National and Kapodistrian University of Athens

School of Health Sciences

Faculty of Medicine

1st Department of Neurology

Director: Professor Leonidas Stefanis

THESIS FOR DOCTORAL DEGREE (PhD)

The role of lysosome function in degradation of proteins that are associated with Parkinson's Disease

Vasileios E. Papadopoulos

ΔΙΔΑΚΤΟΡΙΚΗ ΔΙΑΤΡΙΒΗ

Ο ρόλος της λυσοσωμιακής λειτουργίας στην αποικοδόμηση πρωτεϊνών που σχετίζονται με τη νόσο του Parkinson

Βασίλειος Εμμ. Παπαδόπουλος

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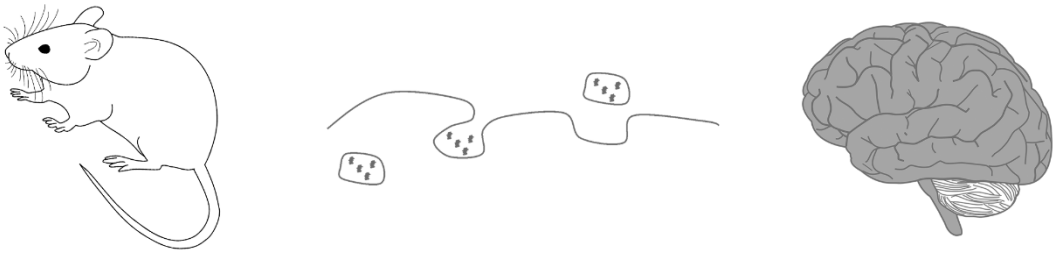
GBA-related lysosomal modulation
and its effect on α-synuclein and exosome secretion



To all the animals that gave their lives

in the name of our hypotheses

Timeline:

13 January 2014 (thesis application)
18 March 2014 (appointment of advisory committee)
17 July 2014 (topic designation)
12 September 2018 (thesis submission)
5 October 2018 (thesis defense) – Grade: Excellent (7/7)

Advisory committee

1. Kostas Potagas, MD, PhD (supervisor)
Associate Professor of Neurology
Faculty of Medicine, National and Kapodistrian University of Athens
2. Leonidas Stefanis, MD, PhD
Professor of Neurology and Neurobiology
Faculty of Medicine, National and Kapodistrian University of Athens
3. Kostas Vekrellis, PhD (scientific supervisor)
Associate Professor in Neurobiology
Biomedical Research Foundation, Academy of Athens (BRFAA)

Examination committee

1. Kostas Potagas, MD, PhD (supervisor)
Associate Professor of Neurology
Faculty of Medicine, National and Kapodistrian University of Athens
2. Georgios Koutsis, MD, PhD
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Faculty of Medicine, National and Kapodistrian University of Athens
3. Panagiota Papazafeiri, PhD
Associate Professor of Animal Physiology
Department of Biology, National and Kapodistrian University of Athens
4. Christos Proukakis, MD, PhD
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Clinical and Movement Neurosciences, Institute of Neurology, University College London
5. Georgia Sotiropoulou, PhD
Associate Professor of Pharmacology
Department of Pharmacy, University of Patras
6. Leonidas Stefanis, MD, PhD
Professor of Neurology and Neurobiology
Faculty of Medicine, National and Kapodistrian University of Athens
7. Kostas Vekrellis, PhD
Associate Professor in Neurobiology
Biomedical Research Foundation, Academy of Athens (BRFAA)

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Petros P. Sfikakis
Professor of Internal Medicine & Rheumatology

Σύνοψη

Οι μεταλλάξεις του γονιδίου της γλυκοσερεβροσιδάσης (GBA1) αποτελούν τον πιο συχνό γενετικό παράγοντα κινδύνου για την εμφάνιση νόσου του Parkinson (PD). Επιπλέον, οι μεταλλάξεις του GBA1 έχουν συσχετιστεί με ελαττωμένη ενεργότητα του λυσοσωμιακού ενζύμου της γλυκοσερεβροσιδάσης (GCase) σε ασθενείς με PD, αλλά και ασθενείς PD που δεν φέρουν μετάλλαξη στο GBA1 παρουσιάζουν επίσης ελαττωμένη ενεργότητα της GCase στο κεντρικό νευρικό σύστημα. Συνολικά, φαίνεται πως η ενεργότητα του συγκεκριμένου ενζύμου διαδραματίζει σημαντικό ρόλο στην παθογένεια της PD. Όσον αφορά τον μηχανισμό δια του οποίου μεσολαβείται η συνεισφορά των μεταλλάξεων του GBA1 στον κίνδυνο εμφάνισης της νόσου, είναι πιθανότερα η μεταβολή της λυσοσωμιακής οδού αποικοδόμησης πρωτεϊνών. Η α-συνουκλεΐνη, η πρωτεΐνη που κατέχει κεντρικό ρόλο στην παθογένεια της PD, έχει δειχθεί ότι εκκρίνεται εξωκυττάρια, τόσο ως ελεύθερη πρωτεΐνη, όσο και ως περιεχόμενο των εξωσωμάτων. Είναι πιθανό η δυσλειτουργία του μονοπατιού της ενδοκυττάρωσης μέσω δυσλειτουργίας της GCase να συμβάλλει σε αλλαγή της έκκρισης εξωσωμάτων και της σχετιζόμενης με εξωσώματα α-συνουκλεΐνης.

Ο σκοπός της παρούσας μελέτης ήταν να εξεταστεί η αλλαγή στη συσσώρευση και την έκκριση της α-συνουκλεΐνης μέσω χειρισμών στη μορφή και την ενεργότητα του λυσοσωμιακού ενζύμου της GCase.

Στο πρώτο στάδιο της διατριβής, κατασκευάστηκαν τέσσερις αδενοϊοί που εμπεριείχαν την αγρίου τύπου (WT), τις μεταλλαγμένες μορφές N370S και D409V της GCase ή το γονίδιο για την πράσινη φθορίζουσα πρωτεΐνη (GFP) ως μάρτυρα. Οι ιοί χρησιμοποιήθηκαν για τη μόλυνση πρωτοταγών καλλιεργειών φλοιϊκών νευρώνων από μύες. Η μεσολαβούμενη από αδενοϊό υπερέκφραση της WT GCase οδήγησε σε σημαντική μείωση της έκκρισης εξωσωμάτων in vitro. Η ποσότητα της α-συνουκλεΐνης τόσο ενδοκυττάρια όσο και εξωκυττάρια παρουσίασε τάση αύξησης η οποία δεν ήταν στατιστικά σημαντική.

Στο δεύτερο στάδιο της διατριβής, σε νέα in vitro πειράματα η συμβολή της ενεργότητας της GCase ελέγχθηκε με την υπερέκφραση της α-συνουκλεΐνης μέσω αδενοϊών, παρουσία ή μη του κονδουριτολικού β-εποξειδίου ομοιοπολικού αναστολέα (CBE), εκλεκτικού αναστολέα της GCase. Εκ νέου, παρουσία του αναστολέα CBE, παρατηρήθηκε τάση αύξησης της α-συνουκλεΐνης, που δεν ήταν στατιστικά σημαντική.

Στο τρίτο στάδιο της διατριβής, διενεργήθηκαν in vivo πειράματα. Επιλέχθηκαν διαγονιδιακοί μύες οι οποίοι ήταν ετερόζυγοι για την μετάλλαξη A53T του γονιδίου της α-συνουκλεΐνης (SNCA). Σε αυτό το μοντέλο ήταν δυνατή η χρόνια αναστολή του ενζύμου της GCase, μέσω της διαπεριτοναϊκής χορήγησης του CBE σε περιοδικά διαστήματα ώστε να προσομοιαστεί η συνεχώς μειωμένη ενεργότητα της GCase σε ασθενείς PD. Πράγματι, η ενδοκυττάρια ολιγομερής μορφή της α-συνουκλεΐνης ήταν στατιστικά σημαντικά αυξημένη στους μύες που έλαβαν τον αναστολέα CBE. Μάλιστα, η αναστολή της GCase προκάλεσε μία αθρόα αύξηση των εγκεφαλικών εξωσωμάτων και της ολιγομερούς α-συνουκλεΐνης που εμπεριέχεται σε εξωσώματα. Στη συνέχεια, για να ελεγχθεί ο ρόλος της μεταλλαγμένης GCase σε ένα χρόνιο μοντέλο της PD, χρησιμοποιήθηκαν ιοί για την υπερέκφραση μεταλλαγμένης GCase στους διαγονιδιακούς για την A53T α-συνουκλεΐνη μύες. Χρησιμοποιώντας τη μέθοδο της διαπίδυσης μπορέσαμε να μετρήσουμε την εκκρινόμενη α-συνουκλεΐνη στο ραβδωτό σώμα ζώντων μυών. Με ιδιαίτερο ενδιαφέρον παρατηρήσαμε ότι οι μύες που υπερεξέφραζαν την μεταλλαγμένη N370S GCase είχαν αυξημένη έκκριση α-συνουκλεΐνης. Σε περαιτέρω πειράματα στα εν λόγω ζώα, φάνηκε ότι αυτή η αύξηση της έκκρισης της α-συνουκλεΐνης οφειλόταν σε δυσλειτουργία του λυσοσώματος, όπως αναδείχθηκε με ανοσοϊστοχημική χρώση για τον δείκτη αυτοφαγίας LC3.

Συνοψίζοντας, η παρούσα διδακτορική διατριβή οδήγησε σε ενδιαφέροντα, καινούρια ευρήματα. Τα αποτελέσματα των ανωτέρω πειραμάτων αποτελούν τα πρώτα στοιχεία για το ότι η μειωμένη ενεργότητα της φυσιολογικής GCase ή η υπερέκφραση μεταλλαγμένης GCase σε ένα χρόνιο in vivo περιβάλλον οδηγούν σε αύξηση της έκκρισης της α-συνουκλεΐνης. Φάνηκε ότι αυτή η μεταβολή οφείλεται, τουλάχιστον εν μέρει, σε δυσλειτουργία της αυτοφαγίας.

Αυτή η δράση της GCase μπορεί να οδηγεί σε ενίσχυση της διάδοσης της α-συνουκλεΐνης η οποία με τη σειρά της να συμβάλλει στην εξέλιξη της παθοφυσιολογίας στην PD που σχετίζεται με μεταλλάξεις στο γονίδιο GBA1. Η σχετιζόμενη με εξωσώματα ολιγομερής α-συνουκλεΐνη μπορεί να αποτελέσει θεραπευτικό στόχο αλλά και να διερευνηθεί περαιτέρω ως πιθανός βιοδείκτης. Η μελέτη των παραγόντων που την επηρεάζουν, όπως η GCase, μπορεί πράγματι να έχει κλινική σημασία.

Abstract

Glucocerebrosidase gene (GBA1) mutations are the most common genetic contributor to Parkinson's disease (PD). Moreover, mutations of GBA1 have been correlated with decreased activity of the lysosomic enzyme glucocerebrosidase (GCase) in PD patients, while PD patients not carrying a GBA1 mutation also exhibit low enzymatic activity of GCase in the central nervous system. In total, it seems that the activity of this specific enzyme is an important factor in PD pathogenesis. The most plausible mechanism through which GBA1 mutations contribute to the risk of developing PD is an alteration of the lysosome pathway of protein degradation. α-Synuclein, a protein with a central role in PD pathogenesis, has been shown to be secreted extracellularly, both as a free form protein and in association with exosomes. A dysregulation of the endocytic pathway through the dysfunction of GCase could result in an alteration of exosome and exosome-associated α-synuclein secretion.

The aim of this study was to examine whether manipulating GCase activity and form both in vivo and in vitro could affect α-synuclein accumulation and secretion.

In the first part of this dissertation, four adenoviruses were constructed containing four different genes respectively; wild type (WT) GBA, mutant N370S GBA, mutant D409V GBA and a gene encoding for green fluorescent protein (GFP) as a positive control. The viruses were used to transduce primary cortical neuronal cultures acquired by embryonic mice dissection. The adenovirus-mediated overexpression of WT GCase resulted in a significant decrease of exosome secretion in vitro. Intracellular and extracellular α-Synuclein levels showed a trend of increase that was not statistically significant.

In the second part of the dissertation, in new in vitro experiments, the contribution of the activity of GCase to α-synuclein levels was investigated by adding conduritol-B epoxide (CBE), a selective inhibitor of GCase, in the presence of adenovirus-mediated α-synuclein overexpression. A non-significant trend of increase of α-synuclein levels was observed in the presence of CBE inhibition.

In the third part of the dissertation, in vivo experiments were performed. Transgenic mice heterozygous for the A53T mutation of the gene encoding for α-synuclein (SNCA) were chosen. In this PD model, a chronic inhibition of the GCase enzyme was achieved by intraperitoneal injections of CBE in specific time intervals. The scheme adopted allowed us to emulate the chronic enzymatic activity decrease of GCase in PD patients. The intracellular oligomeric form of α-synuclein was significantly increased in the mice that received the CBE inhibitor. More specifically, GCase inhibition resulted in profound increase of brain exosomes and oligomeric α-synuclein contained in exosomes. Followingly, in order to examine the role of mutant GCase in a chronic model of PD, adeno-associated viruses were used to overexpress mutant GCase in the striatum of A53T SNCA transgenic mice. Using microdialysis, we were able to measure secreted α-synuclein levels in the same region. Interestingly, mice overexpressing N370S mutant GCase showed an increased secretion of α-synuclein. In further experiments, it was revealed that the α-synuclein secretion increase was due to lysosomal impairment, as observed by immunohistochemical staining for the LC3 autophagy marker.

In conclusion, this dissertation resulted in interesting, novel findings. Our results suggest that, in a chronic in vivo model of PD, GCase activity decrease or mutant GCase overexpression result in α-synuclein secretion increase. It seems that this alteration is, at least in part, due to an autophagy impairment. This GCase effect could lead to increased α-synuclein secretion, driving pathology in GBA-associated PD. Exosome-associated oligomeric α-synuclein, apart from a therapeutic target, may also prove to be a relevant biomarker for PD, and thus the study of its modifiers, such as GCase shown here, may prove to be clinically relevant.

