression and anxiety[28], and loss of

smell (hyposmia)[29] years before being diagnosed with PD. In parallel with the motor symptoms, the non-motor features continue to develop throughout the disease progression, and they become prominent in the late stages of the disease[30]. The pathophysiological mechanisms of the non-motor symptoms are still mostly unknown. Although it has been suggested that dopaminergic pathways underlie the development of non-motor symptoms, dopaminergic medications have poor effects on the majority of non-motor features[25]. Even worse, some dopaminergic therapeutics induce non-motor side effects, including hallucinations and insomnia[31]. Therefore, combined with dopaminergic medications for motor symptoms, symptomatic therapy is used for the treatment of non-motor dysfunctions. The impact of non-motor features on the patient's quality of life is not negligible, effective treatment for non-motor symptoms thus needs to be explored.

1.1.4. Pathophysiology

The progressive loss of midbrain dopaminergic neurons in the substantia nigra and the accumulation of α-synuclein-consisting inclusions called Lewy bodies in the affected brain region are the two main pathophysiology of PD[32]. The impaired DA function due to DA deficiency is the primary cause of the cardinal motor symptoms. Despite the long history of Parkinson's disease research, which was first described two hundred years ago[33], the mechanisms of selective cell death in the substantia nigra have not been fully understood. The principal theory that accounts for the pathophysiology of PD is the abnormal accumulation of neurotoxic α-synuclein[34]. Due to several factors including post-translational modifications (PTMs), enrichment in α-synuclein levels, and interaction with lipid membranes, physiological α-synuclein is misfolded and transformed into pathological states. When the formation of aggregated α-synuclein overwhelms the capacity of clearance, pathological α-synuclein aggregation is piled up and spread across brains, leading to degeneration in specific cell types. Abnormal deposits of α-synuclein are observed in several neurodegenerative disorders other than PD, including dementia with Lewy bodies and multiple system atrophy. Those diseases are defined as synucleinopathy[35,36]. There are numerous theories demonstrating the α-synuclein-linked pathophysiology of PD, which will be described in the following sections.

1.1.5. α-synuclein

α-synuclein is a natively unfolded, soluble monomeric protein abundant in presynaptic nerve terminals[37,38]. A recent study revealed that endogenous α-synuclein also exists as a helically folded tetramer of about 58 kDa[39]. The α-synuclein protein consists of three regions: amphipathic N-terminal region (residues 1-60), hydrophobic non-amyloid-component (NAC) region (residues 61-95), and acidic C-terminal region (residues 96-140)[40]. The lysine-rich N-terminal region, containing repeated apolipoprotein lipid-binding

motifs, is a lipid and membrane binding domain that anchors α-synuclein to membranes[41]. The central NAC region serves as a membrane sensor that determines the membrane binding affinity by transiently binding to membranes[41]. The NAC region is also recognized as the most aggregation-prone region which has the potential to form β-sheet structures[42]. The negatively charged C-terminal region is responsible for Ca^{2+} binding, which is involved in the regulation of membrane binding[43,44].

1.1.5.1. Putative functions of α-synuclein

The physiological roles of α-synuclein remain largely unknown. α-synuclein is associated with various functions, including synaptic plasticity and neurotransmitter release by modulating the assembly of the Soluble N-ethylmaleimide attachment protein receptor (SNARE) complex[45,46]. The genetic studies, using α-synuclein overexpression models, as well as α-, β-, and γ-synuclein triple knockout models, demonstrated the roles of synucleins in a synaptic vesicle (SV) endocytosis[47,48]. Dopaminergic neurons are the neuronal population associated with a high rate of neurotransmitter release and the consequent endocytosis. Given the role of α-synuclein in SV release and trafficking, it is rational to postulate that α-synuclein is involved in synaptic dysfunction in dopaminergic neurons, contributing to the degeneration of dopaminergic neurons[40].

1.1.5.2. Braak hypothesis

α-synuclein is the main component of Lewy bodies, a hallmark of PD and other neurological disorders[49,50]. Lewy bodies are intraneuronal inclusions named after Fritz Heinrich Lewy, who described the presence of Lewy bodies in the brains of PD patients for the first time in 1912[51]. Whether Lewy bodies themselves are deleterious or result from detoxification of pathological α-synuclein aggregation is still inconclusive[52]; while a recent study revealed the process of Lewy body formation and its association with neurodegeneration, emphasizing a detrimental role of Lewy bodies[53]. Not all PD patients exhibit Lewy pathology. For example, Lewy bodies are absent in some rare familial PD cases, such as carriers of *PARK2* and *LRRK2* gene mutations, despite the significant loss of dopaminergic cells[54]. Lewy bodies and α-synuclein fibrils are shown to propagate pathogenic α-synuclein by converting soluble α-synuclein into insoluble forms[55,56]. Earlier, Braak *et al.* described that Lewy body formation starts in the presymptomatic stage, from the enteric nervous system, and the α-synuclein inclusion bodies spread throughout the brain via the vagus nerve as the disease progresses[57]. Accumulating evidence supports the gut-to-brain hypothesis[58,59], which will uncover the potential of the peripheral nervous systems and the gut microbiome as a novel therapeutic and diagnostic target for PD[60].

1.1.5.3. Post-translational modifications

Several mechanisms are proposed for the transformation of physiological α-synuclein (monomer or tetramer) into pathological states (oligomers, fibrils, and Lewy bodies). A variety of PTMs of α-synuclein is observed in diseased human brains and considered pathological. Those include phosphorylation[61], ubiquitination[62], nitration[63], SUMOylation[64], and truncation[65]. Phosphorylation at Serine 129 (pS129) is a particularly well-studied PTM due to its association with Lewy pathology. It was reported that more than 90% of α-synuclein found in Lewy bodies are phosphorylated at the residue[61,66]. Despite the extensive research, the physiological function of pS129 on α-synuclein is still under debate[67]. Some PTMs have been shown to modulate the aggregation tendency, toxicity, and clearance of α-synuclein[68]. For example, Ubiquitination of α-synuclein induces fibril formation in a site-specific manner[69]. C-terminal truncated α-synuclein is considered to increase aggregations and promote prion-like propagation[70]. As PTMs are consequences of environmental stimuli, studying α-synuclein PTMs will help understand the contribution of environmental factors in the pathogenesis of PD[71].

1.1.5.4. Autophagy-lysosomal defects

Defects in the autophagy-lysosomal pathways have been associated with the α-synuclein pathology. Since α-synuclein is partly degraded via autophagy[72–74], impaired autophagy function leads to deteriorated clearance of α-synuclein. Conversely, α-synuclein itself is shown to affect autophagy-lysosomal pathways and mitochondrial functions[73,75,76]. A pathologic bidirectional loop of impaired GCase and α-synuclein accumulation in lysosome was proposed, suggesting the significance of lysosomal functions in GBA1-associated PD[77].

Accumulating evidence emphasizes the contribution of autophagy-lysosomal dysfunction to the propagation of pathologic α-synuclein[78–80]. It is demonstrated that misfolded α-synuclein is secreted via exosomes, and the secretion of α-synuclein is affected by autophagic activity[79,81]. Moreover, exosome-associated α-synuclein is shown as more toxic than exosome-free α-synuclein, indicating the "Trojan horse" function of exosomes propagating α-synuclein pathology[79,82]. On the other hand, lysosome-mediated α-synuclein secretion can be considered a protective response of affected dopaminergic neurons to lower intracellular levels of α-synuclein[83,84]. Fussi *et al.* suggested that exosomal α-synuclein secretion can be a salvage pathway to dispose of excess α-synuclein when autophagy is impaired[84]. They demonstrated that a failure of α-synuclein secretion due to blockage of exosome formation triggers α-synuclein-induced cell death under autophagy inhibition[84]. All in all, autophagy-lysosomal pathways play a pivotal role in α-synuclein regulation, expected to be a potential therapeutic target for PD.

1.1.5.5. Oxidative stress

Dopaminergic neurons, the most affected cell type in PD, are closely linked with oxidative stress. Oxidative stress arises from an imbalance between oxidants and antioxidants when the production of reactive species surpasses the neutralizing capacity of antioxidants. Reactive species are atoms or molecules with unpaired electrons, which makes them unstable and, therefore, "reactive" to other molecules. To become stable, the reactive species attack other compounds and take electrons away, triggering a chain of reaction, which eventually damages the cells[85]. Reactive species, such as reactive oxygen species (ROS) and free radicals, are constantly generated during normal cellular metabolism. While overproduction of ROS induces oxidative stress, low or moderate concentrations of ROS have beneficial effects on various cellular functions, including the maintenance of redox homeostasis[86].

The greatest source of intracellular ROS is mitochondria, and the ROS production is in proportion to the metabolic rate[87]. The nature of dopaminergic neurons demands high energy to sustain their intense neuronal activity, considered to make them particularly vulnerable to mitochondrial dysfunction and oxidative stress[88]. Besides, DA itself is oxidized and yields reactive metabolites, including 3,4-dihydroxyphenylacetaldehyde (DOPAL) and DA quinones, possibly contributing to the selective neurodegeneration in PD[89,90]. Indeed, dysregulated mitochondria and increased oxidative stress are observed in PD brains[91,92]. The link between oxidative stress and the molecular pathogenesis of PD is further supported by the observation that toxins inducing oxidative stress cause PD-like motor phenotypes. Administration of those toxins, including 6-hydroxydopamine (6-OHDA), 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine (MPTP), rotenone, and paraquat, is an established method to induce experimental PD models[93]. The potential of antioxidants as therapeutics for PD has been explored. Yet, none have become successful although several antioxidant drugs have been tested in clinical trials[94]. To overcome the obstacles, improvement in antioxidant stability, bioavailability, and delivery to the brain is crucial.

1.1.5.6. α-synuclein association with lipid membranes

α-synuclein exists in at least two physiological states: a membrane-bound form or a membrane-free disordered form[95]. It has been demonstrated that membrane-bound α-synuclein undergoes a conformational change upon membrane binding, transforming into amphipathic α-helices[96,97]. α-synuclein binds to lipid membranes through its amphipathic N-terminal region. Specifically, residues 6-25 bind to the membrane with high affinity regardless of the lipid composition whereas residues 26-97 work as sensors for determining lipid-specific interactions[41]. Interestingly, all the *SNCA* mutations causing familial PD (A30P, E46K, H50Q, G51D, A53T, and A53E) are found in the sensor domain, indicating the pathological significance of membrane binding[98,99]. Yet, the effect of mutations on the membrane binding is an open question. A53T mutation of α-synuclein

has been shown to reduce membrane binding[100], while the other group found no effect on membrane binding or α-helicity[101]. A recent study examining the impact of all the familial mutations on lipid binding indicated that A30P markedly reduced binding to lipid membranes[102]. The reduced membrane interaction in some of the mutant α-synuclein suggests a protective role of membrane binding. Loss of membrane-bound α-synuclein could indicate a compromised physiological function of α-synuclein and an increased deposit of membrane-free α-synuclein susceptible to aggregation formation[102]. On the other hand, pieces of evidence suggest that α-synuclein-membrane binding is detrimental. It was reported that membrane-bound α-synuclein is prone to aggregate and serves as an aggregation seed[95]. Besides, α-synuclein oligomers are shown to exert neuronal toxicity by interacting with the cellular membrane and disrupting the membrane integrity[103,104]. It has been demonstrated that many other factors, including lipid compositions and the ratio of lipid-to-α-synuclein, influence the affinity of α-synuclein to the membrane and the propensity of aggregation[41,99,104,105]. Although it is challenging to assess the overall effect of the interactions of different factors on α-synuclein behavior *in vivo*, a comprehensive investigation is necessary to elucidate the role of α-synuclein binding to the membrane.

1.1.5.7. α-synuclein secretion

α-synuclein is originally a cytosolic protein, whereas the presence of extracellular α-synuclein has been confirmed in various human bodily fluids and *in vitro* cell culture media[106,107]. The potential of extracellular α-synuclein as a biomarker for synucleinopathies has been explored, yet not established as it is commonly detected in unaffected individuals as well[108]. Recently, the detection of aggregated α-synuclein in cerebrospinal fluid (CSF) with a seed amplification assay (SAA) has drawn attention as a promising diagnostic tool for PD[109]. This finding indicates that extracellular α-synuclein aggregations may represent the progress and development of the disease.

The physiological roles and mechanisms of α-synuclein secretion have been mostly unrevealed. Due to the lack of known signal peptide sequences, α-synuclein is most probably secreted through the endoplasmic reticulum (ER)/Goldi-independent unconventional pathways[110]. To date, multiple pathways have been proposed involving α-synuclein secretion. One such pathway can be multivesicular body (MVB)-mediated unconventional exocytosis. Cytosolic α-synuclein that are either uptaken by neighboring cells or produced within the cell, would be translocated to endosomes[111]. The α-synuclein-contained endosome would be transferred to MVBs, and either degraded by lysosome or released into the extracellular space via exocytosis, possibly mediated by Rab11a[112,113], or via exosomes[79,114]. It is known that α-synuclein is partially secreted through exosome release, and the association of exosomes in transporting α-synuclein toxicity has been indicated[79]. However, as only a small portion of extracellular α-synuclein is secreted via

exosome[79,81,115], exosome-mediated α-synuclein secretion is suggested subsidiary. Some other unconventional α-synuclein secretion pathways have been proposed, involving ER/Goldi-independent exocytosis[116], passive diffusion[117], and misfolding-associated protein secretion (MAPS)[118]. Also, the free extracellular α-synuclein could be alternatively derived from dead cells, or secreted exosomes degraded by extracellular lipase or protease[119].

1.1.6 Etiology

The majority of PD cases are sporadic, and only about 10% account for genetically linked PD[120]. Several gene mutations have been identified as causative for PD. The earliest discovery of familial PD was a missense mutation in the *SNCA* gene, encoding α-synuclein, in 1997[121]. Since then, the connection between PD and α-synuclein has been intensively investigated. Later, duplication and triplication of the *SNCA* gene were also reported, confirming the pathological connection of α-synuclein in PD[122,123]. To date, more than 20 genes are linked to familial forms of PD, including *LRRK2*, *DJ-1*, *Parkin*, and *PINK1*[124,125]. Monogenetic PD often exhibits different clinical presentations and pathology than sporadic PD. For example, *SNCA*, *LRRK2*, *Parkin*, *DJ-1*, and *PINK1* PD are associated with early-onset PD[126]. Interestingly, LRRK2 G6055A (G2019S) mutation is common in both early- and late-onset PD and is also detected among sporadic cases, albeit very rarely [127,128].

The investigation of PD etiology also focuses on identifying behavioral or environmental factors. In 1983, acute parkinsonian syndrome caused by MPTP, a by-product of synthetic heroin, was reported, which raised awareness of the environmental factors of PD[129]. To date, several factors have been identified to modify PD risk. For example, pesticide exposure and brain injury are associated with increased risk, while smoking and caffeine intake are considered protective against PD[120]. In addition to environmental risk factors, the emergence of Genome-wide association studies (GWAS) has unveiled numerous genetic risk factors that contribute to the development of PD[130,131]. Those include *SNCA*, *LRRK2*, *MAPT*, and *GBA1*[132]. Currently, it is commonly understood that both genetic and environmental factors contribute to PD risk and progression.

1.1.7 Selective neurodegeneration in PD

In PD brains, only a specific type of cells are affected. The selective neurodegeneration observed in PD suggests the presence of factors that render certain neuronal populations particularly susceptible. It was reported that neurons that have long, poor- or unmyelinated axons with high energy expenditure are the most vulnerable cell types[57,133]. In contrast, neurons with short axons or highly myelinated long axons are resistant to PD. Based on this observation, it was postulated that inefficient transmission of the action potential and multiple synaptic terminal sites yield excessive metabolic burdens, making

the particular neuronal cells vulnerable[134]. However, it does not explain the resistance of striatal cholinergic interneurons that have long, branched axons with numbers of release sites comparable to dopaminergic neurons in the substantia nigra[135].