Acknowledgements

Doing a PhD in Greece of 2013 was a prospect I had not really considered beforehand. Coming to think of it, a few months after graduating from Medical School, my first option was to start working as a medical doctor. However, a keen interest in science, stemming from my high school years, gradually manifested itself as a desire to actively contribute to scientific knowledge.

A major and perhaps the most important factor in being able to shift direction was my encounter with Kostas Vekrellis, who was to become my supervisor. He was probably the first person to point me towards the direction of pursuing a PhD, at a time when I was more focused on moving abroad to work as a medical doctor. His guidance throughout the years proved essential to my scientific formation: he encouraged me to start thinking as a scientist, to pose the right questions and to work efficiently in answering them.
Nonetheless, my interest in science in general and more specifically in neuroscience dated long before 2013. The great mystery of the human brain and its intriguing complexity was a field that had fascinated me since my early years at Medical School. After meeting professor Kostas Potagas as an undergraduate student, this interest began to appear as the one I should devote my efforts to. His fascinating lectures and intriguing approach to Neurology made me realize that Neurology and Neuroscience were the fields I wanted to delve in. After my graduation, he agreed to become advisor to my thesis and offered me guidance and support valuable to my research and to my overall training in medicine.
Finally yet importantly, I have to stress the contribution of professor Leonidas Stefanis. His insightful perspective, a result of his vast experience as both a researcher and a physician, was essential to my work throughout the years, and I am certain that it will remain vital in my future endeavors.

What I most certainly did not know when I started working on my PhD was how big an effect this period of my life would have on my views and personality. It was a time of great political and social change in Greece and, unfortunately, the academic field was substantially affected by these circumstances. Working

as a researcher and as a PhD student during those years was extremely hard, both mentally and financially. In difficult times however, I was fortunate enough to meet coworkers that would prove to be great friends.

I am certain that my time at the institute would have been a lot less bearable had I not met Giovanna Arianoglou and Vassia Sykioti: my fellow PhD students who taught me everything in the beginning and remained my close friends through the years. The same goes for Eleni Vassilaki, our dear friend and mice expert, who always accompanied us both inside and outside the institute. As for Oeystein Brekk, my great Norwegian friend, one thing is certain: his astute observations and witty comments is something I miss a lot now he has moved on to pursue his dreams in the USA.
Working and living in Athens was an experience these people and I shared together and I am truly happy we did. Yet they are not the only ones to which I am thankful.

Working at the institute allowed me to meet people who had a great impact on my work and views. Mantia Karampetsou, a great scientist and thoughtful coworker, was someone who was always eager for debate, whether it be scientific or political. Together, along with Marina Pantazopoulou, we were able to evolve, act upon our scientific knowledge and take a political stance when times demanded to, something for which I feel proud and indebted to them both.
Antonia Karachaliou was my first and only undergraduate student. Who knew back then that she would prove to be such an amazing student and promising scientist? I most certainly hope I was able to inspire her and contribute to her evolvement to an accomplished researcher.
I cannot omit Georgia Nikolopoulou and Nikos Papagiannakis: the two people who, coming from a similar background, were the first to welcome me to the lab. Their input was vital for me in the beginning and, of course, Georgia's work was the basis that allowed me to build upon and complete a project that would result in a publication.

It seems like a while back now, but I cannot forget Methodios Ximerakis' contribution to my early troubles with cloning. We only worked together for a

brief period before he moved to the USA, but his help was invaluable, for both my PhD and my confidence.
Of course, I have to mention our team's post doc at the time and a professor by now, Evangelia Emmanouilidou. Her work in the lab, her calm presence and insightful contributions were crucial to my efforts, and still are.

It sounds like this lab was a bit crowded and indeed, it was. There, I was to encounter Emmanuela Leandrou, my once classmate in elementary school with whom I was to reunite unexpectedly, and many others along her. Maria Xilouri, the dedicated researcher, Maria Keramioti, my bench neighbor. Katerina Melachrinou, the inspired singer and source of positive thinking. Hardy Rideout, the calm researcher, Georgia Dermentzaki, the ambitious researcher. Matina Maniati, our lab technician. Alexia Polissidis, the happy and polite coworker, Ivi Antoniadou, the founder of our notorious Book Club. Anna Memou and Martina Kamaratou, my fellow cloning survivors. Mary Xylaki, Maria Tsioka, Maria Koronaiou, Vasia Kollia, Maria Nikatou, the inspired master students that worked with us. I am most grateful to have worked with them all.

Finally, I cannot help but pay tribute to the coworkers that stood in solidarity when we had to fight for our rights as PhD students and as scientists who protect the interests of their fellow citizens.

Last but not least, I have to remark the support I received from people close to me. A PhD is more than a full time job that affects all parts of your life. I would not have been able to pull this through had it not been for Phaedra Gavouneli, the person that supported me the most through good times and bad.

My family and friends, who were there when I needed them and backed my choices with love and interest, I thank you once again.

Vassilis E. Papadopoulos
Athens, August 2018

Hippocratic Oath

I swear by Apollo the Healer, by Asclepius, by Hygieia, by Panacea, and by all the gods and goddesses, making them my witnesses, that I will carry out, according to my ability and judgment, this oath and this indenture.

To hold my teacher in this art equal to my own parents; to make him partner in my livelihood; when he is in need of money to share mine with him; to consider his family as my own brothers, and to teach them this art, if they want to learn it, without fee or indenture; to impart precept, oral instruction, and all other instruction to my own sons, the sons of my teacher, and to indentured pupils who have taken the physician's oath, but to nobody else.

I will use treatment to help the sick according to my ability and judgment, but never with a view to injury and wrong-doing. Neither will I administer a poison to anybody when asked to do so, nor will I suggest such a course. Similarly I will not give to a woman a pessary to cause abortion. But I will keep pure and holy both my life and my art. I will not use the knife, not even, verily, on sufferers from stone, but I will give place to such as are craftsmen therein.

Into whatsoever houses I enter, I will enter to help the sick, and I will abstain from all intentional wrong-doing and harm, especially from abusing the bodies of man or woman, bond or free. And whatsoever I shall see or hear in the course of my profession, as well as outside my profession in my intercourse with men, if it be what should not be published abroad, I will never divulge, holding such things to be holy secrets.

Now if I carry out this oath, and break it not, may I gain for ever reputation among all men for my life and for my art; but if I break it and forswear myself, may the opposite befall me.

Hippocrates of Cos (1923). "The Oath". Loeb Classical Library. Translated by James Loeb.

Curriculum Vitae

PERSONAL INFORMATION

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Sex Male | Date of birth 13/10/1989 | Nationality Greek

APPLIED FOR

WORK EXPERIENCE

2/6/2016 – present

Neurology Resident

Laiko General Hospital, Athens, Greece
Eginition University Hospital, Athens, Greece

- Neurological Clinical Examination of patients
- Emergency department - Patient Evaluation
- Diagnostic Lumbar Puncture
- Therapeutic regime planning
- Neurology Outpatient Clinic assistant
- Multiple Sclerosis Outpatient Clinic assistant

5/1/2015 – 1/6/2016

Medical Doctor Volunteer

Médecins du Monde – Greece (MdM-Greece)

- "Emergency support to assist most vulnerable migrants stranded in Greece" - Primary Healthcare Support
- "Public Health: Prevention – Testing – Support" - Primary Healthcare Support

18/3/2014 – present

PhD thesis "The role of lysosomal function in the degradation of proteins associated with Parkinson's disease"

Centre for Basic Research, Biomedical Research Foundation, Academy of Athens, Greece

- Western blotting
- ELISA
- Isolation and characterization of exosomes from cell cultures and tissue
- Viral infection and plasmid transfection of proliferating cell cultures and primary cultures of neurons
- Immunocytochemistry
- Confocal microscopy
- PCR
- DNA cloning
- Adenovirus construction
- Isolation of primary cultures of neurons from dissected mice

- Intraperitoneal injections of mice
- Exosome isolation from tissue and cell culture

EDUCATION AND TRAINING

18/3/2014 - present

"The role of lysosomal function in the degradation of proteins associated with Parkinson's disease", Doctor of Philosophy (PhD)

National and Kapodistrian University of Athens, School of Medicine

- Basic Research skills
- Statistical analysis
- Protocol construction
- Troubleshooting
- Scientific method and thinking

21/9/2007 – 22/7/2013

School of Medicine (MD)

National and Kapodistrian University of Athens, School of Medicine

- 7,7/10
  Top of Class in Neuroscience courses:
- Neurology (10/10)
- Pathology of the Nervous System (10/10)
- Psychiatry (9/10)

1/9/2013 – 31/9/2013

IFMSA Professional Exchange Programme “Pain Management”

Deparment of Pain Management, Royal Cornwall Hospital, Truro, Cornwall, United Kingdom

- Clinical Examination of patients
- Therapeutic regime planning
- Minor operations: Cannula placing, Intubation

1/7/2011 – 31/7/2011

IFMSA Professional Exchange Programme in Neurology

Department of Neurology, American University of Beirut Medical Center, Lebanon

- Clinical Examination of patients
- Case report and journal club seminars

1/7/2011 – 31/7/2011

IFMSA Research Exchange Programme “Ultrasonic Endoscopy and Colonoscopy”

University of Craiova, School of Medicine, Romania

Mother tongue(s): Greek

Other language(s)

	UNDERSTANDING		SPEAKING		WRITING
	Listening	Reading	Spoken interaction	Spoken production	
English	C2	C2	C2	C2	C2
French	B1	B1	B1	B1	B1
Danish	A1	A1	A1	A1	A1

Levels: A1/A2: Basic user - B1/B2: Independent user - C1/C2 Proficient user

ADDITIONAL INFORMATION

Publications

How does an undergraduate pain course influence future physicians' awareness of chronic pain concepts? A comparative study, Pain Med. 2015 Feb;16(2):301-11

Modulation of β-Glucocerebrosidase Increases α-Synuclein secretion and Exosome release in Mouse Models of Parkinson's Disease. Hum. Mol. Genet., 27, 1696-1710.

Presentations

10/2014 Seminar and Workshops on Scientific Debate & Critical Thinking, Turku, Finland **- Lecturer**

06/2015 Alpha-synuclein and the lysosomes in Parkinson's disease: a complicated relationship - **Poster presentation: Examining the role of glucocerebrosidase overexpression in α-synuclein release**

03/2017 11th World Congress on Controversies in Neurology (CONy) - **Poster presentation: Anatomical cause of Gerstmann like syndrome identified through MR DTI Tractography**

09/2017 20 years of alpha-synuclein in Parkinson's Disease and related synucleinopathies: "from the bedside to the bench and back to the patient" - **Oral presentation: Modulation of beta-glucocerebrosidase increases αlpha-synuclein secretion and exosome release in a mouse model of Parkinson's disease**

06/2018 29th National Conference of the Hellenic Neurological Society – **Poster presentation: Meningitis Retention Syndrome and Meningocerebellitis – A rare case**

06/2018 4th Residents' Scientific Conference of the First Department of Psychiaty, National and Kapodistrian University of Athens – **Oral Presentation: Psychosis in extrapyramidal syndromes**

Conferences

10/2008 29th Panhellenic Cardiology Conference, Athens, Greece

10/2009 The Brain: Function, Imaging & Repair, Athens, Greece

12/2011 Advances 2011 & Future Prospects 2012 in Cardiology, Athens, Greece

05/2013 46th Annual Pediatric Therapeutic Meeting, Athens, Greece

06/2015 Alpha-synuclein and the lysosomes in Parkinson's disease: a complicated relationship

12/2016 Hellenic Stroke Organization Stroke Meetings **-03/2017**

03/2017 11th World Congress on Controversies in Neurology (CONy)

09/2017 20 years of alpha-synuclein in Parkinson's Disease and related synucleinopathies: "from the bedside to the bench and back to the patient"

03/2018 Hellenic Stroke Organization Conference

06/2018 29th National Conference of the Hellenic Neurological Society

06/2018 4th Residents' Scientific Conference of the First Department of Psychiaty, National and Kapodistrian University of Athens

Seminars

03/2014 Aphasia Workshop, Eginition University Hospital, Athens, Greece

Honours and awards

03/2017 **CONy Best E-Poster Award**, Best E-Poster presentation, 11th World Congress on Controversies in Neurology (CONy)

09/2017 **AWARDED ABSTRACT**, Best Poster Awards, 20 years of alpha-synuclein in Parkinson's Disease and related synucleinopathies: "from the bedside to the bench and back to the patient"

05/2018 **Scholarship**, Scholarship for Postgraduate Studies in Clinical Neuroscience, 44th Annual Conference of the Athens Medical Society

References

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Citations

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List of Abbreviations

AAV: adeno-associated virus
AChE: acetylcholinesterase
AICD: APP intracellular domain
ALS: amyotrophic lateral sclerosis
ANOVA: Analysis of variance
AP: Anterior – Posterior
APP: Amyloid Precursor Protein
ASYN: α-synuclein
ATP13A2: ATPase cation transporting 13A2 gene
AV: adenovirus
Aβ: Amyloid beta
BRFAA: Biomedical Research Foundation of the Academy of Athens
BSA: Bovine serum albumin
CBE: conduritol-B epoxide
cDNA: complementary DNA
CJD: Creutzfeldt-Jakob disease
CM: condition medium/media
CMA: Chaperone-mediated autophagy
CNS: central nervous system
CSF: Cerebrospinal fluid
DAPI: 4',6-diamidino-2-phenylindole
DIV: day in vitro
DJ1: human protein deglycase gene
DLB: Dementia with Lewy bodies
DNA: Deoxyribonucleic acid
DV : Dorsal – Ventral
ECL: enhanced chemiluminescence substrate
EDTA: Ethylenediaminetetraacetic acid
ELISA: Enzyme-linked Immunosorbent Assay
ER: Endoplasmic reticulum
ERAD: Endoplasmic-reticulum-associated protein degradation
ESCRT: endosomal sorting complexes required for transport

GAPDH: Glyceraldehyde-3-Phosphate Dehydrogenase

GBA\GBA1: Glucocerebrosidase gene

GCase: Glucocerebrosidase

GD: Gaucher Disease

GFP: Green Fluorescent Protein

GPI: glycosylphosphatidylinositol

GTPase: guanosine triphosphatase

GWAS: Genome Wide Association Studies

HEK293/HEK293A: Human Embryonic Kidney Cell Line 293 (A)

HRP: Horseradish peroxidase

IP: intraperitoneal

iPD: idiopathic PD

iPSC: induced pluripotent stem cells

ISF: interstitial fluid

KO: knockout

LAMP-1: Lysosomal-associated membrane protein-1

LC3: Microtubule-associated proteins 1A/1B light chain 3A

LIMP2: lysosomal integral membrane protein type 2

LSD: Least Significant Difference

LRRK2: Leucine-rich repeat kinase 2 gene

MAPT: Microtubule-associated protein tau

miRNA: Micro ribonucleic acid

ML : Medial – Lateral

MOI: Multiplicity of infection

MPTP: 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine

mRNA: Messenger ribonucleic acid

MSA: Multiple system atrophy

MVB: multivesicular body

NBIA1: pantothenate kinase 2 gene

NBIA2: phospholipase A2 group VI gene

NEDD4: neural precursor cell expressed, developmentally down-regulated 4, E3 ubiquitin protein ligase

NGS: Normal Goat Serum

N/S: Normal Saline

PBS: Phosphate-buffered saline

PARK-: Parkinson disease associated genes

PD: Parkinson's Disease

PDD: Parkinson's Disease Dementia

PANK2: pantothenate kinase 2 gene

PINK1: PTEN induced putative kinase 1 gene

PLA2G6: phospholipase A2 group VI gene

PRKN: parkin RBR E3 ubiquitin protein ligase gene

Rab11: a small GTPase of the Rab subfamily of Ras-related GTPases

RBD: REM sleep behavior disorder

REM: Rapid eye movement

RFU: Relative Fluorescent Units

RNA: Ribonucleic acid

sAPPβ: secreted APPβ

SCARB2: scavenger receptor class B member 2 gene

SN: Substantia Nigra

SNCA: synuclein alpha gene

SDS: Sodium dodecyl sulfate

SEM: Standard Error of the Mean

SNARE: Soluble NSF (N-Ethylmaleimide-Sensitive Factor) Attachment

Protein Receptor SUMO: Small Ubiquitin-like Modifier

TBST: Tris Buffered Saline with Tween

TUJ1: Neuron-specific Class III β-tubulin

UCHL1: ubiquitin C-terminal hydrolase L1 gene

UPS: ubiquitin proteasome system

USA: United States of America

USP8: ubiquitin specific peptidase 8

vp: viral particles

VPS35: Vacuolar protein sorting 35

VSP4: Vacuolar sorting protein 4

WT: Wild Type

1. Introduction

1.1 Parkinson's disease

Parkinson's disease (PD) is the second-most common neurodegenerative disorder and affects 3% of the population above 65 years of age. Mortality increases after the first decade of disease onset, eventually doubling compared with the general population (1). Research and consequent improvement in health care has led to longer survival, which is associated with increasing prevalence of PD over time in one 20-year study (2). The number of people with PD is indeed expected to double between 2005 and 2030 (3), while no disease modifying cure is available yet.

The disease is usually diagnosed by the emergence of motor symptoms, which seemingly do not coincide with the onset of pathology in the Central Nervous System (CNS). The diagnosis is based on criteria from the UK PD Brain bank [Table 1.1, (4)].

Slowness of initiation of voluntary movements with progressive reduction in speed and amplitude of repetitive actions (bradykinesia) with one additional symptom, i.e., muscular rigidity, resting tremor or postural instability, are a prerequisite for the diagnosis [Figure 1.1, (4)].

Table 1.1 UK Parkinson's Disease Society Brain Bank clinical diagnostic criteria

Step 1: Diagnosis of Parkinsonian syndrome

Bradykinesia (slowness of initiation of voluntary movement with progressive reduction in speed and amplitude of repetitive action)

And at least one of the following

- Muscular rigidity
- 4–6 Hz rest tremor
- Postural instability not caused by primary visual, vestibular, cerebellar or proprioceptive dysfunction

Step 2: Exclusion criteria for Parkinson's disease

History of repeated strokes with stepwise progression of parkinsonian features
History of repeated head injury
History of definite encephalitis
Oculogyric crises
Neuroleptic treatment at onset of symptoms
More than one affected relative
Sustained remission
Strictly unilateral features after 3 years
Supra-nuclear gaze palsy
Cerebellar signs
Early severe autonomic involvement
Early severe dementia with disturbances of memory, language and praxis Babinski sign
Presence of cerebral tumour or communicating hydrocephalus on CT scan Negative response to large doses of levodopa (if malabsorption excluded)
MPTP exposure

Step 3: supportive prospective positive criteria for Parkinson's disease (three or more required for diagnosis of definite Parkinson's disease)

Unilateral onset
Rest tremor present
Progressive disorder
Persistent asymmetry affecting side of onset more
Excellent response (70–100%) to levodopa
Severe levodopa-induced chorea
Levodopa response for 5 years or more
Clinical course of 10 years or more

Source: Hughes et al. (1992) (4)

Figure 1.1 Illustration of the characteristic facial expression and flexed posture of a Parkinson's disease patient *Adapted from (5).*

Although historically considered a disorder of movement, it has gradually become apparent that non-motor features, such as cognitive impairment, autonomic dysfunction, disorders of sleep, depression, hallucinations and hyposmia, are part of the disease and even precede the onset of motor symptoms. More importantly, these non-motor symptoms add considerably to overall burden of patients, care takers and physicians.

1.1.1 PD genetics and epidemiology

Although heritable forms of PD only represent 5–10% of all cases (Table 1.2), they have provided crucial clues to the mechanisms underlying the neuropathology of PD (Figure 1.2).

Candidate gene association studies were mainly used to identify common variability in loci contributing to a risk of disease. This lead to studies that showed that genetic variability in the SNCA locus, and interestingly at the MAPT locus as well, contributed to disease (6-8). Since then, much progress has been made in association studies by platforms that allow the assessment of genetic variability on the order of 500,000 to 1 million variants (usually single-nucleotide polymorphisms) across the genome with the disease, also known as Genome Wide Association Studies (GWAS) (9). These loci are likely to act through modification of gene expression. Individuals who are high expressers are at marginally increased risk of disease (10) and explain findings that autosomal-dominant kindreds with gene duplications at both loci have true Parkinson's disease [SNCA (11)] or parkinsonism [MAPT (12)].

Table 1.2 Genetic forms of PD

Gene name	Chromosome location	PARK designation	Synucleinopathy
Typical PD			
LRRK2	12q12	PARK8	Lewy related pathology
Earlier onset PD			
SNCA	4q21	PARK1/4	Increased Lewy related pathology
GBA	1q21		Lewy related pathology
Young onset recessive PD			
PRKN	6q25.2-q27	PARK2	None
UCHL1	4p14	PARK5	Not enough cases
PINK1	1p36	PARK6	Not enough cases
DJ1	1p36.23	PARK7	Not enough cases
ATP13A2	1p36	PARK9	Not enough cases
PLA2G6	22q13.1	PARK14/NBIA2	Lewy related pathology, axonal spheroids
PANK2	20p13	NBIA1	Axonal spheroids

Adapted from (13)

PD is thus regarded a sporadic disorder. However, few environmental causes or triggers have so far been identified (14-16). The incidence seems to vary within subgroups defined by race, ethnicity, genotype or environment. Since geography and race are often related, it might be difficult to determine the relative contribution of each to the risk of developing Parkinson disease. In Israel, the prevalence is high, possibly reflecting the higher prevalence of the incompletely penetrant genes associated with Parkinson disease [that is, LRRK2 (which encodes leucine-rich repeat serine/threonine-Protein kinase 2) and GBA (which encodes glucocerebrosidase)] in Ashkenazi Jews (17).

1.2 PD neuropathology

1.2.1 Lewy bodies and the substantia nigra

The pathological hallmark of PD is a region-specific selective loss of dopaminergic, neuromelanin-containing neurons from the pars compacta of the substantia nigra (SN, Figure 1.2). Cell loss exists elsewhere too, with the locus coeruleus, dorsal nuclei of the vagus, raphe nuclei, nucleus basalis of Meynert, and other brain stem structures of catecholaminergic neurons including the ventrotegmental area being affected (18). This nerve-cell loss is accompanied by three distinctive intraneuronal inclusions: the Lewy body, the pale body, and the Lewy neurite. Lewy bodies are divided into classical and cortical types based on their morphology [Figure 1.2, (5)].

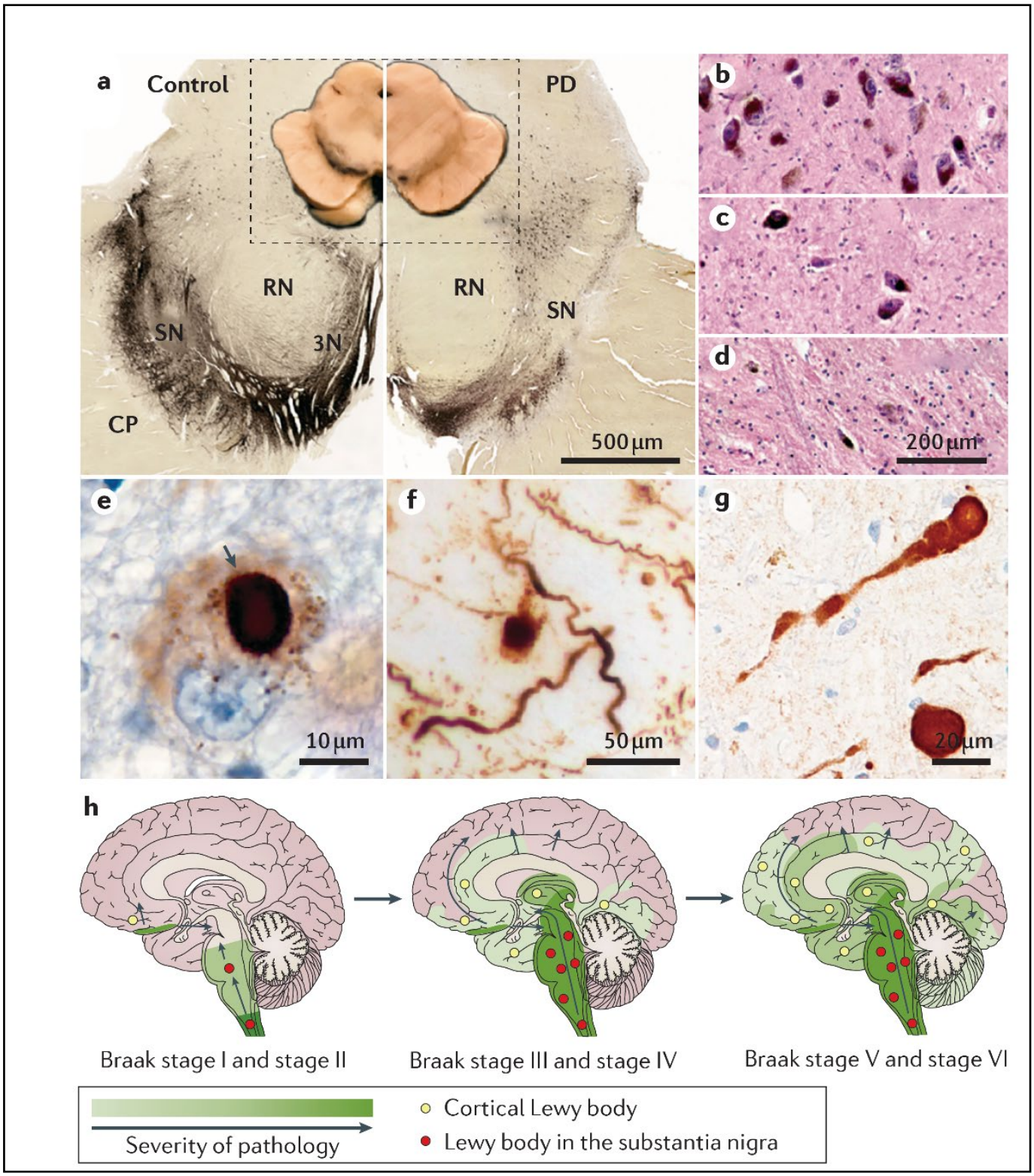


Figure 1.2 PD Neuropathology (a) *Macroscopical and transverse sections of the midbrain stained for tyrosine hydroxylase, the rate limiting enzyme for the synthesis of dopamine. In the transverse histological images, a loss of the ventrolateral parts of the SN is evident. The more medial and dorsal regions are spared. Pigmented neurons in the ventrolateral region of the SN of a healthy control* ***(b)****, showing a normal*

*distribution, and of diagnostically moderate **(c)** and severe **(d)** cell loss in PD, using Haematoxylin/ eosin staining. **(e–g)** Immunohistochemical staining of α-synuclein. Part **(e)** reveals the round, intracytoplasmic Lewy body. Parts **(e)** and **(f)** demonstrate the diffuse, granular deposits of α-synuclein. The α-synuclein deposits in neuronal cell processes and the extracellular dot-like α-synuclein structures are visible in part **(f)**. Finally, α-synuclein spheroids in axons can be seen in part **(g)**. **(h)** The Braak hypothesis of PD progression. In Braak stages I and II α-synuclein inclusions are located in cholinergic and monoaminergic lower brainstem neurons. This pathology concerns yet "asymptomatic" cases. The α-synuclein inclusions then infiltrate similar neurons in the midbrain and basal forebrain. This change represents the time point when the first motor symptoms of PD appear, in Braak stage III and stage IV. Later on, pathology can be found in limbic and neocortical brain regions accounting for cognitive symptoms in Braak stage V and stage VI. 3N: 3rd nerve fibres, CP: cerebral peduncle, RN: red nucleus. Adapted from (19)*

Since degeneration only occurs in specific types of neurons within certain brain regions, a detectable via imaging atrophy of the brain is not present in Parkinson disease. It seems that early on, pathology is restricted to the dopaminergic neurons of the ventrolateral SN with relative sparing of other midbrain dopaminergic neurons (18, 20), but becomes more widespread by end-stage disease (Figure 1.2). The dramatic loss of these dopaminergic neurons even early in the disease suggests that the degeneration in this region starts before the onset of motor symptoms, which is supported by several clinicopathological studies (21, 22).