The selective susceptibility of dopaminergic neurons could be also linked with the neurotransmitter DA. During degradation processes, DA produces several by-products that exert neurotoxicity by inducing oxidative stress, which could result in DA-dependent neurodegenerations. Neuromelanin (NM) is a dark pigment particularly abundant in dopaminergic neurons in the substantia nigra and norepinephrine neurons in the locus coeruleus[136]. NM has been associated with selective neuronal vulnerability in PD since NM-containing neurons show different susceptibilities depending on their NM content[137]. NM pigment contains lipids and peptides as well as melanins[138,139]. The biosynthesis of NM has not been fully elucidated, but its melanic components, eumelanin and pheomelanin, are known to derive from DA oxidation[140]. NM is proposed to have both protective and toxic roles[141]. NM synthesis would be a consequence of an antioxidant mechanism of DA neurons to reduce excess DA, thus NM is considered originally neuroprotective[141–143]. Moreover, it has been demonstrated that NM can accommodate large amounts of iron[139], suggesting its contribution to maintaining redox homeostasis[136]. However, when it is overloaded with irons and other toxic compounds[144,145], NM gradually releases them, exerting neurotoxicity. The dying neurons release NM to the extracellular space, which triggers microglial activation and eventually degenerates surrounding neurons[146,147]. Despite the extensive research, the role of NM in PD pathogenesis is still inconclusive. Therefore, further investigation of the physiological function of NM and NM-induced neurotoxicity is required.

1.2. *GBA1* gene

The *GBA1* gene encodes a lysosomal enzyme GCase, responsible for the hydrolysis of glucosylceramide (GlcCer) and glucosylsphingosine (GlcSph). The *GBA1* gene is located on chromosome 1q21, where a pseudogene presents 16kb downstream of the functional gene[148]. The highly homologous (shared 96% of the exonic sequence) pseudogene is prone to cause homologous recombination between the functional gene, resulting in frequent mutations in the *GBA1* gene[149]. Loss-of-function mutations in the *GBA1* gene cause the accumulation of substrate lipids, leading to the development of Gaucher disease (GD), a rare lysosomal storage disease[150]. Whereas heterozygous mutations less affect the enzymatic activity but are acknowledged as one of the most common risk factors for developing PD. To date, over 300 pathogenic *GBA1* variants have been identified[151,152].

1.2.1 GBA1 and Gaucher disease

GD is an autosomal recessive inherited disease caused by homozygous or compound heterozygous mutations in the *GBA1* gene, resulting in a 10-15% residual activity of the intact enzyme[153]. GD is characterized by the accumulation of GSLs in macrophages. The affected cells enlarged with lipid deposits are called Gaucher cells, named after the French doctor who first described the disease in 1882. Gaucher cells are piled up in multiple organs and tissues, typically in bone marrow, spleen, and liver, impeding the normal function of affected organs[153].

GD is highly heterogeneous in its clinical presentations but has been traditionally subdivided into three subtypes[154]. Type I GD is a non-neuronopathic form that accounts for almost 95% of all GD cases[155]. Type II and III GD present neurological phenotypes, of which type II is the most severe form and is often perinatal lethal[156]. Some mutations in the *GBA1* gene have clear associations with specific subtypes of GD. For example, the L483P (previously known as L444P) mutation is considered a severe mutation that often causes GD type II or III, whereas the N409S (previously known as N370S) mutation is deemed as a mild mutation typically associated with GD type I[157]. Same as other lysosomal storage disorders, there is no cure for GD. The primary treatment for GD is enzyme replacement therapy (ERT), which involves administrating enzymes to alleviate substrate accumulation. ERT has been demonstrated to substantially improve non-neurologic symptoms[158]. In the early 2000s, substrate reduction therapy (SRT) was introduced as an alternative treatment for GD[159]. The concept of SRT, originally proposed by Norman Radin[160,161], is to mitigate the building up of substrates by inhibiting their synthesis. The first SRT drug, miglustat, was approved in Europe in 2002, and then in the US in 2003. However, due to its serious side effects, it has only become a second-line treatment for adults with mild or moderate GD type 1 who do not tolerate ERT[162,163]. Subsequently, eliglustat, which overcame the side effects of miglustat, was approved in several countries for the

treatment of GD type I[164]. ERT does not address the neurological symptoms as the ERT drugs are too large to pass through the blood-brain barrier (BBB). On the other hand, miglustat is in principle permeable across the BBB, and a study has shown its ability to reduce GM1 gangliosides in the CNS of a mouse model[165]. Nevertheless, none of the small chaperone molecules including miglustat have demonstrated efficacy for neurological symptoms of GD[166,167]. Currently, a clinical trial for GD type III of another SRT drug, venglustat, has been underway (NCT04221451).

1.2.2 GBA1 and Parkinson's disease

The connection between the *GBA1* gene and PD was initially discovered through the presentation of parkinsonism among GD patients[168]. The multicenter study revealed that the odds ratio for any GBA1 mutations among PD patients was 5.43, which brought attention to the causative link between GBA1 and PD[169]. Most *GBA1* mutation carriers do not develop PD throughout life, yet the risk of PD is 5-10 fold higher than non-carriers. Among the PD-linked *GBA1* mutations, c.1448T>C (L483P/L444P) and c.1226A>G (N409S/N370S) are the most common mutations across the world[169]. The frequency of specific mutations is ethnically heterogeneous. For example, the N409S mutation accounts for 70% of all *GBA1* variants within the Ashkenazi Jewish (AJ) population whereas the L483P mutation is most common in the non-AJ, European/Asian population[169,170]. The mechanisms of how *GBA1* mutations increase the risk of PD are still elusive. Given the low penetrance of *GBA1* variants in PD, there might be other genetic modifiers contributing to PD pathogenesis.

GBA1-associated PD has been considered clinically indistinguishable from sporadic PD[171]. However, accumulating evidence revealed subtle clinical features of GBA1-associated PD, such as earlier disease onset, higher UPDRS-III scores, and increased frequency of dementia and depression[172–175]. Whether the severity of mutations influences the phenotype is debatable[174,176,177]. It was reported that benign *GBA1* variants also deteriorate particular symptoms, implying yet-to-known roles of GBA1 other than its function as a lysosomal enzyme[174].

1.2.3 Mechanisms underlying increased risk of PD with *GBA1* mutations

Heterozygous *GBA1* mutation carriers show decreased enzyme activity regardless of PD status[178]. Given that GD patients rarely develop PD despite significantly reduced GCase activity[179], the decreased enzymatic activity is not sufficient to trigger PD.

Numerous efforts have been made to identify the molecular mechanism underlying the greater risk of PD among individuals with *GBA1* mutations. One of the major hypotheses is the loss-of-function theory. It was proposed that impaired GCase activity causes the accumulation of GlcCer which induces α-synuclein aggregation, leading to further reduction in GCase activity[77]. Studies have shown that treatment with a molecular

chaperone reduces α-synuclein aggregation by helping the proper folding of GCase and enhancing enzyme activity[180,181]. Those findings ascribe the pathogenesis of GBA1-associated PD to the reduced GCase activity, although the loss-of-function mechanism alone cannot explain the low penetrance of PD among GD patients.

On the other hand, an alternative principal hypothesis highlights gain-of-function machinery wherein mutant GCase gains toxicity through misfolding and retention in the ER. Mutant GCase is shown to have a tendency to be misfolded and accumulate in the ER, which triggers ER stress and unfolded protein response (UPR), leading to the accumulation of α-synuclein[182–184]. Either theory does not exclude the other. Therefore, as Papadopoulos *et al.* proposed, both loss-of-function and gain-of-function mechanisms may contribute to disease development[185].

1.2.4 GBA1 and glycosphingolipids

GCase is involved in the regulation of lipid metabolism through the degradation of GSLs. GSLs are a subgroup of glycolipids composed of oligosaccharide headgroups linked to a ceramide backbone[186]. GSLs are the main component of plasma membranes involving numerous biological processes, including signal transduction and inflammatory responses[187]. The hydrophobic ceramide tail is embedded in the membrane, while the oligosaccharide head groups protrude in the extracellular milieu, where they interact with extracellular molecules[188]. GSLs have been demonstrated to interact with various proteins, contributing to facilitating cell-cell communication[186]. GSLs are sub-grouped into seven series based on their structures; namely, ganglio-, lacto-, neolacto-, globo-, isoglobo-, mollu-, and arthro-series[187]. Particularly, ganglio-, globo-, and neolacto-series are dominant in vertebrates[187]. The expression of GSL species is governed by a tissue- or cell-type-specific manner. In humans, ganglio-series are dominant in the brain, whereas neolacto-series prevail in hematopoietic cells[187]. Gangliosides are further subdivided into 0 (asialo)-, a-, b-, and c-series depending on the number of sialic acids linked to the innermost galactosyl residue[189]. Among them, GM1a, GD1a, GD1b, and GT1b account for more than 90% of the brain GSLs[190] (Figure 1).

Alterations in GSLs are associated with various human diseases, including PD. Several studies have shown that the composition of GSLs, as well as other lipid species, was altered in PD brains[191–193]. Particularly, it was indicated that the main brain gangliosides, including GM1a, GD1a, GD1b, and GT1b, decrease during aging, but more prominently in PD brains[191,194]. However, the complexity of GSL biosynthesis and degradation pathways makes it particularly challenging to identify causal relationships between specific GSL changes and disease development[195]. A possible pathological mechanism linking GSL alterations and PD is the interaction of GSLs and α-synuclein. It has been shown that α-synuclein binds to several GSLs through amino acid residues 34-45[196]. A previous report indicated that GM1 binds to α-synuclein, preventing them from fibrillization[197], while a

subsequent study revealed that vesicles containing GM1 or GM3 accelerate the α-synuclein aggregation[115]. Hence, whether the interaction of GSLs with α-synuclein is detrimental or beneficial remains inconclusive and may be influenced by several factors, such as lipid species and pH.

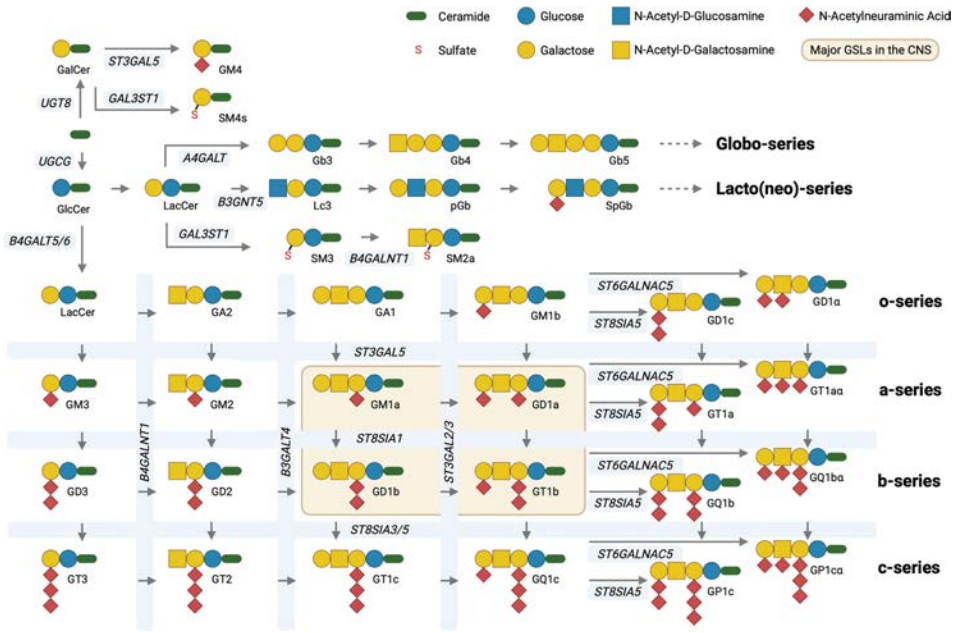

Figure 1. Schematic of GSL biosynthesis adapted from Platt *et al.* (2023)[195] under the Creative Commons Attribution License 4.0 (CC BY).

In light of the association of GBA1 and PD, it was hypothesized that substrate accumulation induced by impaired GCase activity may involve disease pathogenesis. Unlike homozygous mutations seriously compromising GCase function, heterozygous mutations in the *GBA1* gene result in a mild or moderate reduction in GCase activity, with a slight or no significant accumulation of GlcCer and GlcSph in the brain or CSF[193,198–200]. Although decreases in GCase activity and concomitant changes in brain GSLs have been observed in PD brains, the direct pathological connection between GCase activity and GSL alterations is unclear. A recent study showed that ganglioside levels were elevated in four regions of the GBA1-PD brains, suggesting the association of GBA1 with GSL regulation[193]. However, the study did not examine the substantia nigra, the most affected brain region in PD. Therefore, to shed light on the role of GBA1 in GSL regulation within dopaminergic systems, GSL alterations in GBA1-PD iPSC-derived mDA neurons were investigated in Paper II of the thesis.

1.2.5 GBA1 and PSAP/Saposin C

Saposin C is a co-factor of GCase necessary for its enzymatic function. Saposin C, as well as other saposins (A, B, and D), are derived from its precursor protein, prosaposin (PSAP)[201]. The gene encoding prosaposin protein, *PSAP*, is located on chromosome 10q21[202]. PSAP is known to exist in two forms: the 65 kDa of lysosomal form and the 70 kDa of extracellular form[203]. PSAP is primarily synthesized as the 65 kDa form which is routed to the lysosome and cleaved into 15 kDa saposins[204]. Alternatively, PSAP undergoes post-translational modification, yielding the 70 kDa extracellular form[204]. Extracellular PSAP can be found in various bodily fluids, such as blood, saliva, and milk[205,206]. Secreted PSAP is taken up by neighboring cells and directly transported to the lysosome via mannose-6-phosphate receptor[207,208]. Additionally, under stress conditions, the sortilin receptor, primarily used for transporting progranulin (PGRN), is also utilized for PSAP delivery to the lysosome[209–211]. In the lysosome, PSAP undergoes cleavage facilitated by cathepsin D, yielding four saposins[212].

The term "saposin" is derived from "sphingolipid activator protein"[213,214]. As represented by its name, each saposin activates its target lysosomal hydrolases, contributing to maintaining lysosomal homeostasis[215]. Deficiency in saposins induces lysosomal disorders associated with the lack of corresponding enzymes for each saposin[215]. For example, saposin C deficiency has been shown to cause GD among those with intact GCase activity[216–219]. Saposin C's essential role in GCase function and its association with GD pathogenesis posed an assumption that PSAP/saposin C is also involved in PD. However, genetic studies of the *PSAP* gene failed to detect significant associations between *PSAP* variants and PD[220,221]. Whereas CRISPR interference/activation-based screens using human iPSCs discovered *PSAP* as a susceptibility gene for oxidative stress[222]. Moreover, Oji *et al.* revealed that mutations in the saposin D domains of the *PSAP* gene cause familial PD, though it was not confirmed in larger cohorts[221,223]. Thus, a clear genetic connection between the *PSAP* gene and PD has not been established so far.

Saposin C is an essential activator of GCase. Saposin C was first identified in the spleen of a GD patient, as a factor that reconstitutes GCase activity *in vitro*[224]. The mechanism of action how saposin C promotes GCase activity is not fully elucidated. It has been demonstrated that saposin C binds to lipid bilayers and reduces the thickness of the membrane[225]. The mechanism of how saposin C mediates GCase and substrate interaction is under debate. There have been two models originally proposed: the solubilizer model and the liftase model. In the solubilizer model, saposin C extracts GlcCer from the membrane as a soluble complex[225,226]. The saposin C-GlcCer complex is then detached from the membrane and interacts with GCase to transfer the extracted GlcCer. Another theory, the liftase model, describes that saposin C remains associated with the membrane while exposing GlcCer to GCase by "lifting" it from the lipid leaflet, facilitating

GCase binding to GlcCer[227,228]. Currently, the liftase model best describes the function of saposin C[229], although other saposins (saposin B and D) are most likely to work as solubilizers[229,230].