1.2.2 α-Synuclein and neurodegeneration

An abnormal, post-translationally modified, and aggregated form of the presynaptic protein α-synuclein is the main component of Lewy bodies. Although neither the loss of pigmented dopaminergic neurons in the SN (13), (23) nor the deposition of α-synuclein in neurons is specific for Parkinson's disease, these two major neuropathologies are specific for a definitive diagnosis of idiopathic Parkinson disease when applied together [Figure 1.2, (24)].

α-Synuclein was found in Lewy bodies inside neurons and it was assumed that its pathogenic effect occurred at the intracellular level and more specifically in the synaptic function [Figure 1.3, (25)]. However, it was later discovered that α-synuclein is also secreted and could potentially have a pathogenic effect extracellularly (26). Indeed, a number of reports has demonstrated that various species of α-synuclein can induce neurotoxic (26-28) and inflammatory responses (29-36) when applied extracellularly. Interestingly, α-synuclein species are able to propagate from one cell to another [Figure 1.4, (37-42)]. The extracellular presence of α-synuclein is also, in part, due to exosomes (26).

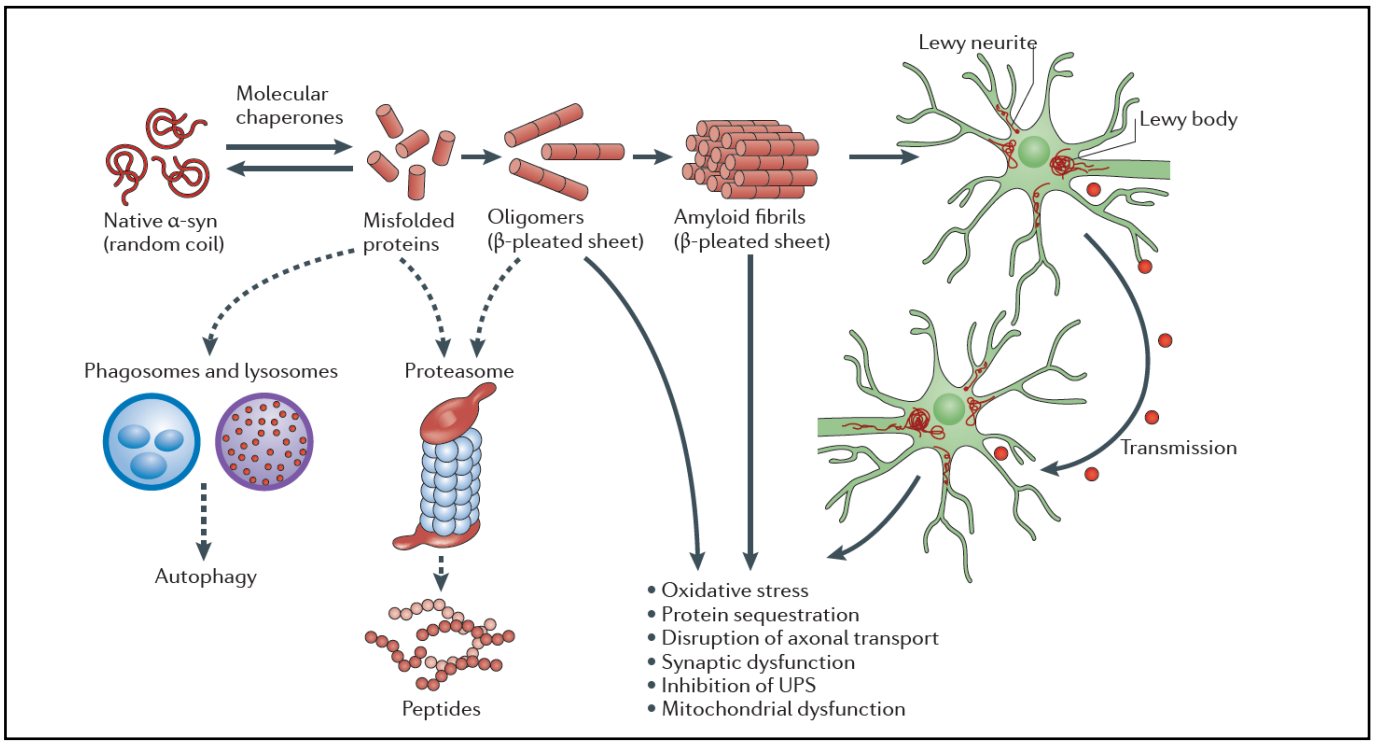


Figure 1.3 Proposed mechanisms of α-synuclein pathology in PD *Normal α-synuclein exists as a monomer, in a soluble random coil state. To acquire pathogenic features, α-synuclein misfolds into dimers, trimers and oligomers. It is not yet clear which species is directly toxic to neuronal cells. These structures further aggregate into protofibrils and amyloid fibrils. These higher-order structures are found in the Lewy bodies. Cell quality control systems such as chaperons, proteasomes and phago-lysosome systems receive a heavy burden of this oligomeric α-synuclein. PD and synucleinopathies disease progression may be dependent of the cell-to-cell transmission of pathogenic α-synuclein. UPS, ubiquitin proteasome system. Adapted from (43).*

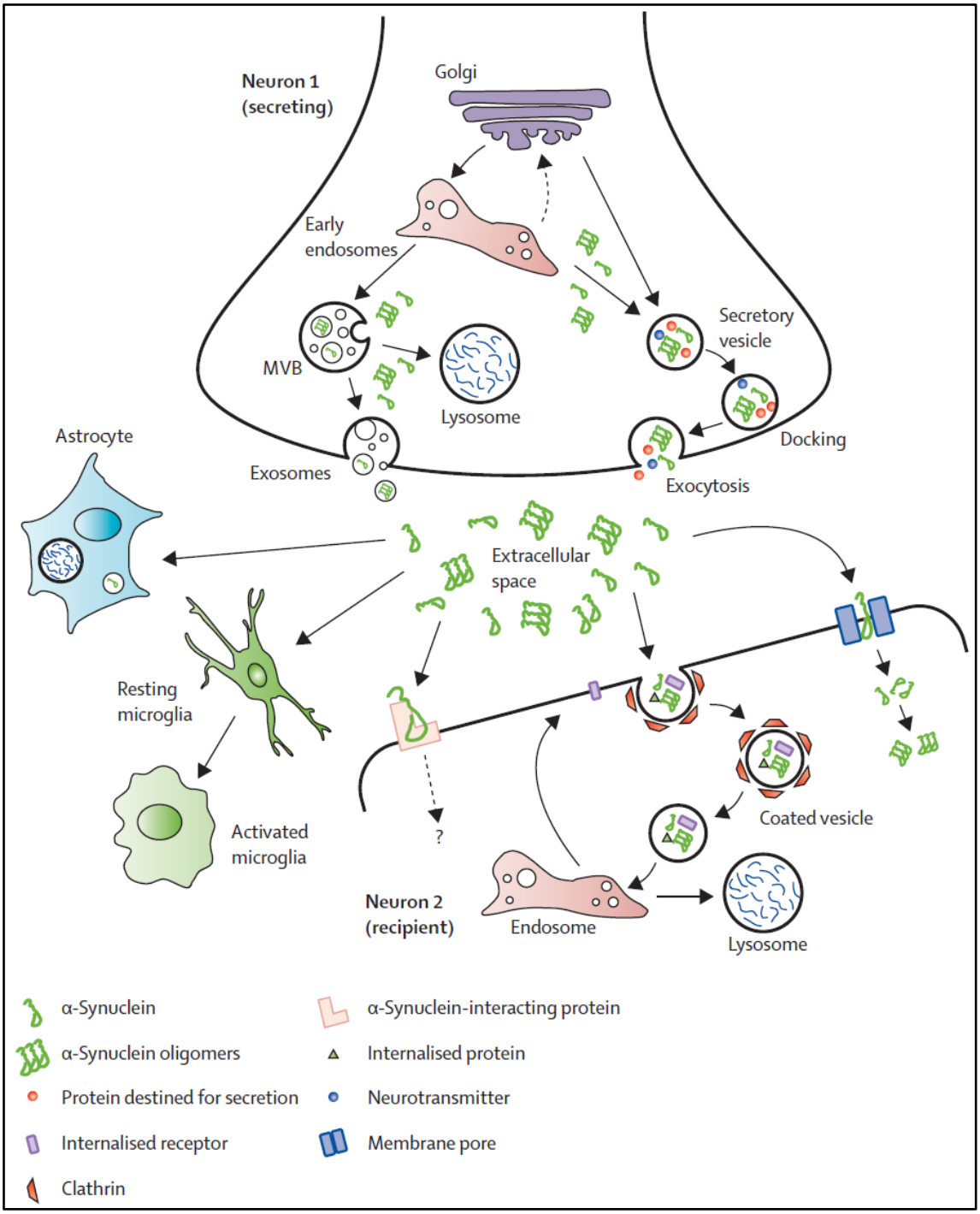


Figure 1.4 α-Synuclein secretion and paracrine actions *Secretion of α-synuclein is mediated through both endocytic and exocytic pathways. Cytoplasmic α-synuclein can enter MVBs though a recycle process of early endosomes where protein material moves to the plasma membrane or to MVBs. After that, MVBs either fuse with lysosomes or with the plasma membrane where they release their contents in exosomes through to the extracellular space. α-Synuclein can also be incorporated into secretory vesicles and be secreted by exocytosis. Extracellular α-synuclein can*

affect recipient neurons by association with unknown membrane proteins or receptors, endocytosis of clathrin-coated pits, or formation of pores. It can also trigger neuroinflammation by microglia activation. α-Synuclein is cleared through endocytosis by astrocytes. MVB=multivesicular body. Adapted from (44).

1.2.3 PD and the synucleinopathies

PD is part of a clinically diverse group of diseases called synucleinopathies. The synucleinopathies share a common neuropathological background of an aggregated, insoluble form of α-synuclein in different populations of neurons and glia (45). Interestingly, α-synuclein immunoreactive lesions may contribute in the phenotype of several other neurodegenerative diseases, without α-synuclein being the major protein constituent of the lesion (46). Thus, formation of insoluble fibrillar aggregates of α-synuclein may lead to a cascade of cellular responses that result in neuronal dysfunction and death. Depending on the areas affected, each disorder expresses different phenotypic traits [Table 1.3, (45)].

Table 1.3 Synucleinopathies
Parkinson's disease (PD) Sporadic Familial with α-synuclein mutations Familial with mutations other than α-synuclein
Dementia with Lewy bodies (DLB) "Pure" Lewy body dementia Lewy body variant of Alzheimer disease Familial Alzheimer disease with amyloid precursor protein mutations Familial Alzheimer disease with presenilin-1 mutations Familial Alzheimer disease with presenilin-2 mutations Down syndrome

Multiple system atrophy (MSA) Shy-Drager syndrome Striatonigral degeneration Olivopontocerebellar atrophy
Neurodegeneration with brain iron accumulation, type 1 Hallervorden-Spatz syndrome Neuroaxonal dystrophy
Other diseases that may have synuclein-immunoreactive lesions Traumatic brain injury Pick disease Amyotrophic lateral sclerosis
Adapted from (46)

PD and Dementia with Lewy bodies (DLB) are two synucleinopathies that are differentiated clinically, by the predominance of extrapyramidal motor features and dementia, respectively. However, as dementia and extrapyramidal signs can occur in close succession, a debate still exists about their nosology (13). It seems indeed that synucleinopathies represent a disease spectrum where clinical features overlap depending on location of pathology (Figure 1.5).

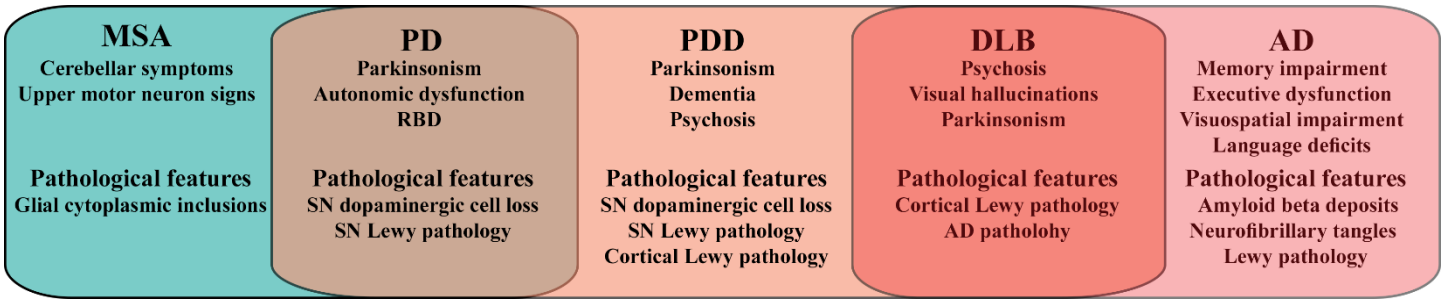


Figure 1.5 The synucleinopathies spectrum *The two most common neurodegenerative diseases, PD and AD, are characterized by extrapyramidal symptoms and memory loss respectively. However, these two clinical presentations overlap, with almost half of PD patients developing cognitive decline and about a third of AD patients developing extrapyramidal symptoms. In between, PDD and DLB patients present with late onset dementia with moderate extrapyramidal symptoms. DLB neuropathology consists of abundant Lewy bodies in the neocortex and the*

subcortex. MSA represents the other side of the spectrum, consisting only of movement symptoms. Adapted from (46).

1.3 Glucocerebrosidase and PD

1.3.1 Gaucher disease mutations – the most common genetic risk factor for PD

The most common genetic risk factor for PD was discovered from an unexpected finding in genetics clinics during studies of patients with Gaucher disease (GD). Initial clinical studies suggested the increased occurrence of parkinsonism in GD patients and their obligate carrier family members (47, 48). Then, through a case–control study, an increased risk of GBA heterozygotes to develop PD was revealed (49). A large collaborative group that analyzed selected GBA1 mutations in more than 5000 PD patients and healthy controls without family history of PD and sequenced the entire coding region in a subset of subjects (50) validated this initial suggestion.

It is quite intriguing that the initial large genome-wide studies on PD patients failed to identify GBA1 as a susceptibility gene. These studies sought to determine common variants and therefore missed the increased frequency of numerous rare GBA1 variants with low penetrance and those that occur on different haplotypes (51). The glucocerebrosidase (GCase) story is now considered a paradigm of ‘‘how an important risk factor for a complex disease can evade detection by systematic analysis; it only came into the radar because of astute clinical observations’’ (51). To date, the carrier status of a heterozygous GBA1 mutation is considered the most common genetic risk factor for synucleinopathies.

1.3.2 GBA PD genotype phenotype correlations

On an individual level, GBA heterozygotes or homozygotes with PD are indistinguishable from idiopathic PD patients. However, rate of motor progression is faster in GBA PD compared to idiopathic PD (52, 53). In addition, GBA mutation carriers are more likely to present with non-motor symptoms which are prevalent in idiopathic PD including cognitive impairment (54), REM sleep behavior disorder (RBD) (55), hyposmia (56), and autonomic dysfunction (54, 57). Moreover, the rate of cognitive change is faster in GBA PD than in idiopathic PD, and therefore, patients tend to have faster motor and cognitive progression than idiopathic PD (Table 1.4).

Table 1.4 Clinical characteristics of GBA PD compared to idiopathic PD		
	GBA PD	**reference**
Age at onset	Younger age at onset	(52, 56, 58-60)
Motor features		
Postural gait instability	Comparable	(52, 59)
Freezing	Unclear	(52, 59)
Dysphagia	More	(52)
Dyskinesia	More	(59)
Response to levodopa	Comparable	(59)
Motor fluctuations	More	(59)
Non-motor features		
Anxiety	Unclear	(54, 59)
Depression	Unclear	(54, 59, 61)
Cognitive impairment/dementia	More	(52, 54, 57, 59, 60, 62, 63)
Hallucinations	More	(56, 57, 59)
Autonomic dysfunction		
Orthostatic hypotension	Unclear	(52, 54, 59)
Constipation	More	
Urinary urgency	More	(54, 59)
Incontinence	Comparable	(54)
Sexual dysfunction	Comparable	(59)
Hyposmia/anosmia	More	(59)
REM sleep behavior disorder	More	(56) (55, 56, 59)
Pathology		
Lewy body density	Comparable	(64)
Adapted from (65)		

1.3.3 Effect of GBA Mutations on Glucocerebrosidase activity

Different GBA mutations differentially affect the enzymatic activity of GCase, as some mutations result in almost no residual activity whereas others show only reduced activity. In several cases, the level of enzymatic activity does not correlate with the severity of GD (66) and the enzymatic activity range of severe and mild GBA mutations can overlap. For instance, the measured enzymatic activity of GCase with the p.N370S mutation, which is always associated with type I GD, may be lower than the measured enzymatic activity of GCase with severe mutations such as p.L444P, p.G390R, p.N382K (67-69). The transport of GCase with the severe p.D409H and p.L444P mutations to the lysosome is also restricted and the transport of GCase with the mild p.N370S mutation was partial (70). They may not reach the lysosome in vivo, and therefore cannot exert their function. Indeed, it is possible that impaired transportation of GCase to the lysosome may also lead to PD. GWAS and other genetic studies have identified variants around SCARB2, the transporter of GCase from the ER to the lysosome, as a genetic risk factor for PD (71-74). GBA mutations may also cause structural effects that can influence the function of GCase. p.N370S GCase demonstrated a reduced capacity of the enzyme to interact with its activator, saposin C, and with anionic phospholipids that are necessary for its proper function (75). Supporting this observation, in a structural model of the interaction between GCase and saposin C, the p.N370S mutation was mapped to the interacting surface of the two proteins (76). In addition, it was suggested that the GBA p.N370S mutation may affect the stability of the helical turn conformation of loop 1 (77).

1.3.4 Proposed mechanisms of GBA in PD

GBA1 mutations contribute to PD pathology in many ways, as suggested by numerous publications focusing on elucidating this pathway (Figure 1.6).

First of all, the defect enzyme's substrate would be the first guess to a pathophysiological link. Indeed, the substrate of GCase, glucosylceramide, may lead to α-synuclein accumulation, and inversely, α-synuclein accumulation may lead to reduced GCase activity and accumulation of its normal substrate (78). Therefore, it is speculated that lipid changes induced by partial glucocerebrosidase deficiency may alter the interaction between lipid microdomains and α-synuclein, which might, in turn, lead to synaptic dysfunction and selective neuronal demise (29, 79).

Biochemical studies have also reported a direct physical interaction between glucocerebrosidase and α-synuclein under acidic conditions, which mimics the lysosomal lumen (80).

As illustrated in the previous section, intracellular homeostasis of α-synuclein is maintained by the actions of the ubiquitin–proteasome system (UPS) and the lysosomal autophagy system. The autolysosomal pathway plays a central role in the degradation of bulky material, including misfolded proteins and damaged organelles; mutations in genes involved in this pathway have increasingly been linked to the synucleinopathies (81, 82).

Several independent studies have reported autolysosomal dysfunction caused by glucocerebrosidase insufficiency. The inhibition of glucocerebrosidase activity by pharmacological inhibitors or mRNA silencing strategies has led to decreased lysosomal protein turnover and accumulation of lysosomal-associated membrane protein-1 (LAMP-1) structures and autophagosomes per LC3-II buildup in various neuronal cell models (78, 83-86). Autophagy and lysosomal dysfunction have also been reported in models and tissue samples from subjects with GBA1-associated PD (85, 87).

Another mechanism by which GBA mutations may result in PD is ER-associated degradation (ERAD) impairment and ER stress-related cell death. α-synuclein accumulation is known to seemingly cause ER stress, impair degradation of ERAD substrates, and inhibit ER to Golgi traffic (88). Along this line, the ER retention detected in experiments with mutated forms of GCase (70, 89-92) may suggest that ER stress is also involved in the pathogenesis of

PD in carriers of some GBA mutations. It was also shown that mutated GCase interacts with parkin, which promotes the accumulation of GCase in aggresome-like structures (93). However, this pathway suggested above is challenged by the fact that null GBA mutations which do not result in a protein product (68, 89, 94), such as 84GG, IVS2+1, R359X (94), and others, also increase the risk of developing PD (61, 95). It is more than obvious that if the protein does not exist, it cannot accumulate or assist in fibrillization. It seems likely that the ER stress observed in models with GBA mutations as described above may be due to α-synuclein accumulation rather than accumulation of GCase itself. It is also likely that GBA mutations increase susceptibility to PD in more ways than one and that both suggested mechanisms contribute to disease development.

To sum up, as described in literature, it “works” both ways; a gain of function effect, which is mediated by the encoded mutant glycoprotein, can be further exacerbated by reduced glucocerebrosidase enzyme activity through a loss of function effect. A vicious cycle of pathology is formed. Thus, both mechanisms may modulate PD susceptibility and lower the observed age of disease onset (96, 97).

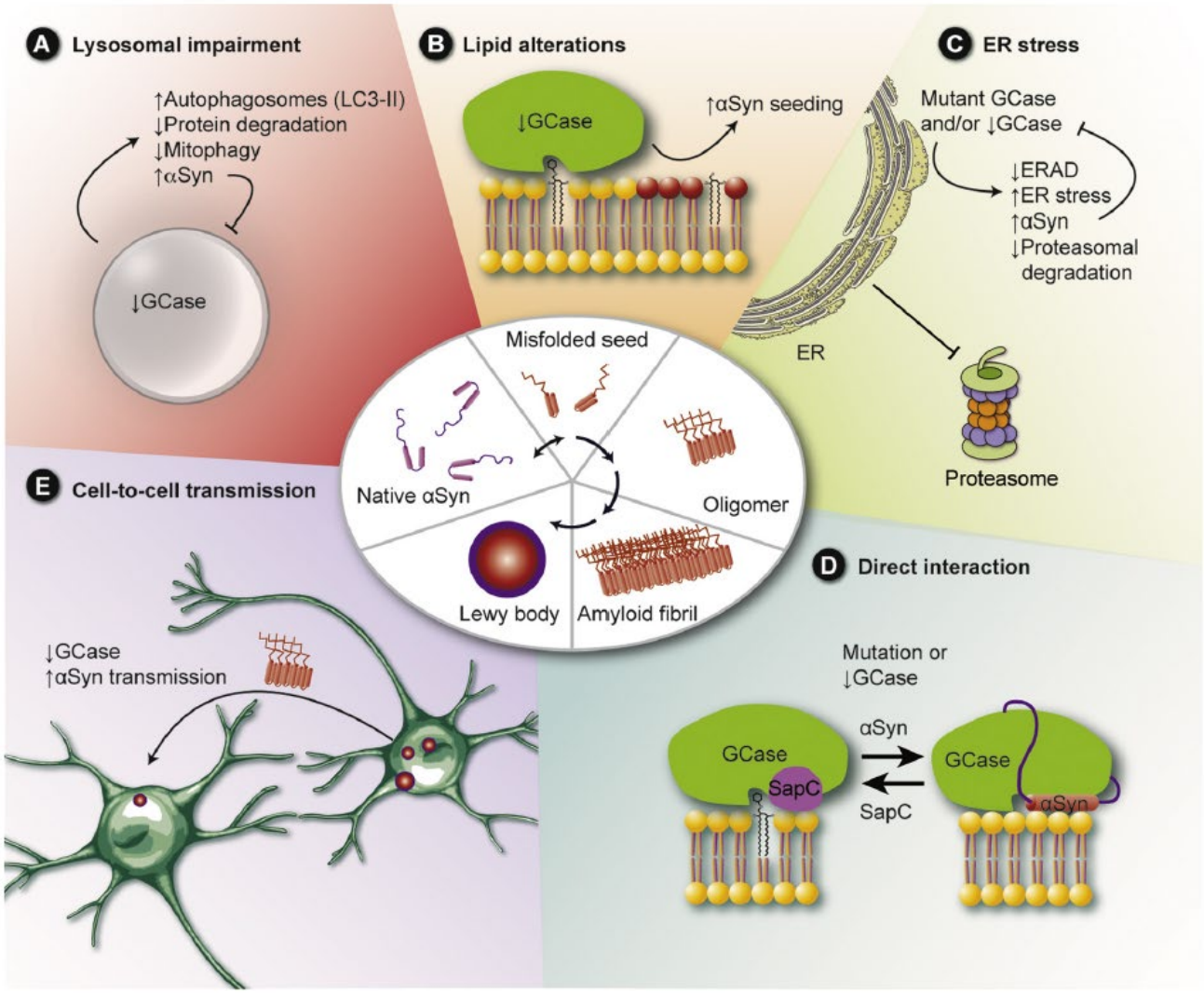


Figure 1.6 Potential roles of glucocerebrosidase in the development of synucleinopathies *Impaired GCase activity can result in reduced autophagosomal function which could result in increased α-synuclein and decreased protein and mitochondrial degradation (A). A reduction of GCase leads to altered lipid membrane composition which promotes α-synuclein seeding (B). A decreased GCase with or without abberant GCase forms can overwhelm the ER and thus cause α-synuclein increase and ER stress (C). Biochemical interaction of α-synuclein and GCase could displace sapocin C and cause further GCase activity decrease (D). A decrease of GCase levels or activity may falicitate cell-to-cell transmission of pathogenic α-synuclein and promote disease propagation (E). Adapted from (98).*

1.4 α-Synuclein secretion and GBA

1.4.1 Prion-like propagation of α-synuclein

An additional mechanism for the development of α-synuclein aggregates has been proposed. The prion-like hypothesis for α-synuclein was first imagined when two groups reported Lewy bodies in fetal grafts of SN tissue in PD patients (99, 100). The hypothesis was later strengthened when a group used brain homogenates from MSA patients to induce neurological dysfunction in transgenic mice via deposition of α-synuclein within neuronal cell bodies and axons (101).

The theory posits that once α-synuclein aggregates have formed in a neuron, they can be transported intra-axonally to other brain regions, be released into the extracellular space (Figure 1.7), be taken up by neighbouring neurons and seed aggregation of endogenous α-synuclein once inside their new cellular host [Figure 1.8, (102, 103)].

As already elaborated, interest in extracellular α-synuclein has risen and a number of studies have demonstrated that α-synuclein can be physiologically secreted to the extracellular space (26, 91). Cell culture studies have demonstrated that lysosome – autophagy impairment leads to increased secretion of α-synuclein into the extracellular space through exosomes and that endocytosis is a key mechanism of uptake of extracellular α-synuclein (104, 105). Thus, initial α-synuclein misfolding in a small number of cells could progressively lead to the spread of α-synuclein aggregates to multiple brain regions over years or decades following the initial insult. This is consistent with the idea that α-synuclein pathology gradually engages more brain regions as the disease progresses, as suggested by Braak *et al*. [Figure 1.2, (106)]. In addition, this model supports the idea that the first sites of α-synuclein aggregation might be in the gut enteric nerves and the olfactory bulb where they underlie the signs and symptoms associated with prodromal Parkinson disease [for example, anosmia and constipation, (107, 108)], before they spread, eventually leading to motor dysfunction once the SN becomes involved (109).