Prosaposin and saposin C have been suggested to exert neuro- and glio-protective effects through binding to G protein-coupled receptor 37 (GPR37) and G protein-coupled receptor 37 like 1 (GPR37L1)[231]. While many studies reported PSAP and prosaptide TX14(a) (PSAP-derived peptides) as endogenous ligands for GPR37/GPR37L1[231,232], the discussion remains inconclusive due to the low reproducibility[233,234]. On the other hand, PSAP may affect the subcellular localization of GPR37 through interaction with gangliosides. It was reported that PSAP promotes the trafficking of GPR37 to ganglioside GM1-containing lipid raft, suggesting the protective effect of PSAP-GM1 toward GPR37 intracellular accumulation[235]. The association between PSAP/saposins and gangliosides has been demonstrated in many studies[212,235–238]. Those findings indicate that PSAP promotes transporting gangliosides to plasma membranes, whereas gangliosides mediate the neuroprotective effect of PSAP through their association with PSAP. PSAP also plays a vital role in sphingolipid metabolism by providing saposins, essential co-factors for several lysosomal sphingolipid-related hydrolases[239]. A recent study showed that loss of PSAP triggers lipid accumulation in neurons, resulting in ferroptosis[222]. Furthermore, dopaminergic PSAP-deficient mice exhibited alterations in brain lipids, suggesting PSAP's role in maintaining lipid homeostasis in dopaminergic neurons[240]. Hence, the therapeutic potential of PSAP/saposins in their association with lipids has been attracting attention. In Paper III of the thesis, we discuss the anti-PD effects of PSAP/saposin C on α-synuclein levels, possibly mediated by the lipid-α-synuclein-saposin C interaction.

1.3. Induced pluripotent stem cell-derived midbrain dopaminergic neurons

1.3.1 Induced pluripotent stem cells in PD research

The scarcity of human tissue is the major obstacle to the investigation of PD. Traditionally, human neuronal cell lines, represented by SH-SY5Y cells, have been widely used to study neurodegenerative disorders, including PD[241]. One of the concerns regarding the use of the cell lines is that they often originate from tumor cells, which may have unwanted genetic/metabolic alterations that will influence the research outcome. Since the advent of human pluripotent stem cells (hiPSCs) technology in 2007[242], increasing numbers of studies have taken advantage of the stem cell-based approach, which can provide more relevant human cellular models. One of the advantages of using iPSC models is that, with the linked clinical data, it enables exploring the phenotype-genotype association of the PD patient. Patient-derived iPSC models provide a tool for understanding the molecular mechanisms underlying the diverse clinical presentation.

hiPSCs have rapidly taken the place of human embryonic stem cells (hESCs), as they can avoid many of the issues that arise from the use of hESCs. Those include immune responses, difficulties in sourcing, and ethical concerns. Unlike hESCs, hiPSCs can be readily generated from various somatic cells, typically fibroblasts and peripheral blood, which accelerated the applications of hiPSCs in drug screening and *in vitro* disease models. The host-derived iPSCs are also expected to mitigate immune rejection, which opened up the potential of hiPSCs for personalized cell therapy. To date, several clinical trials for human stem cell-based cell replacement therapy are ongoing or under preparation, including dopaminergic neuron transplantation for the treatment of PD (hiPSC: UMIN000033564, hESC: NCT04802733 and NCT05635409). Although there are some concerns to be addressed regarding the clinical use of iPSCs for cell therapy, such as tumorigenesis and long-term safety, the iPSC technologies will broaden the therapeutic horizons of numerous diseases, including PD.

Thus, advances in iPSCs technologies have been significantly fostering PD research, both molecular mechanism investigations and clinical applications. For either purpose, the successful derivation of the target cells, midbrain dopaminergic neurons, plays a pivotal role in the research of PD. In this thesis, we developed an optimized protocol to differentiate mDA neurons from iPSCs. The backgrounds and strategies for the protocol development will be discussed in the following section.

1.3.2 Classification of human midbrain dopaminergic neurons

Midbrain dopaminergic (mDA) neurons are one of the most affected neuronal cell types in the brain of PD. Historically, mDA neurons have been subdivided into anatomically and functionally distinctive subpopulations, termed A8, A9, and A10[243]. Of these, A9 clusters

of mDA neurons are found in the substantia nigra pars compacta, where the most severely degenerated brain region in PD. A9 neurons are molecularly characterized by the expression of G-protein-regulated inward-rectifier potassium channel 2 (GIRK2)[244] and Aldehyde dehydrogenase 1 family A1 (ALDH1A1)[245]. A8 and A10 clusters of mDA neurons are located in the retrorubal field (RRF) and ventral tegmental area (VTA), respectively. A10 subpopulations are distinguished from A9 subpopulations by the expression of Calbindin (CALB), which is usually absent in the A9 subpopulations[246]. By contrast with susceptible A9 neurons, A10 subpopulations are relatively intact in the brain of PD despite its spatial proximity to A9 subpopulations[247]. Both A9 and A10 clusters of mDA neurons innervate the striatum. Specifically, A9 neurons predominantly project to the dorsolateral striatum while A10 neurons project to the ventral striatum[248].

Recent advances in single-cell transcriptomics allow refinements of the traditional anatomical definitions of mDA neuron subtyping by molecularly characterizing heterogeneous cell populations within mDA systems[249]. Spatial transcriptomic approaches will facilitate correlating anatomical properties and transcriptomic characteristics[250,251]. Although more comprehensive studies are required to fully elucidate the mDA neuron diversity, the single-cell transcriptomic approach has fostered an understanding of mDA systems at higher resolution.

1.3.3 Directed differentiation and direct reprogramming

Technologies to differentiate mDA neurons have been remarkably advanced in the last 20 years. Different strategies have been adopted to obtain mDA neurons from stem cells[252]. Currently, directed differentiation and direct reprogramming are the two major approaches in practice.

Directed differentiation is a method to confine the direction of stem cell differentiation to a specific lineage/cell type by modulating the environment. Initial protocols for directed differentiation relied on embryoid body formation, stromal cell co-culture, and neural rosettes[253–256]. More recently, mDA differentiation has been achieved by mimicking the *in vivo* brain development with the supplement of small molecules and growth factors at the specific time point during the differentiation. Detailed strategies for chemical-based directed differentiation are discussed in the following section 1.3.5.

Direct reprogramming, on the other hand, is a method to convert terminally differentiated somatic cells into other cell types without going through a pluripotent state. It is accomplished by forced expression of specific transcription factors that play crucial roles in cell fate determination. For example, direct reprogramming of mDA neurons is achieved by introducing combinations of several essential transcription factors, such as *ASC11*, *NGN2*, *SOX2*, *LMX1A*, *EN1*, *NURR1* (also known as *NR4A2*), and *PITX3*[257–259].

Both strategies have advantages and disadvantages. A study suggested that directed differentiation is well recapitulating the endogenous gene regulatory networks[260]. In contrast, direct reprogramming can retain the epigenetic traits of the source cells, which are generally erased during the reprogramming steps in directed differentiation[261]. The impact of rejuvenation during iPSC reprogramming is not negligible, particularly when studying late-onset diseases like PD. Hence, to trigger a diseased state of PD in stem-cell-derived models, additional manipulations, such as inducing stress and imitating aging phenotypes, are often employed. Those include exposure to toxins (6-OHDA, MPTP, and rotenone) and overexpression of α-synuclein.

1.3.4 Monoculture, co-culture, and organoid models

Many differentiation protocols have been developed intending to generate a single, specific cell type. For instance, differentiation protocols for pure mDA neurons have been developed for cell transplantation therapies or studying dopaminergic neurodegeneration. However, monoculture systems do not represent the brain environment where various cell types interact. Therefore, co-culture systems containing different cell types are often utilized to evaluate cell-cell interactions. Nowadays, more complex 3D cell cultures, consisting of multiple cell types are employed to model specific tissues or organs. Those are called organoids[262]. Monotypic culture provides simpler and more reproducible models, whereas heterotypic culture offers more physiologically relevant models[263]. Hence, suitable iPSC models need to be selected depending on the purpose of the study.

1.3.5 Directed differentiation towards midbrain dopaminergic neurons

Directed differentiation protocols for mDA neurons are based on three stages: neural induction by dual-SMAD inhibition, mDA specification by midbrain floor plate (mFP) induction, and mDA maturation. The strategy has been developed inspired by neurodevelopmental insights in mostly murine brains. A recent study analyzing human and murine ventral midbrain (VM) development provided greater details of molecular, temporal, and spatial similarities and differences between the developing human and mouse brain[264] (summarized in a review by Ásgrímsdóttir and Arenas[265]). The uncovered differences in murine and human VM development emphasize the importance of protocol refinement based on human brain development. Therefore, understanding human mDA neuron development is vital for further improvement of the differentiation protocol.

1.3.5.1. Neuralization

Neuralization is the first step of the mDA differentiation inducing an ectodermal, neural fate in the cells. The dual-SMAD inhibition method, introduced by Chambers *et al*, has been widely used for neural induction[266]. The method involves fostering neuralization by restraining somatic fate using transforming growth factor β (TGFβ) and bone

morphogenetic protein (BMP) signaling inhibitors. This approach also enabled feeder-free culture systems, overcoming the problem with the previous highly-undefined culture systems relying on stromal cells[255,267,268]. Synthetic small molecules SB431542[266,269–272] or A83-01[273] are commonly used to inhibit TGFβ signaling. LDN193189[270–273] or Noggin[266,269] are often employed for the inhibition of BMP signaling.

1.3.5.2. Specification

After the acquisition of neural fate, cells subsequently obtain regional identities; in the case of mDA neurons, ventral midbrain. Particularly, it has been revealed that mDA neurons originated from neurogenic radial glia-like cells that reside within the mFP[264,274,275]. In the developing brain, mFP is formed at the ventral midline of the neural tube through the orchestrated regulations of the two organizing centers, the midbrain-hindbrain boundary (MHB) and the FP[276]. The mFP specification is accomplished by the activation of several signaling pathways, which are temporally and spatially controlled by morphogenic gradients along the anterior-posterior (AP) and the dorsal-ventral (DV) axis (Figure 2[277]).

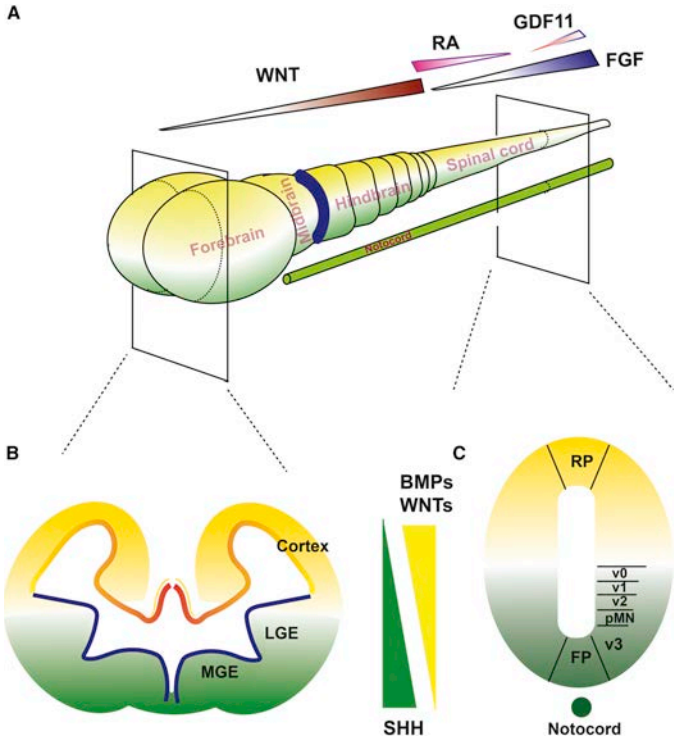

Figure 2. Neural Patterning Principle for Neural Progenitor Subtype Specification. **A** A–P patterning is under the regulation of several morphogens during development. The gradient of WNTs dictates the regionalization of the forebrain, mid-hindbrain, and anterior spinal cord, whereas gradients of RA and FGFs govern the spinal cord segmentation. **B** and **C** D–V patterning in the forebrain (B) and spinal cord (C) is set by the dorsally derived morphogens WNTs and BMPs (yellow color) and the ventrally derived SHH (green color). This figure was published in Cell Stem Cell, Vol 19, Issue 5, Yunlong Tao and Su-Chun Zhang, Neural Subtype Specification from Human Pluripotent Stem Cells, Pages 573-586, Copyright Elsevier (2016)[277]. Reprinted with permission.

AP patterning is governed by the two morphogens secreted from the MHB, WNTs and fibroblast growth factors (FGFs)[277]. Expression of *Wnt1* and *Fgf8* is regulated by the mutually suppressing transcription factors *Otx2* (expressed in the fore- and midbrain)[278,279] and *Gbx2* (expressed in the hindbrain)[279–281], respectively. *Wnt1* regulates several genes involved in the mDA differentiation and maintenance, including *Otx2*[282], *Lmx1a/b*[282], *Engrailed 1* (*En1*)[283], *Nurr1*[282,284], and *Pitx3*[282,285,286]. Knockout experiments of *Wnt1*[285,287] or *Lmx1a/b*[288] led to a loss of mDA neurons, indicating that the expression of *Wnt1* and *Lmx1a/b* are essential for mDA specification. Especially, Lmx1a is crucial for the specification of mDA fate[289]. Another key morphogen FGF8 guides the hindbrain fate by activating the Ras-ERK signaling pathway under *Gbx2* expression[290,291]. Lower FGF8 concentration drives midbrain identification[291,292], which requires the expression of *Wnt1*[285].

DV patterning is regulated by the sonic hedgehog (SHH) protein and BMP gradient, derived from the floor plate (FP) and the roof plate (RP), respectively[293]. SHH induces the expression of *Foxa1/2*, which plays an essential role in the ventral patterning of mFP progenitors[294,295]. *Foxa1/2* regulates multiple essential genes in mDA specification, including *Ngn2*[296], *Nurr1*[296], *Lmx1a/b*[297], and *Th*[296]. Thus, the coordinated activation of *Wnt1*-induced-*Lmx1a* and *Shh*-induced-*Foxa2* is the fundamental event for the specification of mDA progenitors in mFP.

In vitro mFP specification is achieved based on the morphogen-governed patterning through the activation of the Wnt/β-catenin and the SHH signaling pathways, typically with CHIR99021 and Purmorphamine. CHIR99021 is a glycogen synthase kinase 3β inhibitor that exerts dose-dependent caudalization effects through modulating WNT signaling[269]f. Purmorphamine is a purine derivative activating the SHH pathway by targeting Smoothened[298]. Retinoic acid and/or FGF8β are often employed to promote caudalization[299–301]. Recently, biphasic activation of the WNT signaling pathway was shown to induce robust EN1 expression, leading to better mFP patterning[271]. The concept of biphasic WNT activation is based on the two distinct roles of WNT during midbrain development. In the earlier stage, the AP gradient of WNT guides the original forebrain fate toward the midbrain and hindbrain identities[302]. Whereas in the later stage, strong WNT expression in the Otx2-expressing cells at MHB defines the midbrain identity[303]. The initial concentration of 0.7 μM CHIR99021 in the biphasic WNT activation mimics the early dose-dependent caudalization, while 7.5 μM "CHIR boost" imitates the promoted WNT signaling at the anterior border. Also, the strategy does not use FGF8 during mFP induction to avoid the risk of hindbrain contamination, which occurs due to improper timing and dosage of exogenous FGF8 delivery[304].

Another key factor for stem cell differentiation and maintenance is the extracellular matrix (ECM), a component of the stem cell niche regulating the behavior of stem cells[305]. Various ECM proteins have been utilized for stem cell differentiation, including Matrigel,

Geltrex, and Laminins. An ECM protein, Laminin 511, has been shown to promote mDA neuron survival and differentiation by enhancing midbrain patterning[272,306].

Several transcription factors are recognized as essential for mDA progenitor specification, including *Foxa2*[307], *Lmx1a*[308], *En1*[309], and *Otx2*[310]. Particularly, the co-expression of *Foxa2* and *Lmx1a* has been traditionally used for mFP specification[311]. However, it was suggested that *Foxa2* and *Lmx1a* alone are not sufficient to discriminate mDA neuronal lineage from the subthalamic nucleus (STN) lineage[312]. Therefore, a more precise molecular definition of mDA progenitors needs to be identified. Understanding the transcriptional characteristics of human mDA progenitors is vital for distinguishing mDA identity from other closely related lineages and developing differentiation protocols to generate bona fide mDA neurons.