As already mentioned, it has also been shown that α-synuclein release is achieved in part via exosomes (26, 110). Interestingly, oligomeric species can be found in exosomes (26) suggesting that increased exosome – associated secretion of α-synuclein could aid toxic seed formation perhaps via the proposed prion-like mechanism of pathology progression. Moreover, upregulation of exosome secretion has been correlated with conditions of impaired lysosomal function and increased cytosolic cargo of a misfolded protein (26, 110-113). In agreement with these data, levels of α-synuclein were recently found to be increased in the media of GBA-N370S Parkinson's induced pluripotent stem cells (iPSC)-derived dopamine neurons which also exhibited autophagic/lysosomal disturbances (91).

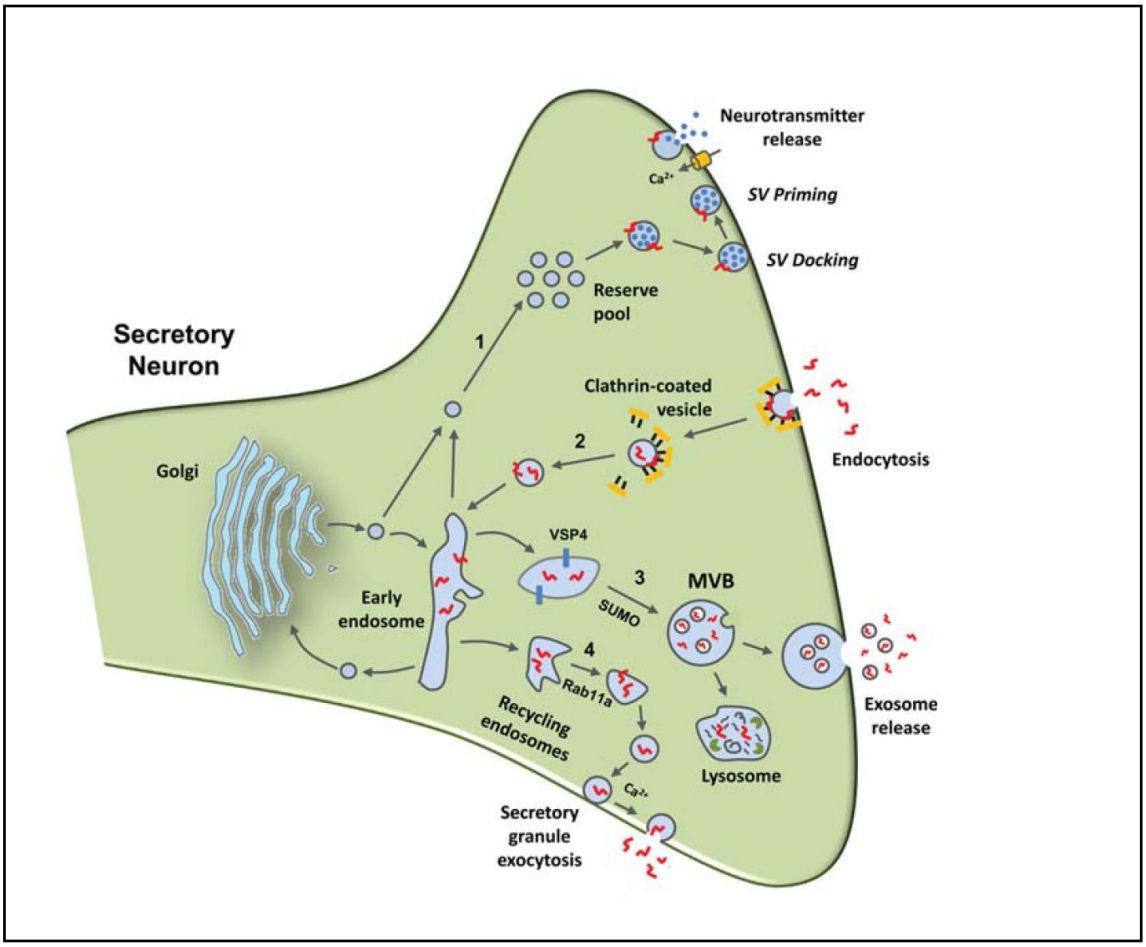


Figure 1.7 Proposed pathways for α-synuclein exocytosis *α-Synuclein, depicted here as a red line, promotes the SNARE complex assembly by association with synaptic vesicles and thus facilitates neurotransmitter release. Synaptic vesicles can enter early endosomes via Golgi or clathrin-mediated endocytosis. Followingly,*

endosomal α-synuclein can be secreted via two pathways. First, it can be incorporated in MVBs assisted by VSP4 and SUMO and be externalized as exosomal α-synuclein. Secondly, it can be recycled in the endosomal compartment and be released later in a Rab11a-dependent pathway. Adapted from (114).

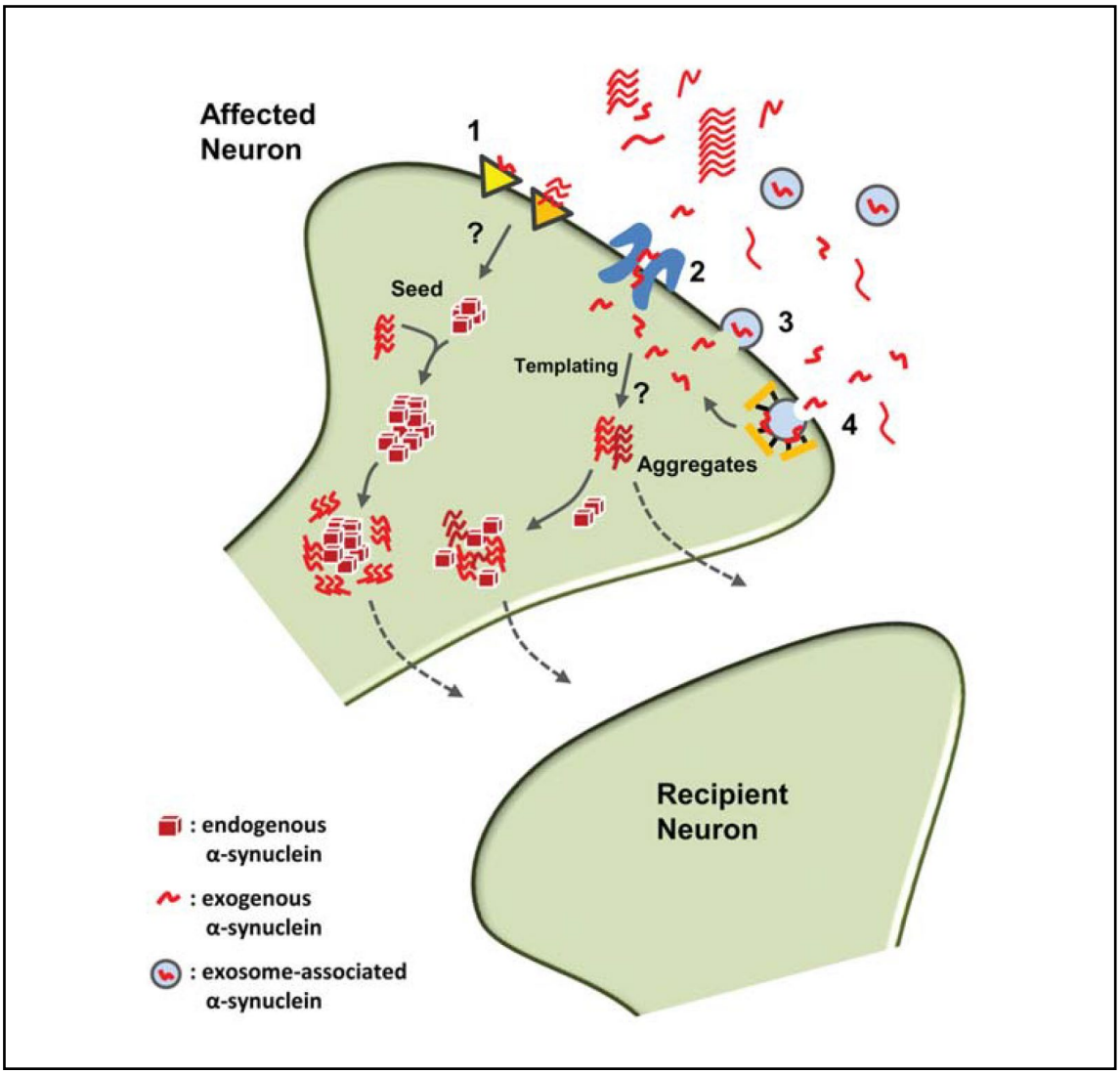


Figure 1.8 Possible mechanisms of uptake and propagation of α-synuclein *The pathway via which exogenous α-synuclein (red line) enters neurons is not yet identified. A translocation via a pore in the plasma membrane or a protein complex, a fusion of exosomes containing α-synuclein with the membrane, and endocytosis seem plausible mechanisms. Once in the intracellular space, α-synuclein can act as a seed to facilitate production of oligomers and fibrils in a process where endogenous α-synuclein (red cube) is involved. Alternatively, extracellular α-synuclein can signal the seeding of the endogenous protein by interacting with a receptor in the plasma*

membrane. Whatever the mechanism of uptake, α-synuclein can be then externalized again and affect neighboring cells. Adapted from (114).

1.4.2 Exosomes in neurodegenerative diseases

Secretion of extracellular vesicles (EVs) is a well-recognized means of communication between cells of the same or different type. The majority of cell types in the body releases exosomes into the extracellular environment. Originally identified to be involved in the non-degradative removal of the transferrin receptor during the maturation process of reticulocytes (115, 116) exosomes have now also been recognized as an important communication and signaling pathway in the body in both normal and disease settings. Exosomes differ from other EVs based on the secretion pathway used and the size of the vesicle released. Unlike other EVs that can bud from the plasma membrane, such as microvesicles (117), exosomes are created from intraluminal vesicles that form within multivesicular bodies (MVBs, or multivesicular endosomes). The subsequent fusion of the MVB at the plasma membrane releases these vesicles into the extracellular milieu where they are known as exosomes (Figure 1.9).

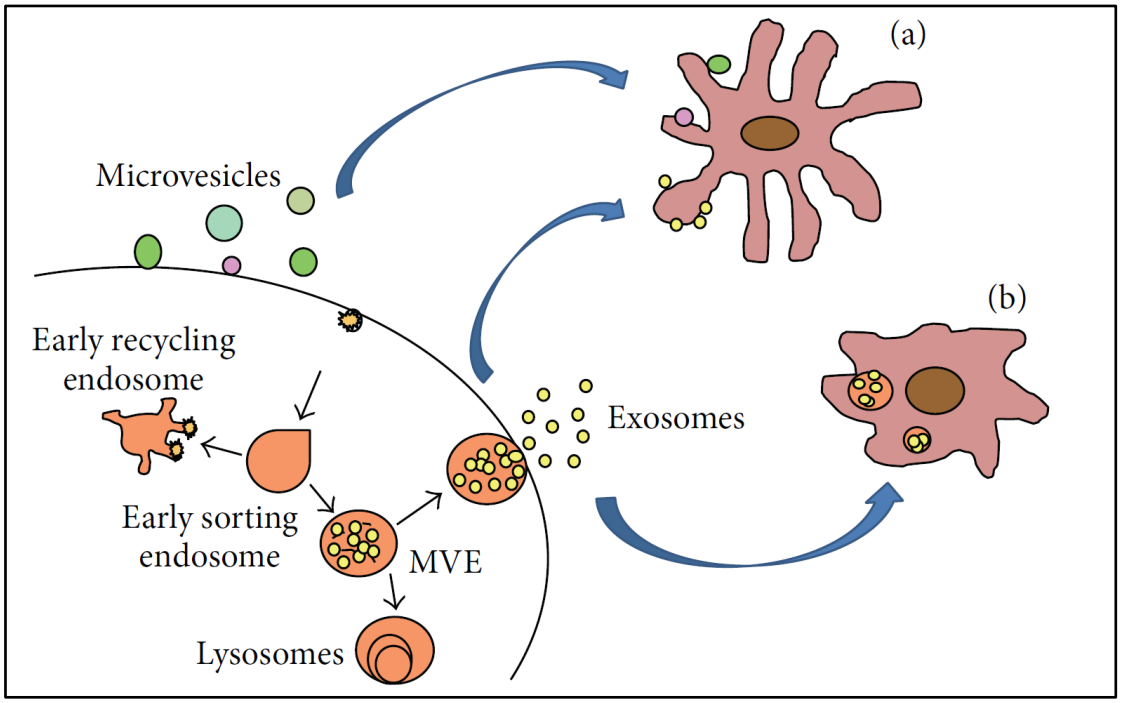


Figure 1.9 Exosomes merge with and release their contents into recipient cells *Exosomes are released from a host cell and are uptaken by recipient cells. Exosomes are generated in the host cell by the merging of MVBs with the cell membrane and then released into the extracellular space. These exosomes can be fused with the plasma membrane (a) or be internalized (b) by recipient cells. Adapted from (118).*

Initial studies showed that exosomes contain a specific subset of cellular proteins. These proteins seem to represent a specific subcellular compartment and not random cell fragments. Interestingly, EVs and exosomes are also enriched in small RNA, including mRNA, and miRNA. It is thus possible that they can modulate gene expression in distant cells.

The richest area of exosome research has come from disease studies, in particular the cancer field. Recent evidence has highlighted a role for exosomes to promote metastasis and regulate tumor immune response (119). The ability of exosomes to promote the spread of disease is also thought to play a role in neurodegenerative disorders (Figure 1.10). Exosomes containing aggregation-prone proteins involved in PD, Alzheimer's disease (AD), Creutzfeldt-Jakob

disease (CJD), and amyotrophic lateral sclerosis (ALS) have all been found in the cerebral spinal fluid and blood of patients affected by these disorders.