1.3.5.3. Maturation

Once the cells establish their identities, they are terminally differentiated into mature cell types, typically accompanied by the cell cycle exit. After the specification step of the *in vitro* differentiation, mFP progenitors undergo mDA neurogenesis and maturation for typically >30 days. Maturation is facilitated by various growth factors such as brain-derived neurotrophic factor (BDNF), glial cell line-derived neurotrophic factor (GDNF), and TGFβ, as well as small molecules including ascorbic acid (AA), dibutyryl cyclic adenosine monophosphate (dbcAMP), and DAPT. A notch inhibitor, DAPT, promotes neuronal differentiation by impeding glial fate[313,314]. Recently, the activation of Liver X Receptor (LXR) by an LXR ligand GW3965, combined with the inhibition of FGF signaling by MEK/ERK pathway inhibitors, SU5402 and PD0325901, was demonstrated to promote mDA neurogenesis[272].

Mature mDA neurons are characterized by the expression of genes essential for dopaminergic functions, such as *Th*, *Ddc*, *Dat* (*Slc6a3*), and *Vmat2* (*Slc18a2*)[249]. Additionally, mDA neuronal identity and survival are maintained through the expression of *Nurr1*[315,316], *Pitx3*[317], *En1*[309,318,319], *Foxa1/2*[320], and *Lmx1a/b*[321]. Those are also developmental genes that discriminate mDA neurons from other DA neurons with different origins than mFP[249]. However, it has been reported that the mDA neuronal lineage has a transcription factor profile overlapping with the STN lineage[312]. The contamination of undesired cell types is the major hindrance in transplantation, leading to poor graft outcomes and graft-induced dyskinesia[304,322]. Developing a better differentiation method, fulfilled through an in-depth understanding of mDA neurogenesis and specification, will make stem cell therapy promising.

2 RESEARCH AIMS

Genetic variants in the *GBA1* gene are among the most prevalent risk factors for developing PD. However, the specific role of GCase, a lysosomal hydrolase the *GBA1* gene encodes for, in the pathogenesis of PD is only partially known. This thesis generally aims to investigate the molecular mechanisms underlying GBA1-associated PD. Specifically, the general objective is broken down into the following aims:

Aim 1. To elucidate the association of the *GBA1* gene mutation and PD by focusing on alterations in secretome and glycosphingolipids.

Aim 2. To investigate the therapeutic potential of PSAP and saposin C on PD, specifically their interaction with α-synuclein.

Aim 3. To establish a robust and efficient chemical-based mDA differentiation protocol that provides a reliable experimental model of PD applied in the research project.

The research aims are pursued in the three constituent papers and the thesis. Aim 1 is fulfilled in Papers I and II, Aim 2 is fulfilled in Paper III, and Aim 3 is fulfilled in the result section of the thesis.

3 MATERIALS AND METHODS

A thorough description of materials and methods used in the thesis is found in the materials and methods section of each constituent paper. In this section, some of the key methods are picked up and discussed in depth.

3.1. Cell culture experiments

3.1.1. Patient-derived induced pluripotent stem cell models of PD

iPSCs generated from patients have been used for disease modeling. iPSCs can be reprogrammed from diverse somatic cells, typically fibroblasts. The reprogramming is achieved by introducing subsets of key transcription factors named Yamanaka factors (*OCT4*, *SOX2*, *KLF4*, and *C-MYC*)[242] or Thomson factors (*OCT4*, *SOX2*, *NANOG*, and *LIN28*)[323]. Generated iPSCs can be genetically engineered for various purposes. For example, to study the impact of specific gene variants, isogenic control iPSCs are generated by introducing specific gene mutations or correcting them. This approach is highly advantageous as it allows a close examination of the role of gene mutations, which is often hindered by variability in genetic backgrounds when using iPSCs from different individuals as controls. Moreover, patient-derived iPSCs can be associated with clinical information obtained from the patients, bridging *in vitro* findings to clinical observations.

In this thesis, we employed an iPS cell line derived from a PD patient carrying *GBA1* N409S heterozygous mutation (GBA1-PD: NH50187). For the control, we utilized an isogenic control iPS cell line (ISO-PD: NH50186), as well as iPSCs from a healthy individual for non-PD control (EPIPSC: ND41865). The *GBA1* N409S heterozygous mutation in the isogenic control iPSCs was corrected with CRISPR/Cas9. All the iPSCs were acquired from the Rutgers University Cell and DNA Repository and passed the quality control for editing (off-target editing and homogeneity) and pluripotency (sterility, identity, pluripotency, residual expression of reprogramming factor, genetic stability and viability).

3.1.2. Directed differentiation for midbrain dopaminergic neurons

iPSCs were differentiated into midbrain dopaminergic neurons using a directed differentiation strategy. To achieve the best efficiency with our iPSCs, an optimized differentiation protocol was developed based on a protocol reported by Nishimura *et al.*[272,324] The protocol consists of three steps: neuralization and specification, differentiation, and maturation.

The very first step of differentiation, neuralization, was accomplished by dual-SMAD inhibition[266]. Undifferentiated iPSCs have the potential to differentiate into three embryonic germ layer lineages, including ectoderm, endoderm, and mesoderm. To direct the cells into the neural lineage, which is derived from ectoderm, we treated the cells with two SMAD inhibitors, 10 μM SB431542 and 250 nM LDN193189 for 6 days. At the same

time, FP fate was induced by the administration of 1 μM Purmorphamine and different concentrations of CHIR99021. Purmorphamine is a SHH activator promoting ventralization, while CHIR99021 is a WNT activator mediating caudalization. In our optimized protocol, we employed the biphasic WNT activation strategy reported by Kim *et al.*[271]. This method involves three days of the low CHIR phase (0.7 μM) and six days of the high CHIR phase (7.5 μM), followed by one day of the end phase (3 μM). The biphasic WNT activation promotes efficient FP patterning by mimicking strictly regulated WNT signaling during midbrain development, enabling improved mDA neuron specification. For the neuralization and specification step, cells were plated onto a Geltrex+LN511 double-coated plate at a density of 200K cells/cm^2. LN511 has been shown to enhance mDA differentiation and survival[272,306]. The combination of LN511 and Geltrex improved cell viability and attachment, particularly at the late stage of specification.

On day 11, mFP progenitors were replated at a high seeding density (800K cells/cm^2) and cultured for five days to be differentiated into immature mDA neurons. Various growth factors and small molecules were supplemented to stimulate differentiation. Those include 20 ng/mL BDNF, 20 ng/mL GDNF, 1 ng/mL TGFβ3, 200 μM dbcAMP, and 200 μM AA. Following Nishimura *et al.*'s protocol, we added 10 μM GW3965, an LXR ligand facilitating cell cycle exit, to promote neurogenesis[272]. For the differentiation step, cells were plated onto a LN511-alone coated plate, which facilitates mDA differentiation while keeping the cells easy to dissociate at the time of final replating on day 16.

For the maturation step, immature mDA neurons were plated onto an LN511+Poly-L-ornithine (PLO) double-coated plate at a density of 800K cells/cm^2. PLO enhanced cell adhesion to the plate and enabled even distribution of mDA neurons. 10 μM DAPT on top of the sets of growth factors and small molecules continued to be supplemented throughout the maturation step. For the first four days of maturation, 1 μM PD0325901 and 5 μM SU5402, MEK/ERK inhibitors, were added to promote further neurogenesis[272]. The mDA neurons were cultured until around day 60 and subjected to various experiments.

Throughout the whole mDA differentiation, Neurobasal supplemented with L-Glutamine and B27 was used for cell culture media. 1x N2 supplement was added to the media until day nine of the differentiation. Cell culture media was replaced every day until day 23 and every other day with half-medium change afterward. A sufficient volume of cell culture media was added, typically 1 mL/well for a 24-well plate and 2-4 mL/well for a 12-well plate. Cells were treated with 10 μM Y27632, Rock inhibitor, for 48 hours after each replating to suppress apoptosis[325].

3.1.3. Generation of stable overexpression cell lines

Cells stably overexpressing specific genes are widely used to study the function of the gene of interest. One of the advantages of stable overexpression cell lines compared with transient overexpression is that the expression levels are typically moderate and consistent after several passages, which enables relatively long-term experiments with high reproducibility. On the other hand, a drawback is a lengthy process that takes several weeks to select colonies and establish a stable overexpressing cell line[326]. Stable overexpression cell colonies can be obtained through weeks of antibiotic selection of the cells whose genome was integrated with the recombinant DNA. PSAP-GFP stably transfected SH-SY5Y cells and control EGFP stably transfected SH-SY5Y cells were generated by forced expression of the vector plasmids containing hygromycin- or geneticin-resistance gene followed by antibiotic selection. PSAP-GFP plasmids (HG16224-ACG, Sino Biological) and EGFP plasmids (13031, Addgene) were transfected to SH-SY5Y cells (CRL-2266™, 70019544, ATCC) using Lipofectamine 2000 (12143, Qiagen). After four weeks of antibiotic selection with 200 µg/mL Hygromycin B or 200 µg/mL Geneticin, PSAP-GFP or EGFP stable overexpression cells were obtained, respectively.

The colony screening was done by observation of fluorescent proteins under a fluorescent microscope. The selected colonies were dissociated and transferred to a 24-well plate. The colonies were further expanded in a 6-well plate, and once they reached 80% confluence, the cells were harvested and the protein levels of exogenous PSAP-GFP or EGFP were confirmed by Western Blot. The established stable overexpressing cell lines were cultured with low concentrations of antibiotics (50-100 µg/mL) to maintain the integration of recombinant DNA, and the protein expression from recombinant DNA was routinely checked by fluorescent microscope.

3.1.4. Immunocytochemistry

Immunocytochemistry is a technique for visualizing cellular proteins to study the expression levels and localization of the protein. As indicated by the term, the method is based on immunological techniques using antibodies that specifically recognize the protein of interest. First, cells were plated on a cell culture plate/dish with a glass bottom and cultured until they were attached. Cells were then fixed with 4% paraformaldehyde (PFA) for 20 minutes at room temperature (RT). The fixation procedure is done to preserve the morphological structure and immobilize antigens. Next, fixed cells were permeabilized by incubated in PBS with 0.02% Triton X-100 for 30 minutes. Permeabilization is a process of making holes in cell membranes, which enables antibodies to access intracellular epitopes. After the permeabilization, cells were incubated with 5% donkey serum for 1 hour at RT. The process is called blocking, which aims to reduce the non-specific binding of antibodies. Cells were then incubated with a primary antibody overnight at 4°C. The following day, cells were washed out with PBS three

times and incubated with a secondary antibody for 1-2 hours at RT. The secondary antibodies are conjugated with fluorophores that emit fluorescence when excited with a specific wavelength of light. Multiple proteins can be visualized in the same sample by combining different host species and fluorophores. Visualization of immunostaining was performed on Zeiss LSM 880 or 900 (Carl Zeiss AG, Oberkochen, Germany).

3.2. Protein experiments

3.2.1. Immunoblotting

Immunoblotting, also known as Western Blotting, is a semi-quantitative method to quantify proteins. The process of immunoblotting consists of three steps: SDS-PAGE, blotting/transfer, and immunological detection.

SDS-PAGE is a method to separate different sizes of proteins by polyacrylamide gel electrophoresis (PAGE). Proteins are denatured and unfolded with sodium dodecyl sulfate (SDS) and reducing agents such as 2-mercaptoethanol, which allows the binding of negatively charged SDS to proteins, proportional to their polypeptide chain length. Then, the negatively charged proteins are loaded onto a polyacrylamide gel and migrated by electrophoresis. The mesh-like structure of the polymerized gel allows the separation of proteins based on their molecular weight. Typically, small proteins migrate faster and *vice versa* because of the sieving effect of the gel.

After proteins are separated in the gel, the proteins are transferred onto membranes. There are many transfer methods available, of which electro-transfer is most widely used. The electro-transfer involves the application of an electric field to elute proteins from the gel to membranes. Depending on the character of the protein of interest and the following detection methods, either nitrocellulose membranes or polyvinylidene difluoride (PVDF) membranes are used. Nitrocellulose membrane is less sensitive with less background noise, whereas PVDF gives higher sensitivity with a lower affinity for protein. The transfer step is followed by an immunological detection step that involves blocking, antibody incubation, and fluorescence/chemiluminescence detection. Optionally, an optimal detection for α-synuclein requires a fixation step before blocking to improve the immobilization of α-synuclein to membranes[327]. Fixation is done by incubation in 0.4% PFA for 30 minutes at RT. Then, the membrane is blocked with 5% bovine serum albumin or skim milk in tris-buffered saline with 0.1% Tween 20 (TBS-T) for 30 minutes at RT.

The blocked membrane is incubated with primary antibody for 2 hours at RT or overnight at 4°C, depending on the antibodies and targets. Appropriate secondary antibodies need to be applied depending on the detection methods. For example, a secondary antibody labeled with a fluorescent probe is used for fluorescent detection, while a horseradish peroxidase-conjugated secondary antibody is employed for chemiluminescent detection. The protein is visualized on LI-COR Odyssey CLx (LI-COR Biosciences) or ChemiDoc™ MP Imaging System (Bio-Rad). Densitometric analysis of protein band intensity is performed on the Image Studio software version 5.2.5 to quantify relative protein levels to housekeeping proteins such as GAPDH or β-actin. Detailed information about antibodies and dilution factors used in the studies can be found in each constitutive paper.

3.2.2. GCase activity assay

The enzymatic activity of GCase can be experimentally assessed in several ways. The most widely used method is the *in vitro* GCase activity assay measuring the degradation ability of GCase toward an artificial fluorogenic substrate, 4-methylumbelliferyl β-D-glucopyranoside (4-MUG). 4-MUG is cleaved by GCase and yields 4-methylumbelliferone (4-MU) that emits blue light at 449 nm when excited by 360 nm light. The activity is expressed as the amount of 4-MUG converted to 4-MU per unit of time, which can be calculated as a linear increase in fluorescence intensity.

In vitro GCase activity with 4-MUG is often carried out using cell/tissue lysates. As the whole cell lysate also contains GBA2, a cytoplasmic enzyme that shares substrate specificity with GCase, the result of the GCase activity assay reflects the contribution from GBA2 activity. To remove the impact of GBA2 activity, a selective GCase inhibitor, Conduritol B epoxide (CBE), is added to the reaction, and the CBE-treated values (=residual GBA2 activity) are subtracted from the measurements as a background signal. Alternatively, treating the lysate with N-(5-adamantane-1-yl-methoxy-pentyl)-Deoxynojirimycin (AMP-dNM), a selective GBA2 inhibitor, and subtracting the AMP-dNM sensitive component from the total activity allows the evaluation of GBA2 activity.

Several factors influence the measurement of *in vitro* GCase activity[328]. The presence of detergent in lysis buffer can affect the experimental activity of GCase. Thus, mild detergents, such as TNT buffer, are used for cell lysis. Alternatively, the cell membrane is mechanically disrupted by freeze-thaw. The enzyme activity is also affected by pH. For example, both GCase and GBA2 cleave 4-MUG as well as natural substrate glucosylceramide, but the optimal pH for each enzyme is different. The difference in optimal pH is derived from their intracellular localization, where GCase exists in lysosome while GBA2 is cytoplasmic. Therefore, the reaction buffer is prepared at optimal pH for GCase, which is around pH 4.7-5.9[329], to suppress non-specific activity.

While *in vitro* GCase activity assay is a robust method to measure GCase activity and has been employed in many studies, it must be considered that the assay condition does not reflect an actual lysosomal environment. To overcome the drawbacks, the *in situ* live-cell GCase activity assay has been developed. A cell membrane permeable substrate for GCase, 5-(Pentafluorobenzoylamino)Fluorescein Di-β-D-Glucopyranoside (PFB-FDGlu), enables the measurement of in situ lysosomal GCase activity in living cells[330]. PFB-FDGlu emits green fluorescence when the quencher is cleaved by GCase. PFB-FDGlu is taken up by pinocytosis and trafficked toward lysosomes, the environment where GCase exerts innate activity.

To measure GCase activity from cell lysates, cells were lysed with TNT buffer (100 mM Tris-HCl (pH 7.4), 100 mM NaCl, 0.2% Triton X-100) for 30 minutes on ice. Cell lysates were centrifuged for 30 min at 16,000 x g, 4°C, and the supernatant was subjected to

protein quantification with BCA assay. 10 µg of total protein was mixed with the assay buffer (Citrate–Phosphate buffer pH 5.4, 1% bovine serum albumin, 0.25% Triton X-100, 0.25% Sodium taurocholate, 0.1% EDTA) and loaded into a black 96-well plate in duplicate. 1 mM CBE or 5 nM AMP-dNM was added to the control wells. The reaction was initiated by adding 1 mM 4-MUG. The fluorescence from the degradation product of 4-MUG was read on a Tecan Spark 10 M (Ex: 360 / Em: 449) every 10 minutes for 6 hours. Background signals from CBE or AMP-dNM treated wells were subtracted to calculate specific GCase or GBA2 activity, respectively. GCase/GBA2 activity was expressed as the slope of linear regression calculated from the measured fluorescence intensity from 100-300 minutes (21 data points).

3.2.3. Proximity extension assay

In Paper I, the secretome of CSF and cell culture supernatant was analyzed using the proximity extension assay (PEA) technology at Olink proteomics[331]. PEA is a plate assay based on the immunological detection of target proteins. The unique feature of this technology is the use of oligonucleotide-labeled antibodies, which bind to the antigens pair-wisely. When a pair of matched oligonucleotide-labeled antibodies bind to the target protein, they are brought to a distance close enough to hybridize. The hybridized template DNA is amplified and measured by real-time qPCR. The PEA technology eliminates the cross-reactivity of antibodies, enabling the quantification of multiple proteins with high specificity.

The CSF and cell culture supernatant were analyzed with the Olink Metabolism 96 panel. The list of pre-selected target proteins in the panel is available online (https://olink.com/products-services/target/biological-process/, accessed on 24 February 2024).

3.3. Clinical evaluation of Parkinson's disease patients

Clinical evaluations of PD patients included in the study in Paper I were conducted by a movement disorder specialist in neurological clinics within Region Stockholm. The disease severity of the patients was evaluated with the UPDRS-III[332] and the HY[9]. UPDRS consists of four parts of assessments, including non-motor experiences of daily living (Part I), motor experiences of daily living (Part II), motor examination (Part III), and motor complications (Part IV). The HY scale is a staging scale widely used for evaluating the progression of PD. The cognitive impairment was assessed using the Montreal Cognitive Assessment (MoCA)[333] scale. MoCA is frequently used for evaluating mild cognitive impairment in PD patients. PD medications are summarized as L-dopa equivalent doses (LEDD)[334].

3.4. Glycosphingolipid analysis by NP-HPLC

GSLs in iPSC-derived mDA neurons were analyzed using normal-phase high-performance liquid chromatography (NP-HPLC). HPLC is a chromatography technique that separates analytes based on the speed of travel in the column, which is determined by the physiological properties of the analytes (*e.g.* size, polarity, electric charge). Analytes are injected into a stream of eluent (mobile phase) and separated while flowing through a column (stationary phase). The separated components are detected as electric signals and a chromatogram of the signals versus time is generated.

HPLC is widely employed to identify and quantify individual components of various analytes. NP-HPLC is a form of HPLC using a polar stationary phase (typically silica gel) and a less polar mobile phase (non-aqueous). NP-HPLC is particularly suitable for separating lipid classes. The GSL detection method used in the thesis employs a fluorescent compound, anthranilic acid (2-AA), to label oligosaccharides before NP-HPLC[335]. The detailed protocol is available online[336] (https://protocols.io/view/analysis-of-glycosphingolipids-from-human-plasma-busvnwe6.html, accessed on 24 February 2024), modified using 0.2 mg protein equivalent of freeze-thawed cells in dd H_2O for the cell pellet analysis in Paper II.

3.5. Statistical Analysis

The statistical analysis was performed with GraphPad Prism v9.00 unless stated otherwise, and v9.00, 7.03, or 5.04 for Paper III. For comparisons between two groups, a two-tailed Student's t-test or a Mann-Whitney U test was used. For comparisons between three or more groups, a One-Way analysis of variance (ANOVA) with Dunnett's multiple comparison test was used. For comparisons between groups with two independent variables, a Two-Way ANOVA with Tukey's multiple comparison test was used. For a series of comparisons between two groups, two-tailed multiple t-tests with or without Benjamini, Krieger, and Yekutieli's false discovery rate (FDR) correction were used. The FDR cutoff of <0.05 was used for the determination of the significance. For correlation analyses, Spearman's rank correlation coefficient was used. For outlier detection, the robust regression and outlier removal (ROUT) method (Q=1 %) was used. Data represented as mean ± the standard error of the mean (SEM). Significance was set as follows: * = $p < 0.05$; ** = $p < 0.01$; *** = $p < 0.001$.

3.6. Ethical Considerations

The human study in Paper I was conducted in accordance with the Declaration of Helsinki and has been approved by the Swedish Ethical Review Authority (dnr 2020-03684, dnr 2019-04967)[337]. The patients included in the study gave written consent to the storage of their samples for future use in studies. The patients were clinically assessed by a movement disorder specialist and fulfilled the clinical diagnosis criteria for PD[7]. CSF

samples from patients were collected by lumbar punctures given by a movement disorder specialist in neurological clinics within Region Stockholm, where the collected samples had been stored.

Figure 1 is reprinted from Platt *et al.*[195] under the Creative Commons Attribution (CC BY) 4.0 International (https://creativecommons.org/licenses/by/4.0/, accessed on 8 March 2024). Figure 2 is reprinted from Tao and Zhang[277] with Permission. Paper I and Paper II are reprinted from Kojima *et al.*[338,339] Under the CC BY 4.0 license.

4 RESULTS AND DISCUSSION

The factors driving the increased risk of developing PD among *GBA1* mutation carriers are still elusive. To delineate the roles of GBA1 in PD pathogenesis, we employed GBA1-PD iPSCs (GBA1-PD) derived from a PD patient carrying *GBA1* N409S heterozygous mutation. Particularly, we examined GBA1-specific alterations by using isogenic control iPSCs (ISO-PD), in which the mutation present in the GBA1-PD iPSCs was corrected with CRISPR/Cas9.

Protocol development for midbrain dopaminergic neuronal differentiation

First, to establish the iPSC-derived GBA1-PD model being used in the study, we developed an optimized differentiation protocol for midbrain dopaminergic neurons. The original protocol is based on the FP induction method reported by Nishimura *et al*[272,324]. (Figure

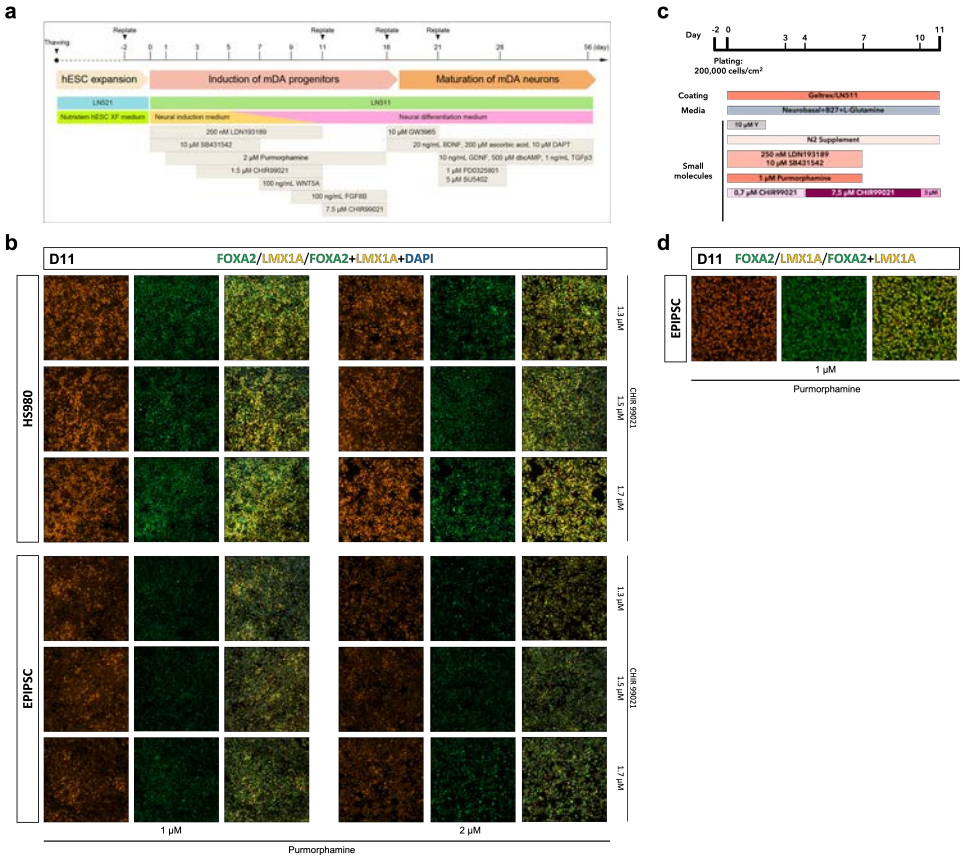

Figure 3. Immunostaining for floor plate markers of HS980 or EPIPSC cells on day 11 of differentiation under CHIR99021 and Purmorphamine titration. **a** Schematic of differentiation protocol developed by Nishimura *et al.* (2023) **b** comparison of FOXA2/LMX1A staining on hESCs (HS980, upper) and hiPSCs (EPIPSC, bottom) differentiated using Nishimura *et al.* (2023) protocol with different combinations of the indicated CHIR99021/Purmorphamine dosage. **c** Schematic of the optimized midbrain floor plate induction protocol adapted from the biphasic WNT activation reported by Kim *et al.* (2021) **d** FOXA2/LMX1A staining on differentiated hiPSCs using the optimized protocol. Images are representative of at least two independent experiments.

3a). As the protocol was principally developed for hESCs, it did not give optimal results on the iPSCs we used. Specifically, low Lmx1a/Foxa2 expression during the FP patterning was the major trouble. To optimize the differentiation protocol, we used healthy control hiPSCs (EPIPSC) as a reference cell line. First, to adjust the Wnt/SHH activation for iPSCs, we modulated the concentration of CHIR99021 and Purmorphamine (Figure 3b). We titrated 1.3-1.7 µM CHIR99021 in combination with 1 or 2 µM Purmorphamine and evaluated the mFP patterning outcome for each condition. However, the CHIR99021 and Purmorphamine dosage within the titrated range did not improve the pattering efficiency of iPSCs to a level comparable to that of hESCs. Then, we applied the biphasic WNT activation reported by Kim *et al.*[271], which involves four days of 0.7 µM CHIR99021 treatment followed by the 7.5 µM "CHIR boost" condition. For the adaptation of the biphasic WNT activation to the original protocol, we examined the following conditions: initial seeding density, use of Purmorphamine for SHH activation, and plate coating. After several conditions were tested, the optimized mFP induction was developed for iPSCs (Figure 3c and 3d). The initial seeding density of 200K/cm^2 cells worked best for our iPS cell lines. We followed the original protocol using 1 µM Purmorphamine instead of 500 ng/mL SHH C24II, since Purmorphamine minimized variability in patterning efficiency across experiments and cell lines, giving consistent outcomes. We employed a double-coating with Geltrex and LN511 instead of a single-LN511 coating to enhance the cell adhesion. The double-coating method helped reduce cell detachments without affecting the pattering efficiency.

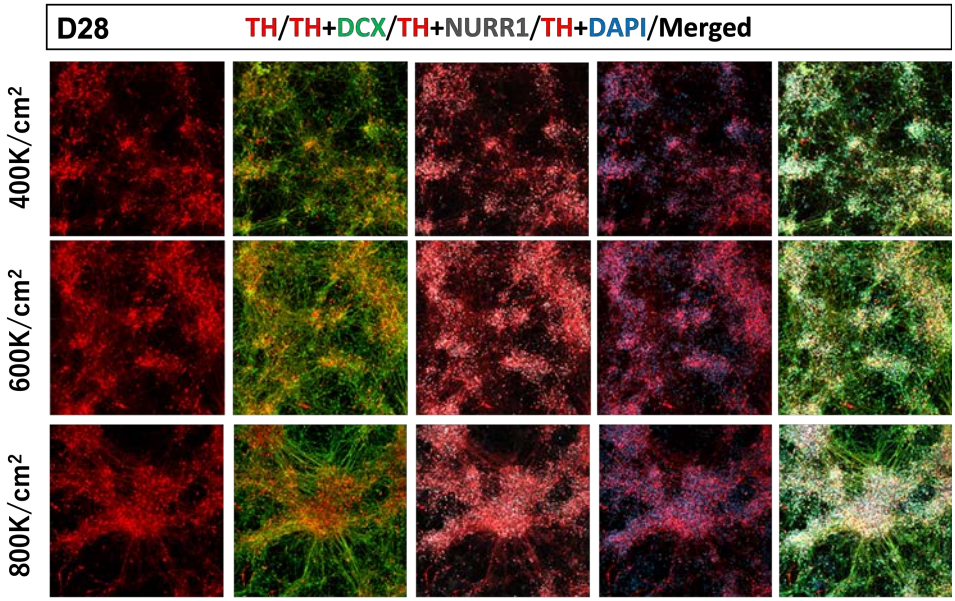

Figure 4. Immunostaining for dopaminergic markers of EPIPSC cells on day 28 of differentiation under different seeding densities. Comparison of TH/DCX/NURR1 staining and cell distribution on hiPSCs plated at the indicated seeding density. Images are representative of at least two independent experiments.

Next, to determine the optimal cell density at final replating on day 16, we tested different seeding densities and analyzed immunostaining of dopaminergic markers on day 28 (Figure 4). Seeding density did not alter the differentiation efficiency, whereas we found that the cell viability was improved when the progenitor cells were plated at 800K/cm^2 on day 16.

Nishimura *et al.* suggested the activation of LXR and FGF inhibition improves mDA neurogenesis and differentiation[272]. To evaluate the efficacy of this approach with the optimized protocol, we differentiated the hiPSCs with LXR activator, GW3965, and FGF inhibitor, SU5402 and PD0325901. Based on the increased signal intensity from immunostaining of Nurr1+ cells with the treatment, we concluded that treatment with 10 µM GW3965 (day 12-15), 5 µM SU5402, and 1 µM PD0325901 (day 16-21) enhanced the differentiation efficiency (Figure 5). On the other hand, the supplement of GW3965 and SU5402/PD0325901 induced uneven cell distributions and clump formations. To solve this problem, we applied double-coating with PLO and LN511. The pre-coating with PLO improved cell distributions and diminished cell clumping.

Finally, we successfully developed an optimized mDA differentiation protocol for our iPSCs (Figure 6a). The immunostaining and mRNA expression of mDA neuron marker genes demonstrated that the established protocol efficiently generated mDA neurons from iPSCs (Figures 6b and 6c). The bulk qPCR analysis confirmed increased *TH* and *NURR1* expression on day 60. The elevated expression of *FOXA2*, *LMX1A*, and *EN1* indicated a proper mFP patterning of the differentiated cells. Moreover, an increase in *GIRK2* expression, a marker for A9 neurons, was also observed. The robustness of the optimized protocol was validated in several cell lines, including the *GBA1* N409S iPSCs and its isogenic control iPSCs, confirming the high reproducibility of the protocol (Figure 6d).