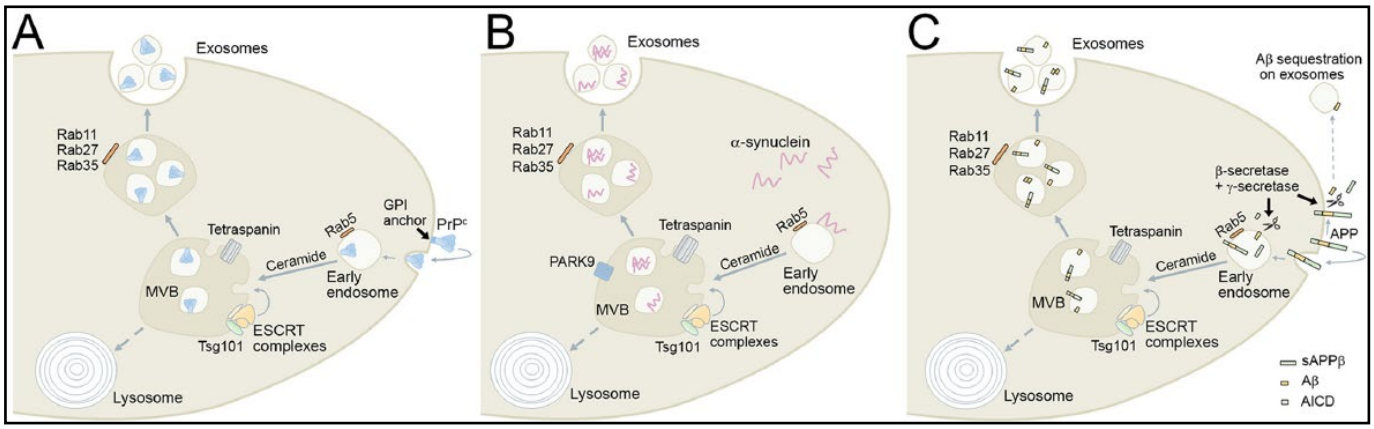


Figure 1.10 Biogenesis of exosomes containing proteins associated with neurodegenerative diseases *Prion protein (PrP^c) is found on the cell surface anchored by a glycosylphosphatidylinositol (GPI) linker to lipid rafts. Internalized through early endosomes and then by a ceramide-dependent process, it can enter MVBs. When MVBs fuse with the cell membrane, PrP^c is released in exosomes (A). α-Synuclein can be found on early endosomes. Through a process involving ceramide, tetraspanins, or endosomal sorting complexes required for transport (ESCRT) components, α-synuclein enters the MVBs. PARK9 and Rab11 can then promote the exosomal release of α-synuclein (B). APP is also found on early endosomes, where it can be cleaved by secretases to form secreted APPβ (sAPPβ), Aβ, and APP intracellular domain (AICD). While the AICD fraction moves to the nucleus, Aβ can be trafficked into MVBs through a process involving ceramide, tetraspanins, or ESCRT components as with α-synuclein and PrP^c. Fusion of MVBs with the cell membrane results in release of exosomes containing Aβ. Extracellular Aβ can also be sequestered by exosomes, which can then be degraded by microglia (C). Adapted from (120).*

A failure of cell quality control systems is a core feature of PD. Interestingly, failure of autophagy-lysosome pathway has been found to be important for the release of extracellular α-synuclein, and, remarkably, lysosomal dysfunction by either pharmacological or genetic manipulation increases exosomal release of α-synuclein (110, 113, 121).

Evidence for transmission of exosomal α-synuclein has up until now mainly been limited to in vitro studies, although it is not clear whether studies using brain extracts that resulted in the propagation of α-synuclein pathology also contained exosomal α-synuclein (42). Recently, exosomes derived from the CSF of PD patients have been shown to transmit α-synuclein aggregation using a reporter cell line (122), but as yet, no direct proof for in vivo exosomal transmission of α-synuclein has been observed. Data from our lab demonstrated that SH-SY5Y cells expressing α-synuclein released both monomeric and oligomeric forms of α-synuclein. This was either through a free form secretion in the medium or within exosomes, the latter in a process that was calcium-dependent (26). Subsequent studies confirmed these findings in cell lines and also showed that exosomal release of α-synuclein and uptake by recipient cells was increased when the lysosome was inhibited (91, 110, 123). When compared to free α-synuclein oligomers derived from conditioned media, exosome-associated α-synuclein oligomers were more likely to be taken up by recipient cells and cause toxicity (123).

α-Synuclein is targeted to endosomes by ubiquitination by the E3 ligase Nedd4 (124, 125) and this process is negatively regulated by USP8 (126). It is therefore possible that when lysosomal impairment exists, the endosomal fraction of α-synuclein could escape degradation and be released either by the recycling endosome in a process involving Rab11a and Hsp90 (121, 127) or within exosomes from MVB (Figure 1.11).

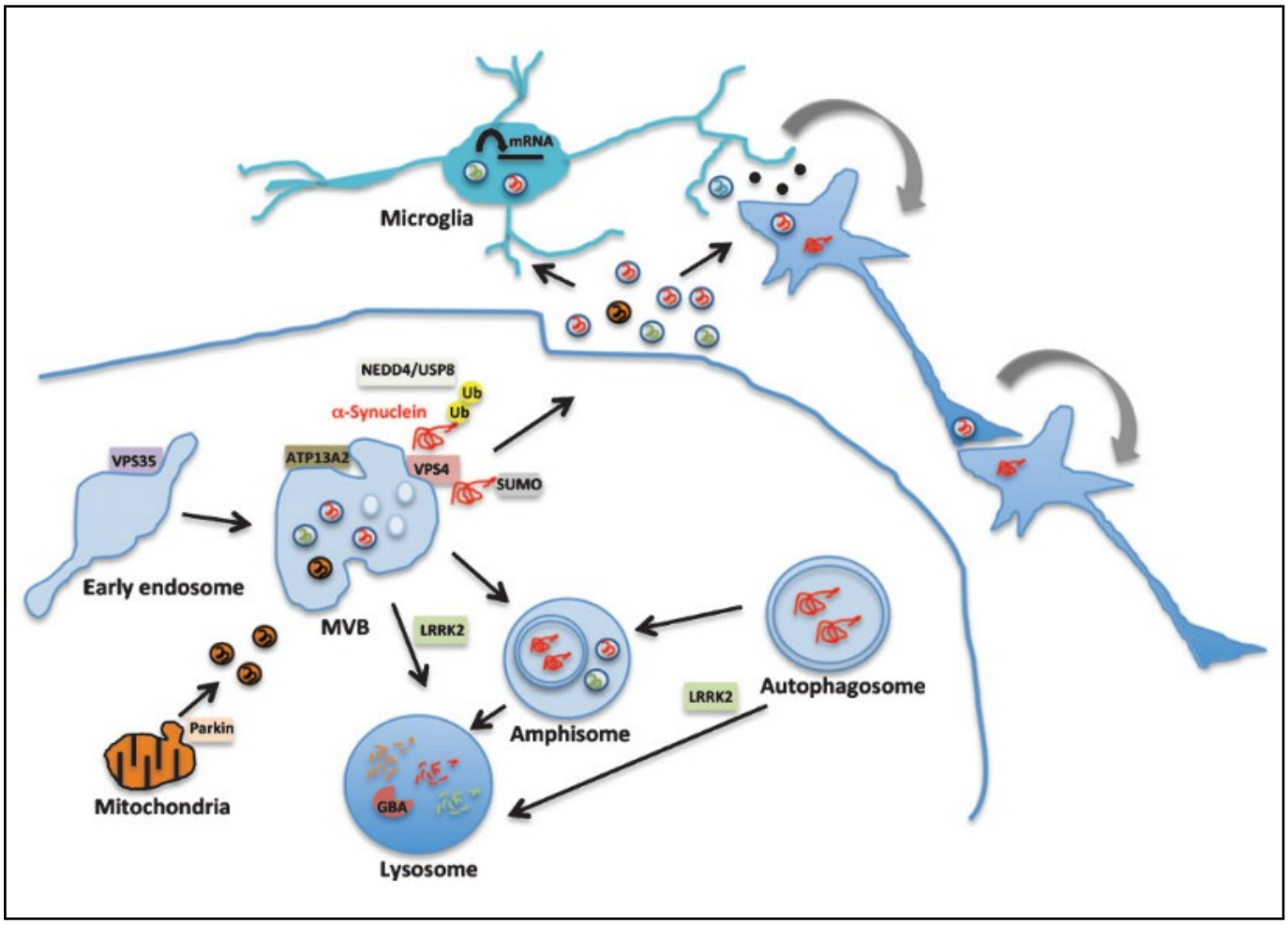


Figure 1.11 Exosome signaling in PD pathology *Exosomes are the end-result of MVBs fusion with the plasma membrane. MVBs, part of the endosomal-lysosomal pathway, can be affected by genes involved in familial PD such as VPS35, LRRK2, ATP13A2 and Parkin. GBA mutations impair lysosomal function and can also affect this pathway. LRRK2 (green line) has been shown to be contained in exosomes and can also regulate this pathway. α-Synuclein (red line) is trafficked via the endosomal pathway and released via exosomes, assisted by NEDD4, USP8 ubiquitination or SUMO protein sumoylation. Once released, exosomal α-synuclein can promote PD pathology by microglial cell activation or via uptake and signaling in neighboring neuronal cells. Adapted from (128).*

1.5 Aims of the study

Given the intriguing hypothesis that PD can be traced back to a single starting point and evolve by propagating in neighboring or distant CNS regions, we sought to investigate a role of GCase dysfunction in this cascade of events. The role of GBA, a gene encoding for a lysosomal enzyme and a common genetic risk factor for PD, has not been thoroughly investigated in its involvement in α-synuclein secretion, especially in vivo. Dysregulation of extracellular α-synuclein levels could be critical for the initiation or the progression of Parkinson's disease. To this end, the effects of altered GCase activity and expression in the secretion of α-synuclein in PD have not been identified.

We set out to perform experiments mimicking both suggested pathways of GBA-PD pathology; briefly, a gain of toxic function by GBA point mutations and a loss of function of GCase using a pharmacological compound.

1.5.1 Experimental approach

We performed:

1. Adenovirus mediated overexpression of mutant forms of GBA:
    a. in vitro, in primary cortical neuronal cultures
    b. in vivo, by intrastriatal stereotactic injections in mice
2. Pharmacologic inhibition of central nervous system (CNS) GCase activity achieved by administering conduritol B epoxide (CBE):
    a. in vitro, in primary cortical neuronal cultures
    b. in vivo, via intraperitoneal injections in mice

Using these models we were able to:

1. Measure secreted α-synuclein levels:
    a. in CM of neuronal cultures

   b. in ISF of mice, through in vivo microdialysis

2. Measure exosomes levels and exosome – associated α-synuclein:

   a. In CM of neuronal cultures

   b. by whole brain exosome isolation in mice

3. Investigate underlying pathology leading to alterations in secreted α-synuclein levels by measuring:

   a. lysosomal turnover of the autophagosomal markers LC3-II and p62

   b. GCase activity

   c. proteasomal activity, including estimating levels of polyubiquinated proteins.

2. Materials

2.1 Animals

Eight-weeks old male and female Tg mice expressing human mutant A53T α-synuclein under the control of the prion promoter (Jackson Laboratories, Bar Harbor, ME) (129) were used in this study. All efforts were made to minimize animal suffering and to reduce the number of the animals used, according to the European Communities Council Directive (86/609/EEC) guidelines for the care and use of laboratory animals. All animal experiments were approved by the Institutional Animal Care and Use Committee of BRFAA (permit number 1153/12-05-2015).

2.2 Cortical neuronal cultures

Cultures of C57/ Bl6 (embryonic day 16, E16) cortical neurons were prepared as previously described (130). Dissociated cells were plated onto poly-D-lysine-coated 6-well or 12-well dishes at a density of approximately 1.5×10^6 cells/cm^2. Cells were maintained in Neurobasal medium (Gibco, Rockville, MD, USA; Invitrogen, Carlsbad, CA, USA), with B27 supplement (Gibco; Invitrogen), L-glutamine (0.5 mM), and penicillin/streptomycin (1%). More than 98% of the cells cultured under these conditions represent postmitotic neurons (130). The time in culture of cells was calculated using days in vitro (DIV), with the day of harvesting designated as 0 DIV.

2.3 Adenoviral transduction of primary cortical neurons

Cultures of cortical neurons were transduced with WT, D409V, N370S GBA and WT SNCA AVs on 5 DIV. A multiplicity of infection (MOI) of 10 to 15 was used. CM were removed and replaced with fresh Neurobasal medium containing the AVs. On 6 DIV CM containing the AVs were removed and CM of 0-5 DIV cultures was reused. Cells were collected on 9 DIV.

2.4 Inhibition of GCase activity in primary cortical neurons using conduritol-B-epoxide (CBE)

CBE was added in cultures of cortical neurons on 6 DIV in a concentration of 200μM. Cells were collected 72h later, on 9 DIV.

2.5 Western Immunoblotting

Embryonic mouse cortical neurons were washed twice in cold PBS and then harvested in STET lysis buffer (50 mM Tris, pH 7.6, 150 mM NaCl, 1% Triton X-100, 2 mM EDTA) with protease inhibitors. Lysates were centrifuged at 10.000g for 10 min at 4°C. Protein concentrations were determined using the Bradford method (Bio-Rad, Hercules, CA, USA). Twenty to fifty micrograms of lysates were mixed with 4x Laemmli buffer prior to running on 10-13% SDS–polyacrylamide gels. Following transfer to a nitrocellulose membrane, blots were probed with the following antibodies: polyclonal α-syn C-20 (1:1000; Santa Cruz Biotechnology), SNCA/syn-1/α-Synuclein monoclonal (1:1000; BD Biosciences), anti-alpha-synuclein phospho S129 antibody monoclonal [EP1536Y] (1:500; Abcam), Anti-Glucocerebrosidase (C-terminal) antibody polyclonal (1:500; Sigma-Aldrich), monoclonal b-actin (1:1000; Santa Cruz Biotechnology), monoclonal Anti-γ-Tubulin antibody(1:1000; Sigma-Aldrich), Anti-GAPDH antibody polyclonal (1:1000; Abcam), polyclonal LC3 (1:1000; MBL Life Sciences), polyclonal P62 (1:1000; MBL Life Sciences), polyclonal ubiquitin [Z0458] (1:1000; Dako), monoclonal human specific anti -GBA antibody (1:1000; gift of Pablo Sardi, Sanofi Genzyme; 12). Blots were probed with horseradish peroxidase-conjugated secondary antibodies and visualized with enhanced chemiluminescence substrate (ECL) following exposure to Super RX film (FUJI FILM, Europe GmbH, Germany). After scanning the images with Adobe Photoshop CS6 (Adobe Systems, USA), Gel analyzer software 1.0 (Biosure, Greece) was used to quantify the intensity of the bands.