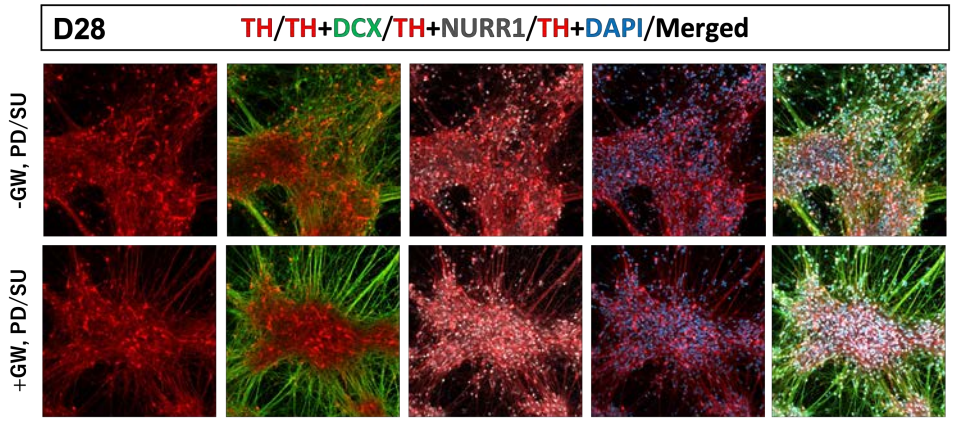

Figure 5. Immunostaining for dopaminergic markers of EPIPSC cells on day 28 of differentiation with or without LXR/FGF treatment. Comparison of TH/DCX/NURR1 staining and cell distribution on hiPSCs plated at 800K/cm^2 density treated with or without 10 µM GW3965, 5 µM SU5402, and 1 µM PD0325901 treatment. Images are representative of at least two independent experiments.

More thorough evaluations of the optimized differentiation protocol, including single-cell transcriptomic characterizations of differentiated cells, are ongoing.

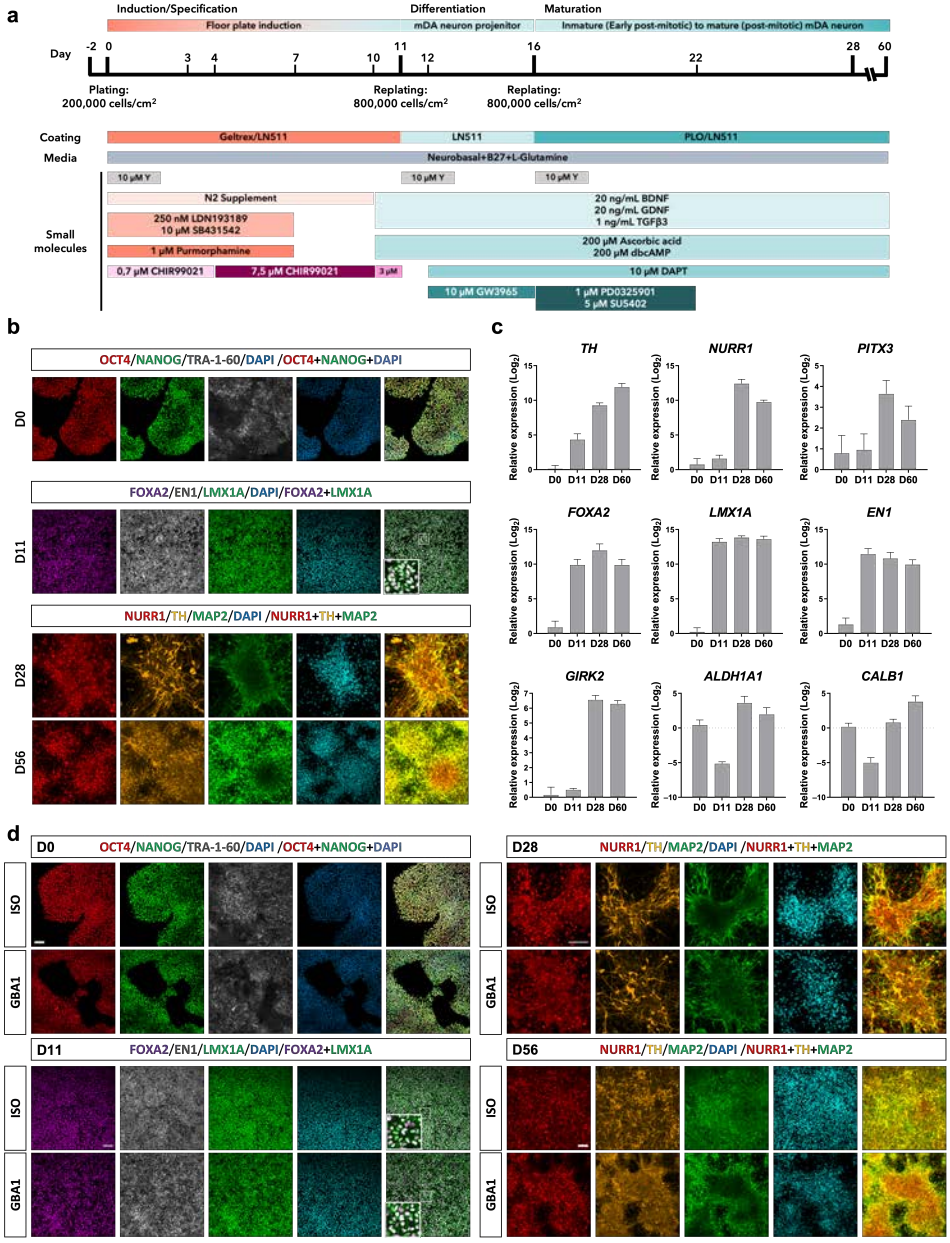

Figure 6. Optimized differentiation protocol generates mDA neurons from iPSCs. **a** Schematic of the optimized differentiation protocol. **b** Immunostaining for pluripotency markers (D0), floor plate markers (D11), and dopaminergic neuronal markers (D28 and D56) of EPIPSC iPSCs differentiated using the optimized protocol. **c** qPCR quantification of dopaminergic marker gene expression levels of EPIPSC iPSCs at the indicated time point during differentiation (n=2). Data expressed as Log_2(delta delta Ct value) normalized to day 0. *GAPDH* was used as an endogenous control for quantifications of relative mRNA levels. **d** Immunostaining for pluripotency markers (D0), floor plate markers (D11), and dopaminergic neuronal markers (D28 and D56) of GBA1 N409S mutant and isogenic iPSCs differentiated using the optimized protocol. Images are representative of at least three independent experiments.

General characterization of GBA1-PD mDA neurons (Paper II)

First, the mature GBA1-PD mDA neurons obtained through a 60-day-long differentiation underwent dopaminergic neuronal characterization. Immunoreactivity of FOXA2, LMX1A, and EN1 at day 11 confirmed proper mFP patterning (Figure 6d). Co-staining of TH, NURR1, and MAP2 demonstrated that both ISO-PD and GBA1-PD iPSCs were differentiated into mDA neurons in comparable efficiency (Figure 6d). The dopaminergic functionality of the differentiated neurons was confirmed by quantifying DA release upon stimulation (Figure 7a). To examine the expression levels of dopaminergic marker genes, bulk qPCR analysis was performed (Figure 7b). Among the dopaminergic marker genes we examined, mRNA levels of *DDC* and *COMT* were significantly lower in GBA1-PD compared to ISO-PD mDA neurons. The *DDC* gene encodes AADC, also known as DDC, an enzyme responsible for catalyzing L-3,4-dihydroxyphenylalanine (DOPA) to DA. The *COMT* gene, encoding COMT, is a key enzyme involving the degradation of several catecholamines, including DA and epinephrine. Given both *DDC* and *COMT* play a pivotal role in DA synthesis and degradation, the observed reduction in mRNA expression levels suggests a possible disturbance in DA metabolism in the GBA1-PD neurons.

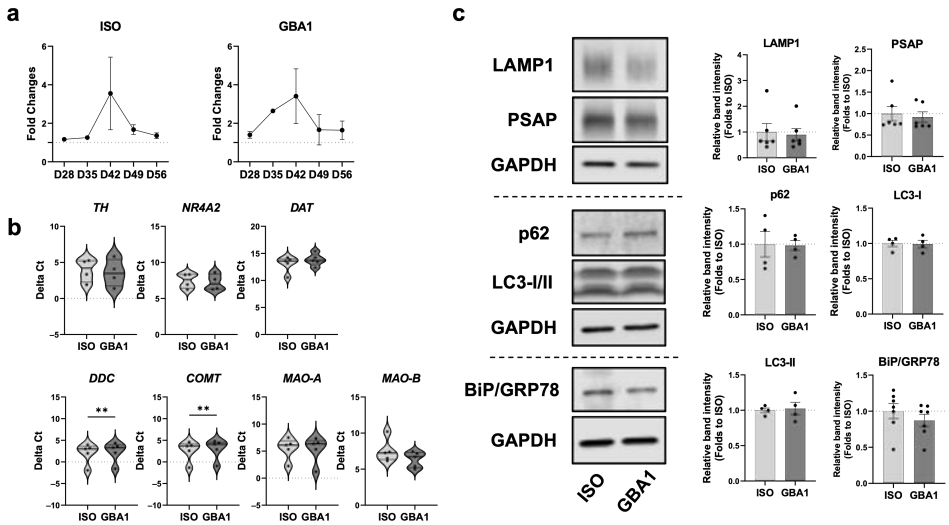

Figure 7. General characterization of mDA neurons differentiated from GBA1-PD and ISO-PD iPSCs. **a** DA release upon KCl stimulation on days 28-56. Data expressed as fold changes to basal DA levels (n=2-5). **b** qPCR quantification of mRNA levels for dopaminergic marker genes on day 60 (n=4). Data represented as delta Ct values. **c** Representative immunoblots for lysosomal proteins (LAMP1, PSAP), autophagy-associated proteins (p62 and LC3-I/II), and ER stress marker protein (BiP/GRP78) on day 60 (Left). Bar graphs of densitometric quantifications of relative protein levels (Right, n=4-7). GAPDH was used as an endogenous control for quantifications of relative mRNA and protein levels.

Autophagy-lysosomal pathway associated-protein levels in GBA1-PD mDA neurons

The impaired autophagy-lysosomal pathway is reported in GBA1-associated PD studies using patient-derived iPSCs and neural crest stem cells[183,340,341]. To assess the impact of *GBA1* mutations on autophagy-lysosomal pathways in our cellular models, we measured the levels of proteins involving autophagy-lysosomal functions and ER stress, including LAMP1, PSAP, p62, LC3-I/II, and Binding immunoglobulin protein/Glucose-regulated protein 78 (BiP/GRP78) (Figure 7c). Under basal conditions, we did not observe any changes in the protein levels of these markers in GBA1-PD mDA neurons. The result suggests that the *GBA1* N409S heterozygous mutation alone may not induce obvious defects in the autophagy-lysosomal pathway under physiological conditions with low cellular stress levels.

The link between α-synuclein, GSL, and GCase in GBA1-PD mDA neurons (Paper II)

α-synuclein is the most prevalent hallmark of PD and its specific role in the context of GBA1-PD has been explored. GlcCer, a substrate of GCase, is shown to interact with α-synuclein and promote its aggregation[77,342,343]. Recently, it has been revealed that GSL levels and composition are altered in the post-mortem PD brain and plasma[191,344]. GCase plays an important role in GSL homeostasis through the degradation of GlcCer, while the impact of dysfunctional GCase on the whole GSL metabolisms is unknown. Thus, the association of GCase to α-synuclein and GSLs was investigated,

Glycohydrolase activities in GBA1-PD mDA neurons (Paper I and II)

To investigate the roles of defective GCace in α-synuclein and GSLs, we first assessed the GBA1 (Lysosomal GCase) activity in GBA1-PD mDA neurons. Consistent with previous reports[345–347], GBA1 activity was halved in the *GBA1* mutant neurons, accompanied by reduced GCase protein levels (Figures 8a and 8b). Moreover, the enzyme activity of the GBA2, a non-lysosomal GCase, also showed a decline in GBA1-PD neurons, which is consistent with the previous studies in substantia nigra of idiopathic PD, GBA1-PD iPSC-neurons, and GD type II fibroblasts[191,347,348] (Figure 8c). It has been reported that GBA2 activity depends on GBA1 activity, but not *vice versa*[349]. Given that decreased GBA2 activity is observed along with a reduction in GBA1 activity among PD patients, an interplay between GBA1 and GBA2 is possibly involved in the pathogenesis of PD.

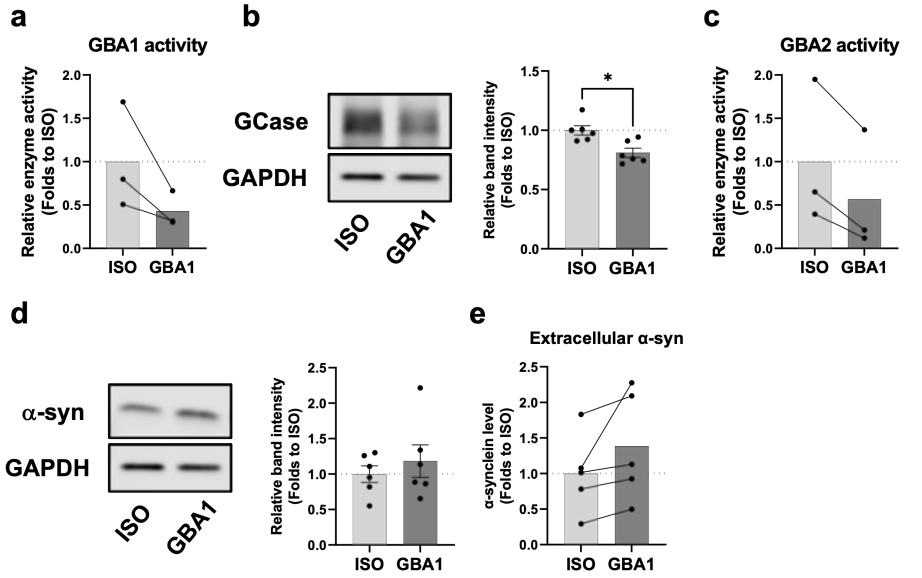

Figure 8. Reduced GCase activities and protein levels accompanied by 1.39-fold elevation of extracellular α-synuclein in GBA1-PD mDA neurons compared with ISO-PD at day 60. **a** Relative GBA1 activity (n=3). **b** Representative immunoblots for GBA1 (Left). A bar graph of densitometric quantifications of relative protein levels (Right, n=6). **c** Relative GBA2 activity (n=3). **d** Representative immunoblots for α-synuclein (Left). A bar graph of densitometric quantifications of relative protein levels (Right, n=6). **e** Relative extracellular α-synuclein levels quantified by ELISA (n=5). Data represented as fold changes to ISO-PD. GAPDH was used as an endogenous control for quantifications of relative protein levels. Two-tailed Student's t-test, *=p<0.05.

α-synuclein pathology in GBA1-PD mDA neurons (Paper II)

Next, we investigated the α-synuclein pathology in GBA1-PD mDA neurons. Contradictory to previous studies[346,347,350], we did not see the intracellular accumulation of α-synuclein protein in our GBA1-PD mDA neurons (Figure 8d). It has been suggested that reduced GCase activity does not trigger total α-synuclein accumulation or pathological α-synuclein but rather aggravates pre-existing α-synuclein pathology[351]. Our Western blot approach could only detect monomeric α-synuclein. Therefore, a more comprehensive analysis of pathological α-synuclein species, including phosphorylated α-synuclein and α-synuclein fibrils, is required to closely evaluate the association of GBA1-PD and α-synuclein pathology. This will be achieved through a combination of different detection methods (*e.g.* immunostaining, seed amplification assay).

It has been shown that secreted α-synuclein contributes to the propagation of pathology, and GCase depletion promotes the transmission of α-synuclein aggregation[352]. To investigate whether the *GBA1* mutation affects α-synuclein secretion, we measured the released α-synuclein levels in the cell culture media (Figure 8e). The result demonstrated that the extracellular α-synuclein levels were 1.39-fold elevated, although the change was not statistically significant. Notably, our findings of increased α-synuclein release but unchanged intracellular levels are consistent with the earlier findings of Fernandes *et al.*[183].

Glycosphingolipid alterations in GBA1-PD mDA neurons (Paper II)

α-synuclein has been shown to interact with lipid membranes, and the membrane-α-synuclein association is considered one of the essential physiological functions of α-synuclein. As GSLs are the major components of plasma membranes, we investigated the alterations of GSLs in GBA1-PD neurons. Despite the significant decrease in GCase activity, no deposit of total GSLs and GlcCer was observed in GBA1-PD mDA neurons compared with ISO-PD controls (Figures 9a and 9b). Deposit of GCase substrates is typical in GD patients, but whether it occurs in PD brains has been debatable. There is a discrepancy between several post-mortem PD brain studies[191,198,353], which could be derived from the difference in the brain region and the analytical methods. Huebecker *et al.* revealed a substrate accumulation in the substantia nigra of PD brains, accompanied by a decrease in multiple lysosomal hydrolase activities, including GCase[191]. On the other hand, Gegg *et al.* found no correlation between GCase activity and GlcCer levels in the putamen and cerebellum of GBA1-PD brains[198]. An *in vitro* study also demonstrated an increase in GlcCer levels in dopaminergic neurons differentiated from GBA1-PD iPSCs compared with isogenic controls or healthy controls[346,347], while another GBA1-PD iPSCs study did not reproduce this result[183]. Hence, there is a clear need for further investigations to elucidate the association between reduced GCase activity and substrate accumulation in PD.

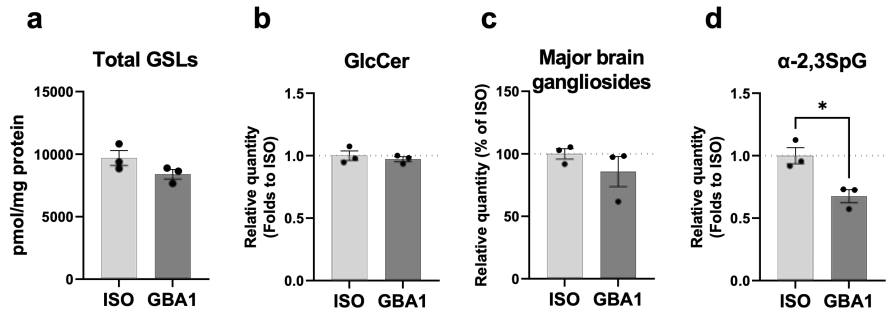

Figure 9. No accumulations of total GSLs and GlcCer, but decreased α-2,3SpG levels were observed in GBA1-PD mDA neurons compared with ISO-PD at day 60. **a** Total GSL levels (n=3). **b** Relative GlcCer quantity (n=3). Data represented as fold changes to ISO-PD. **c** Relative quantity of major brain gangliosides (GM1a, GD1a, GD1b, and GT1b) (n=3). Data expressed as percentages to ISO-PD. **d** Relative α-2,3SpG quantity (n=3). Data represented as fold changes to ISO-PD. Two-tailed Student's t-test, *=p<0.05.

It has been reported that the levels of major brain gangliosides, including GM1a, GD1a, GD1b, and GT1b declined with age, and the age-related loss of gangliosides was more pronounced in PD brains[191]. To examine whether GBA1 is involved in the reduction of brain gangliosides, we performed a detailed quantitative analysis of complex GSLs in GBA1-PD

neurons and ISO-PD neurons. In addition to the major brain gangliosides, a total of 12 species of GSLs were analyzed, including GlcCer, LacCer, α-2,3SpG, GM3, GD3, GM2, GM1a, GD1b, GM1b, GD1a, GT1a, and GQ1b (Figure 10). The result showed no statistically significant difference in major brain ganglioside levels between GBA1-PD and ISO-PD neurons (Figure 9c). This is partially consistent with the previous study reported that GSL analysis of GBA1-PD brains was statistically undistinguishable from idiopathic PD brains[191]. Surprisingly, we found that sialyl(α2-3)paragloboside (α-2,3SpG) levels were decreased in GBA1-PD mDA neurons (Figure 9d). α-2,3SpG is a neolacto-series GSL enriched in human periphery nerves. Since α-2,3SpG is not common in the adult human brain, its function in the CNS has not been explored. Therefore, the specific roles of α-2,3SpG in PD and its association with GBA1 deficiency need to be assessed in future studies. Also, while the result showed no statistically significant differences in other ganglioside levels, GM1b, GD1a, GD1b, GT1b, and GQ1b showed a decreasing trend in GBA1-PD neurons compared with ISO-PD neurons (Figure 10). Further investigations of GSL alterations in GBA1-PD will elucidate the GBA1-specific regulation of GSLs in PD, which contributes to bridging the gap between GBA1 and PD.

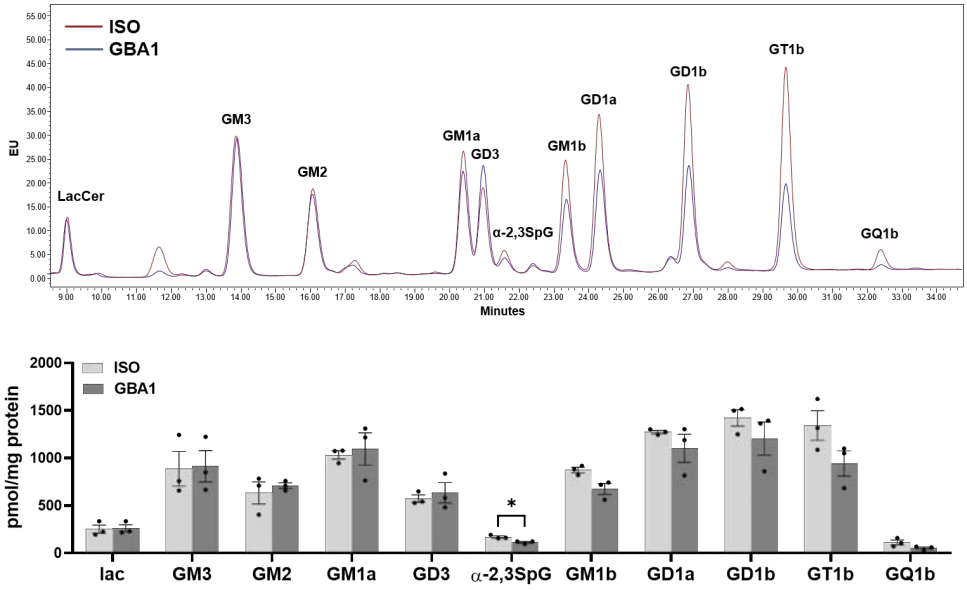

Figure 10. Representative HPLC profile for GSLs of GBA1-PD and ISO-PD mDA neurons (Top) and quantification of individual GSLs (Bottom, n=3). Two-tailed Student's t-test, *=p<0.05.

Secretome alterations in GBA1-associated PD CSF and iPSC-derived mDA neurons (Paper I)

Protein secretion is an essential intercellular communication tool. CSF is a bodily fluid circulating through the brain and the spinal cord, transporting nutrients and removing wastes. CSF also contains diverse proteins secreted from neurons and glia, thus disturbances in the CSF secretome may represent pathological conditions in CNS. Therefore, investigating secretome alterations would help identify relevant biomarkers and therapeutic targets. To examine GBA1-specific secretome changes, we analyzed the CSF of GBA1-PD and idiopathic PD (iPD) patients using a proximity extension assay (PEA) available at the Olink platform. The result demonstrated that several protein levels were significantly altered in GBA1-PD CSF compared with iPD (Figure 11a). In parallel, we also analyzed the cell culture supernatant from iPSC-derived neurons (Figure 11b). A comparison of the CSF and iPSC-derived neuron analysis identified the five hit proteins significantly altered in both CSF and GBA1-PD iPC-derived neurons.

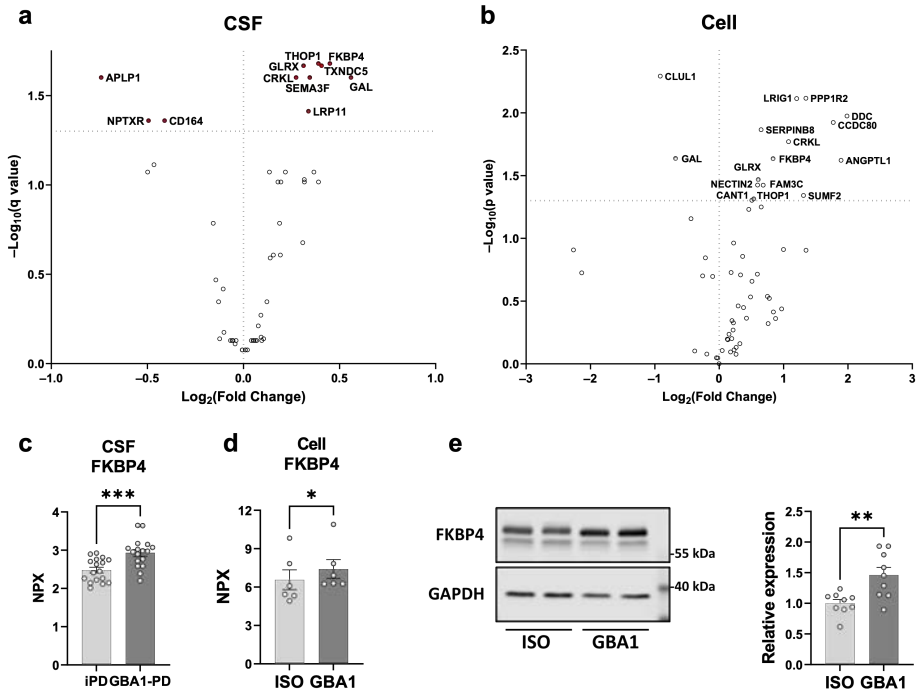

Figure 11. Secretome analysis of CSF and cell culture supernatant from GBA1-PD patients identified GBA1-specific alterations in FKBP4. **a** Volcano plot of CSF secretome analysis comparing GBA1-PD and idiopathic PD (n=17) Data represented as changes in protein levels (log_2(fold change), x-axis) versus statistical significance ($-log_{10}$(q value), y-axis). **b** Volcano plot of cell culture supernatant secretome analysis comparing GBA1-PD and ISO-PD (n=6) Data represented as changes in protein levels (log_2(fold change), x-axis) versus statistical significance ($-log_{10}$(p value), y-axis). **c** A bar graph of FKBP4 levels in CSF (n=17), and **d** cell culture supernatant (n=6). Data expressed as normalized protein expression (NPX) values. **e** Representative immunoblots for FKBP4 (Left). A bar graph of densitometric quantifications of relative protein levels (Right, n=9). Data represented as fold changes to ISO-PD. GAPDH was used as an endogenous control. Two-tailed Student's t-test, *=p<0.05, **=p<0.01, ***=p<0.001.

Among these, we opted for FKBP4, the most significantly altered protein in the CSF analysis also found in the cell culture supernatant, as a target of further investigation in iPSC-derived neurons (Figures 11c and 11d). To examine if the intracellular FKBP4 was also changed, we measured FKBP4 levels in iPSC-derived mDA neurons (Figure 11e). Immunoblot analysis showed that protein levels of FKBP4 were significantly increased in GBA1-PD neurons, demonstrating GBA1-specific regulation of FKBP4 in dopaminergic neurons.

FKBP4 is a member of the immunophilin protein family involving a wide range of cellular processes through its chaperone activities and interaction with steroid receptors. Particularly, its association with immunoregulation suggests the significance of the immune system in GBA1-associated PD, which was indicated in the recent work of Kaiser *et al.*[354]. Also, the upregulation of FKBP4 has been suggested as a biomarker for psychiatric disorders[355]. Interestingly, GBA1-PD is associated with a greater frequency of hallucinations and depression[172], implying an involvement of FKBP4 with the increased occurrence of psychiatric symptoms in GBA1-PD. While the identification of FKBP4 proposes a novel molecular mechanism in GBA1-PD, further investigation is required to determine whether elevated FKBP4 levels were specific to GBA1-PD or common in GBA1 mutation carriers regardless of PD status.

Furthermore, we correlated secretome changes in CSF and clinical data, including demographic and PD scales (the HY, MoCA, and UPDRS), to associate CSF proteins with clinical manifestations. We demonstrated that one of the upregulated proteins in GBA1-PD, CRKL, exhibits a negative correlation with UPDRS scores in GBA1-PD, whereas correlation in iPD showed the opposite trend. This finding indicates that the CSF levels of CRKL are oppositely associated with motor functions in GBA1-PD and iPD. CRKL is an adaptor protein involving diverse biological processes. The association of CRKL with PD has not been well-explored yet. However, cell type-specific CRKL expression in adult human mDA neurons has been recently revealed, implying a specific function of CRKL in mDA neurons[356]. Also, the association of CRKL in mDA neuron development through Reelin downstream signaling has been reported[357,358]. Our finding suggests an association of CRKL with motor functions, shedding light on the unrevealed role of CRKL in PD.

To conclude, by comparing the secretome of CSF and iPSC-derived neurons, we succeeded in extracting mDA neuron-attributed alterations from CSF analysis. The identified CSF protein, FKBP4, suggests its association with immune function and psychiatric symptoms in GBA1-PD. Additionally, we showed the association between secreted protein and clinical manifestations by correlating the clinical data with the secretome changes. Also, through this study, we demonstrated the potential of iPSC-derived neuron models as a tool for studying CSF alterations at cellular levels. Our iPSC models will help further investigate the pathological role of the identified proteins, including FKBP4, in GBA1-associated PD.

Role of prosaposin and saposin C in PD and its association with GCase (Paper III)

Saposin C is an essential activator of GCase. Saposin C derives from its precursor protein, PSAP, a known neurotrophic factor. Deficiency in saposin C due to a mutation in the *PSAP* gene triggers atypical GD. Recently, the link between genetic mutations in saposin D domains of the PSAP gene and PD has been suggested[223]. Given their association with GCase and neuroprotective function, PSAP and saposin C have been attracting attention as a therapeutic target for GD and PD.

To investigate the therapeutic potential of PSAP and saposin C, we generated GFP-conjugated-PSAP stably overexpressing (PSAP-OE) SH-SY5Y cells and assessed the effect of PSAP upregulation on α-synuclein pathology. We demonstrated that monomeric α-synuclein levels were significantly decreased in PSAP-OE cells compared with control cells which stably overexpressed EGFP (EGFP-OE) (Figures 12a and 12b). Moreover, we showed that extracellular α-synuclein levels were also reduced by PSAP overexpression (Figure 12c).

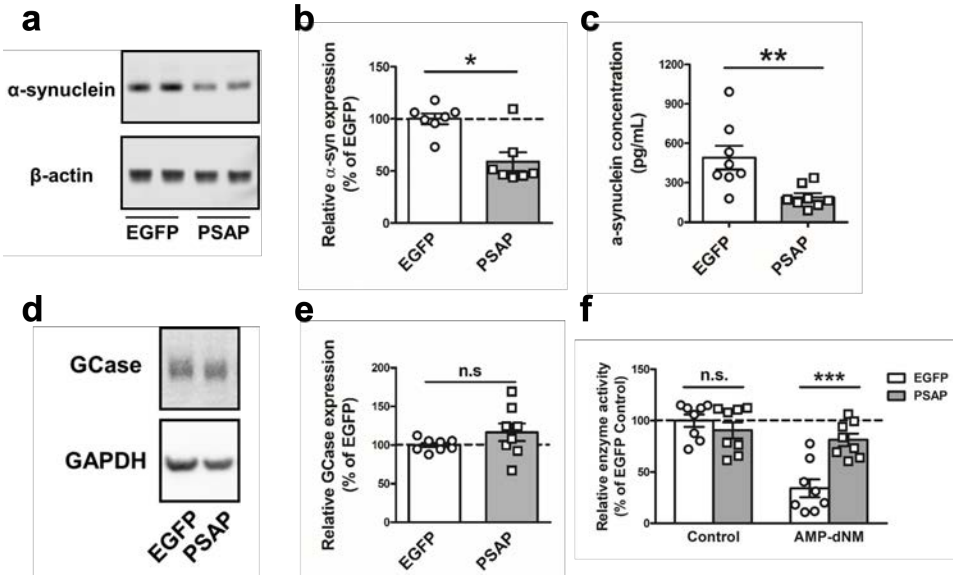

Figure 12. PSAP overexpressing SH-SY5Y cells (PSAP-OE) show reduced α-synuclein levels and upregulated GBA1 enzyme activity compared with EGFP-overexpressing control cells (EGFP-OE). **a** Representative immunoblots for α-synuclein. **b** A bar graph of densitometric quantifications of relative protein levels (n=7). Data represented as percentages to EGFP-OE. **c** Quantification of extracellular α-synuclein by ELISA (n=8). **d** Representative immunoblots for GCase. **e** A bar graph of densitometric quantifications of relative protein levels (n=8). Data represented as percentages to EGFP. **f** Relative non-specific GCase activity (Control, n=8) and selective GCase activity (AMP-dNM, n=8). Data expressed as percentages to EGFP-OE control. β-actin or GAPDH was used as an endogenous control for quantifications of relative protein levels. Mann-Whitney U-test (b, c, e) and Two-way ANOVA with Tukey's multiple comparison test (f), *=p<0.05, **=p<0.01, ***=p<0.001.

Next, we examined If PSAP overexpression affects GCase function. The protein levels of GCase and non-specific GCase activities were not changed in PSAP-OE cells (Figures 12d and 12e). However, specific GCase activity, assessed by inhibiting GBA2 activities with AMP-dNM, was significantly increased by PSAP overexpression (Figure 12f). Since GBA1-dependent GBA2 activity has been suggested, our result may represent the interplay between GBA1 and GBA2, possibly mediated by PSAP. To examine if the alteration in α-synuclein levels was caused by the upregulated GCase activity, we impeded GCase activity with CBE, an irreversible GCase inhibitor. 5-days of 100 μM CBE treatment did not alter α-synuclein levels of PSAP-OE cells, although it robustly decreased the non-specific GCase activity. Therefore, we showed that the observed reduction in α-synuclein levels was independent of GCase activity.

To further investigate the mechanisms of PSAP regulating α-synuclein levels, we assessed the alterations in autophagy-lysosomal pathways. The autophagy function was evaluated by the assessment of autophagy markers, such as LC3-I/II and p62 under basal or autophagy-impaired conditions (Figures 13a-c). For autophagy inhibition, we employed Bafilomycin A1, an autophagy inhibitor inducing an accumulation of LC3-II by blocking autolysosome formation. The accumulation of LC3-II caused by Bafilomycin A1 treatment was significantly smaller in PSAP-OE cells compared with EGFP-OE cells, implicating that PSAP affects autophagy flux. We also examined the influence of autophagy inhibition on α-synuclein levels, but no alterations were observed by Bafilomycin A1 treatment compared with dimethyl sulfoxide (DMSO) treated condition. Thus, PSAP overexpression affected autophagy while autophagy inhibition did not change α-synuclein levels.

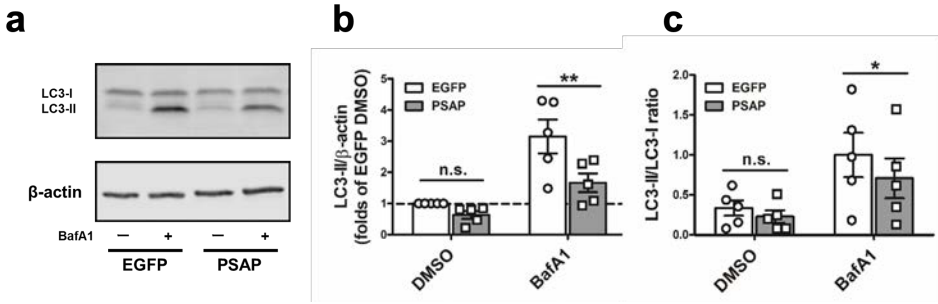

Figure 13. Altered autophagy under autophagy inhibition in PSAP-OE cells. **a** Representative immunoblots for LC3-I/II under 100 nM Bafilomycin A1 or DMSO treatment. **b** A bar graph of densitometric quantifications of relative protein levels (n=5). Data represented as fold changes to DMSO-treated EGFP-OE. β-actin was used as an endogenous control for quantifications of relative protein levels. **c** A bar graph of LC3-II/LC3-I ratio (n=5). Two-way ANOVA with Tukey's multiple comparison test, *=p<0.05, **=p<0.01.

As PSAP overexpression reduces α-synuclein levels both intra- and extracellularly, we examined if PSAP knockdown has an opposite effect on α-synuclein. We depleted endogenous *PSAP* expression by treating the cells with siRNA. Both mRNA and protein levels of PSAP were downregulated about by 50% in siPSAP-treated SH-SY5Y cells compared with control siRNA-treated cells (Figure 14a). Notably, the knockdown of PSAP led to elevated α-synuclein levels (Figure 14b). In contrast to protein levels, reduced α-synuclein mRNA levels were observed, suggesting a compensatory inhibition in *SNCA* transcription towards the α-synuclein accumulation. Hence, the upregulation and downregulation experiments of PSAP suggest that PSAP is involved in the regulation of α-synuclein levels but through a machinery independent of neither GCase activity nor autophagy function.

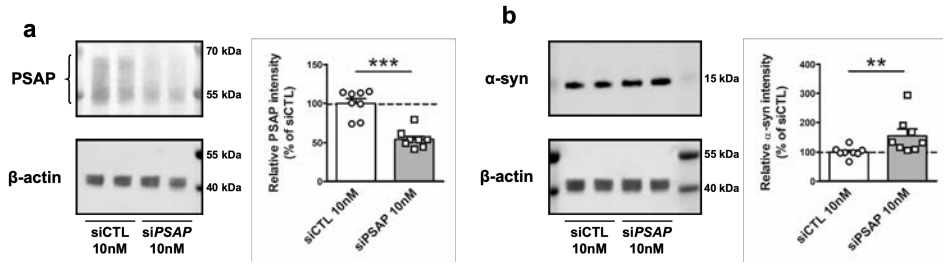

Figure 14. siRNA-mediated PSAP knockdown increased α-synuclein levels in SH-SY5Y cells. **a** Representative immunoblots for PSAP under 10 nM siCTL or siPSAP treatment (Left). A bar graph of densitometric quantifications of relative protein levels (Right, n=8). **b** Representative immunoblots for α-synuclein under 10 nM siCTL or siPSAP treatment (Left). A bar graph of densitometric quantifications of relative protein levels (Right, n=8). Data represented as percentages to siCTL-treated SH-SY5Y cells. β-actin was used as an endogenous control. Two-way ANOVA with Tukey's multiple comparison test, **=p<0.01, ***=p<0.001.

Previous studies indicate that saposin C can interfere with α-synuclein-GCase interaction by competing for GCase binding[359,360]. Saposin C is considered to interact with both GCase and the lipid membrane containing GlcCer, a substrate for GCase, and put them together to promote the enzymatic reaction[216]. To investigate whether there is an interaction between α-synuclein and saposin C towards the lipid membrane, we measured the amount of α-synuclein bound with the artificial lipid membrane under the presence of saposin C or saposin C-derived peptide Tx14(a) (Figures 15a and 15b). The amount of α-synuclein retained with the membrane was significantly decreased when saposin C was co-incubated, and this was observed in the buffer with the lysosomal pH 5.4, but not the cytoplasmic pH 7.4. We also showed that neurotrophic peptide Tx14(a) did not alter the binding of α-synuclein to the membrane, implicating that full-length saposin C is required for the interaction with α-synuclein. This finding suggests that saposin C can interfere with α-synuclein-lipid membrane binding at the lysosomal pH.

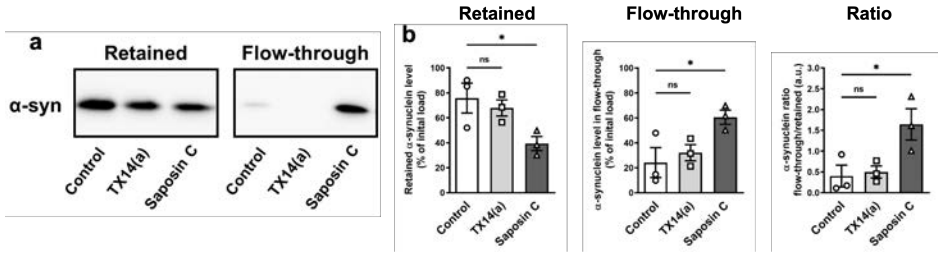

Figure 15. Saposin C but not TX14(a) detached α-synuclein from GlcCer-enriched lipid vesicles at lysosomal pH 5.4. **a** Representative immunoblots for α-synuclein retained with vesicles (Left) or detected in flow-through fractions (Right), incubated with control peptides, Tx14(a) or Saposin C. **b** Bar graphs of densitometric quantifications of relative protein levels in each condition (n=3). Right, levels of α-synuclein retained with vesicles. Middle, levels of α-synuclein detected in the flow-through fractions. Right, the ratio of flow-through/retained α-synuclein. Data represented as percentages of initial α-synuclein load. One-way ANOVA with Dunnett's multiple comparison test, *=p<0.05.

To conclude, we demonstrated that PSAP reduces α-synuclein levels, which may result from the competitive lipid binding of saposin C with α-synuclein. Given that the lipid membrane could serve as a scaffold for α-synuclein aggregation, saposin C may improve α-synuclein clearance by preventing α-synuclein from aggregation through its competitive lipid binding. However, the exact pathological role of α-synuclein binding to lipid membranes is still uncertain, thus the causal relationship between the reduced α-synuclein-membrane binding and enhanced α-synuclein clearance by PSAP needs to be investigated. Our study also revealed that altered GCase activity by PSAP overexpression or knockdown was not the principal factor modulating the α-synuclein levels. Although a more detailed investigation of the specific role of PSAP/saposin C in α-synuclein pathology is crucial, our findings suggest a therapeutic potential of PSAP/saposin C for PD.

5 CONCLUSIONS AND PERSPECTIVES

The *GBA1* gene and its encoding protein GCase have been investigated for their roles in the development of GD and PD. The great frequency of recombination between its highly homologous pseudogene adjacent to the *GBA1* gene gives rise to numerous variants, resulting in heterogeneous clinical presentations rooted in complex genotype-phenotype interactions. Particularly, although the genetic link between GBA1 and PD has been clearly demonstrated, their essential association in pathogenesis remains ambiguous. One of the most controversial arguments has been whether the reduced GCase enzyme activity accounts for PD pathogenesis. The low penetrance of PD among GD patients negates the significance of compromised enzyme activity. Nevertheless, numerous studies have demonstrated the critical role of dampened GCase activity in disease formation, particularly in association with α-synuclein pathology. This thesis reported that neither CBE-induced nor mutation-induced loss of GCase activity did not cause α-synuclein accumulation. On the other hand, we showed that the activator of GCase, PSAP/saposin C, regulates α-synuclein, possibly through interfering α-synuclein-membrane association. Our findings propose PSAP/saposin C as a potential therapeutic target for α-synuclein pathology.

iPSC-based neuronal models have been widely utilized to study neurodegenerative diseases, including PD. In this thesis, we developed a refined differentiation protocol that efficiently yields mDA neurons with high reproducibility across different cell lines. The optimized protocol allowed us to study GBA1-associated PD with mDA neurons differentiated from patient-derived iPSCs. Further sophistication and detailed validation of the differentiation protocol are currently ongoing. The established differentiation protocol for mDA neurons provides a valuable tool for studying the molecular mechanisms of PD. Moreover, the advancement in mDA neuron differentiation methods will facilitate the applications of iPSC-derived neurons in drug screening and development, even cell replacement therapy.

With iPSCs derived from a GBA1 N409S PD patient, we discovered GBA1-PD-specific alterations in CSF secretome. The identified protein FKBP4 implies the association of psychiatric symptoms prominent in GBA1-PD, bridging clinical observations and in vitro studies. Detailed investigations into the connection between FKBP4, PD, and clinical phenotypes are crucial to reveal the role of FKBP4 in GBA1-PD. Moreover, we found a GBA1-specific alteration in α-2,3SpG, a GSL that has not been explored in the context of PD. Our finding suggests either direct or indirect involvement of GCase in the regulation of GSL homeostasis, particularly α-2,3SpG. More studies are warranted to substantiate the pathological relationship between GSL alterations and PD. Also, our results are produced solely on a GBA1 N409S iPS cell line and its isogenic control iPS cell line, thus requiring further validations in other GBA1-PD-derived iPSCs in the future.

Conclusively, the findings in this thesis highlight the contribution of various factors, including PSAP/saposin C, FKBP4, and α-2,3SpG, possibly influencing the resilience of GBA1-deficient cells. Whether it falls into pathological states relies on the overall consequence of the interactions among relevant factors, with GCase potentially playing a pivotal role in the process. Identifying GBA1-associated key factors involving disease formation and elucidating their roles will help find a way to prevent developing PD and/or slow down the disease progression.

6 ACKNOWLEDGEMENTS

This work would not have been completed without the support of many people. I would like to express my gratitude and appreciation to the following people for helping me throughout this journey.

To Xiaoqun Zhang, Per Svenningsson, and Ernest Arenas, for welcoming me as a doctoral student in your group and for your supervision and support throughout my doctoral study, without which I could not achieve this work.

To Guochang Lyu, my friend and colleague, for your tremendous contribution to the stem cell work, your kind support, and the enjoyable talks we had all the time.

To Wojciech Paslawski for your committed scientific support and supervision and for being a chairperson of my defense.

To Tianyi Li for your friendly help and supervision during the early years of my doctoral study.

To Frances Platt, Kerri-Lee Wallom, and Qiaochu Zhang, for your contribution to the GSL analysis and manuscript writing, and your helpful feedback on the manuscript.

To Jean-Michel Fustin for being my mentor and for your wholehearted support for years.

To past and present Svenningsson lab members, Xavier Morató Arús, Carl-Fredrik Bowin, Daniel Dautan, Ellen Hertz, Vesna Lazarevic, Dejan Mamula, Vasco Sousa, Peter Söderberg, Johan Wallin, Yunting Yang, Justyna Zareba-Paslawska, and Mark Zurbrügg, for your kind support/supervision and fruitful discussion during seminars.

To Ji-Seon Seo for your friendly help and scientific mentoring, and for always cheering me up.

To Alonso Abney, Ellen Appleton, Niclas Branzell, Anderson Camargo, Daniel Doyon, Ivana Flais, Yachao He, Maria Kalomoiri, Shervin Khosousi, Ioannis Mantas, Akshay Naraine, Marcus Saarinen, Andrea Sturchio, for being great lab mates and for your friendly help inside/outside of the lab.

To Carmen Saltó, thank you for introducing me to the Arenas lab and providing training for stem cell culture and differentiation.

To past and present Arenas lab members, Carmen Abaurre, Emilia Síf Ásgrímsdóttir, Sandra Gellhaar, Ka Wai Lee, Kaneyasu Nishimura, Alessandra Rigamonti, Chiara Tremolanti, Natalie Welsh, Anqi Xiong, and Shanzheng Yang, for having me in the group and for your kind support. I appreciate all the fruitful discussions during meetings and the scientific knowledge/techniques you shared with me.

To Markus Karlsson and Caroline Lindblad, for being nice office mates and inviting me to wonderful Japanese/Swedish dinners you cooked.

To my Japanese former senior colleagues in Karolinska Institutet, Shunichiro Tsuji and Takeo Mukai, for introducing me to life in Sweden, giving me a fantastic rice cooker and other helpful stuff away, and for having great talks with me.

To my Japanese colleagues in Sweden, for sharing experiences and knowledge of doing PhD in Sweden as an international student, and for enjoying meals and fika together.

To my friends in Europe and Japan for having a fantastic time with me either on-site or remotely throughout stressful PhD studies, particularly during the pandemic.

To my family for supporting and watching over me from a distance with love.

To Riki, for always being on my side, for your continuous help, and for spending an enormous amount of time with me over the phone. I am so proud of both of us for getting over such a hardship together.

To Japan Student Services Organization, Shigeta Education Foundation, and Scandinavia-Japan Sasakawa Foundation for the financial support that facilitated my doctoral study at Karolinska Institutet.

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