2.6 Statistical Analysis

The data are shown as the mean ± SEM. Unless stated otherwise, statistical analysis was carried out with GraphPad Prism 6 (GraphPad Software, San Diego, CA, USA) using Student's t-test or one-way analysis of variance (ANOVA) followed by Tukey's or Fisher's LSD multiple comparisons post-test. Values of $p<0.05$ were considered significant.

2.7 GCase Activity Assay

GCase enzyme activity was assessed using a standard fluorimetric assay (131). Briefly, primary cortical neurons or mouse brain tissue were lysed in 20-50 µl of GCase Activity Assay Buffer (50 mM Citric Assay, 176 mM K_2HPO_4, 10 mM sodium taurocholate, 0.01% Tween-20, final pH=5.9). Following incubation on ice for 30 minutes, samples were centrifuged for 10 minutes at 4°C. Neuron cell lysate (5 µl) was incubated with either 5 µl of assay buffer or 5 µl of 40 mM CBE (Sigma), for 15 min at room temperature. Subsequently, 25 µl of 5 mM 4-methylumbiferil β-glucopyranoside (4MU-β-Glc) substrates were added to samples, followed by 25 min incubation at 37°C. Samples were then cooled on ice and reactions were stopped with 465 µl Stop Buffer (1M NaOH, 1M Glycine, final pH=10). Relative Fluorescent Units (RFU) were measured at 450 nm in a Perkin-Elmer LS-55 luminescence Spectrometer (Perkin-Elmer, Norwalk, CT, USA). Each sample was measured in duplicate, the average nonspecific activity was subtracted from each reading and the final result was normalized to the total protein concentration.

2.8 Adenoviral vector construction and virus production

Full-length human wild-type (WT), D409V and N370S GBA cDNA were cloned into a modified version of the PENTR.GD entry vector and introduced into the pAd/PL-DEST Gateway vector (Invitrogen). Second-generation E1, E3, E2a-deleted recombinant human serotype 5 adenoviruses (rAd) were generated, as described previously (132). Viral vector stocks were amplified from plaque isolates in order to guarantee homogeneity of the production. Final vector

stocks were purified and concentrated using double discontinuous and continuous cesium chloride (CsCl) gradients.

2.9 Primary cortical neuron cultures survival assay

To evaluate the toxicity of the viral induction we infected primary cortical neurons with the above mentioned AVs. Cells were infected on the fifth DIV and collected on the seventh and eighth DIV. Cells were lysed in a buffer that preserved cell nuclei from living cells and the lysate was used to calculate living cells with a Neubauer chamber.

2.10 Proteasomal activity assay

Neuronal tissue was lysed as described earlier in lysis buffer. The supernatants were collected for the measurement of the enzymatic chymotrypsin-like activity of the proteasome, essentially as described (133), based on cleavage of proteasomal substrate III (Calbiochem), using a Perkin Elmer fluorimeter LC-55 with excitation at 380 nm and emission at 438 nm. Three independent reactions were performed for each sample, in the presence or absence of the selective proteasomal inhibitor epoxomicin (1 μM), and the mean of the difference between these measurements was recorded.

2.11 Partial inhibition of glucocerebrosidase activity in WT and PrP-A53T-SNCA mice using conduritol-B-epoxide (CBE)

A partial reduction in CNS glucocerebrosidase activity in *PrP-A53T-SNCA* mice (animals with WT alleles of *Gba1*) was achieved by intraperitoneal administration of a covalent inhibitor of the enzyme, conduritol-B-epoxide (CBE, 100 mg/kg, *ip*, 3 times per week for 8 weeks) (Figure 2.1). Daily administration of CBE has been reported to result in a murine model that mimics the enzyme deficiency in GD patients (134). Previous studies indicated that an administration interval of 48 to 72 hs was optimal to achieve a residual brain glucocerebrosidase activity close to 50% (135). Therefore, in order to achieve

a partial reduction in CNS glucocerebrosidase activity a 3 times per week dosage was adopted.

Figure 2.1 Intraperitoneal injection

2.12 Ultra-sensitive ELISA for α-synuclein

For the sandwich ELISA, the monoclonal Syn-1 antibody (BD Biosciences), raised against amino acids 15–123 of the human, mouse or rat α-synuclein sequence, was used as capture antibody. This antibody recognizes a conserved epitope in human and rodent α-synuclein (residues 91–99) whereas it shows no reactivity for the β- or γ-synuclein isoforms [perrin 2003]. The polyclonal C-20 antibody (Santa Cruz), raised against a C-terminus peptide of human α-synuclein, was used for antigen detection through direct conjugation with HRP (Pierce). Each ELISA plate (Corning Costar) was coated for 24 hrs at room temperature with 0.5 µg/ml of Syn-1 (50 µl per well) in 100 mM $NaHCO_3$, pH 9.3. The plates were washed three times in wash buffer (50 mM Tris-HCl, 150 mM NaCl and 0.04% Tween-20) and 50 µl of sample or recombinant α-synuclein (as standard), appropriately diluted in TBST/BSA (10 mM Tris-Cl, pH 7.6, 100 mM NaCl, 0.1% Tween-20 and 1% BSA) was added. To allow antigen binding, plates were incubated at 37°C for 2 ½ hrs. After washing three times

with wash buffer, 50 µl of HRP-conjugated C-20 antibody (3000-6000x diluted in TBST/BSA) were added to each well and further incubated for 1 hr at ambient temperature. The wells were washed and 50 µl of chemiluminogenic HRP substrate (ultrasensitive luminol reagent, BioFX Laboratories) were added to each well. The wells were incubated for 10 min at room temperature and the chemiluminescence was integrated for 1 s. Spark™ 10M multimode microplate reader (Tecan) was used for reading the plates.

2.13 In vivo microdialysis

Guide cannulas were stereotaxically implanted in the striatum under isoflurane anesthesia (4–2.5%) as previously described (136) (Figure 2.2). Animals were kept anesthetized during the whole procedure. Breathing was kept stable using an oxygen / air ratio of 0.5. Bore holes were made above the right striatum according to the mouse atlas of Paxinos and Franklin (coordinates, AP=+0.5 mm, ML=−2.2 mm, DV=−2.4 mm). CMA 12 guide cannulas were inserted and fixed to the skull with stainless steel screws and dental cement. Mice were removed from the stereotaxic device and allowed to recover in individual cages. 72–96 hrs after surgery, mice were moved to the microdialysis cage. During microdialysis, mice were awake and had free access to food and water (CMA 120 System for Freely Moving Animals) (Figure 2.2). CMA 12 custom made probes were manually inserted and connected to the CMA 402 syringe pump with a constant flow rate of 0.6 µl/min. Prior to sample collection, the probe was allowed to equilibrate for at least 2 hrs with the same flow rate. Samples were collected bihourly for 12-14 hrs using a CMA 170 refrigerated fraction collector and stored at −80°C until analyzed by ELISA.

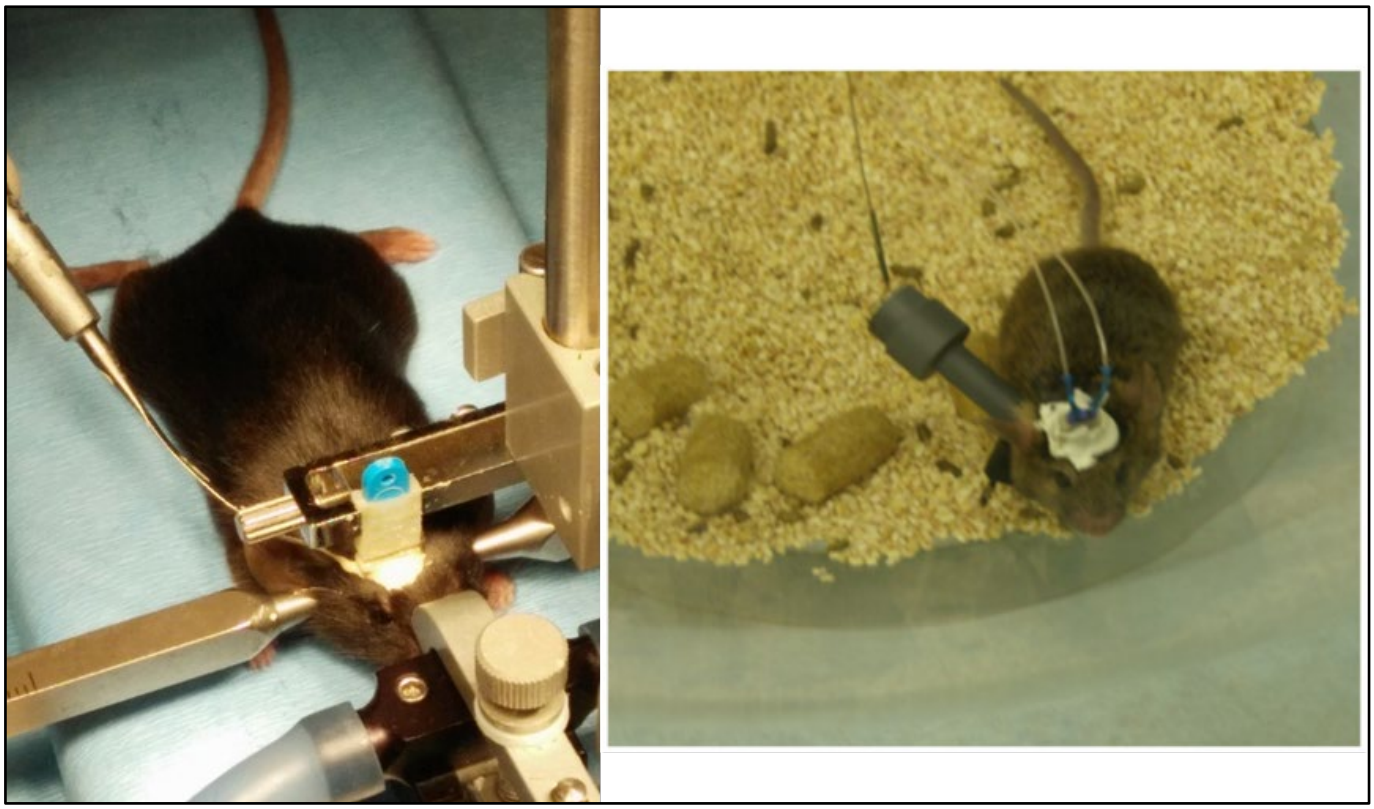


Figure 2.2 Implant of guide cannula and in vivo microdialysis

2.14 Isolation of externalized membrane vesicles

The isolation of the externalized membrane vesicles was performed as described previously (137). Briefly, the condition medium was first centrifuged at 4000g for 10 min at 4°C to remove dead cells and debris, and the supernatant was further centrifuged at 100,000g for 2 h at 4°C. The supernatant (S100) was collected, and the pellet (P100) containing the externalized vesicles was reconstituted in 50 µl of PBS.

2.15 Preparation of exosome-depleted medium

The depletion of the medium from secreted exosomes released during the first four days of culture was performed as described previously (138). Briefly, Neurobasal medium containing 10% B27, penicillin/streptomycin, and l-glutamine was centrifuged at 100,000g for 2 h at 4°C. The supernatant was carefully removed and sterilized by filtering through a 0.2 µm filter (Whatman) and stored at 4°C until reused in primary neuronal cultures.

2.16 Exosome Quantification

The amount of exosomes released was assessed based on the activity of acetylcholinesterase, an enzyme specific to these vesicles (139). Acetylcholinesterase activity was determined as described by Savina et al. (140). Briefly, 5 µl of the exosome fraction was suspended in 20 µl of PBS. 5 µl of this PBS-diluted exosome fraction was then added to individual wells on a 96-well flat bottomed microplate. 1.25 mM acetylthiocholine and 0.1 mM 5,5′-dithiobis(2-nitrobenzoic acid) were then added to exosome fractions in a final volume of 250 µl, and the change in absorbance at 405 nm was monitored. The data presented represent acetylcholinesterase enzymatic activity after 30 min of incubation, normalized to the amount of total protein of the exosome fraction measured through a Bradford protein assay.

2.17 Whole Brain Exosome Isolation and Purification

Exosomes were isolated from mouse brain tissue as previously described (141), with slight modifications (Figure 2.3). Frozen mouse brain was dissected and treated with 20 units/ml papain (Worthington) in Hibernate E solution (3 ml/hemi-brain; BrainBits, Springfield, IL) for 15 min at 37 °C. The brain tissue was gently homogenized in 2 volumes (6ml/hemi-brain) of cold Hibernate E solution. The brain homogenate was sequentially filtered through a 40-m mesh filter (BD Biosciences) and a 0.2-m syringe filter (Thermo Scientific). Exosomes were isolated from the filtrate as described previously (138). Briefly, the filtrate was sequentially centrifuged at 300g for 10 min at 4 °C, 2000g for 10 min at 4 °C, and 10,000g for 30 min at 4 °C to discard cells, membranes, and debris. The supernatant was centrifuged at 100,000g for 70 min at 4 °C to pellet exosomes. The exosome pellet was resuspended in 60 ml of cold PBS (Invitrogen), and the exosome solution was centrifuged at 100,000g for 70 min at 4 °C. The washed exosome pellet was resuspended in 2 ml of 0.95 M sucrose solution and inserted inside a sucrose step gradient column (six 2-ml steps starting from 2.0 M sucrose up to 0.25 M sucrose in 0.35 M increments, with the 0.95 M sucrose step containing the exosomes). The sucrose step gradient was centrifuged at 200,000g for 16 h at 4 °C. One-ml fractions were collected

from the top of the gradient, and fractions flanking the interphase separating two neighboring sucrose layers were pooled together for a total of seven fractions (a, top 1-ml fraction; b, 2-ml; c, 2-ml; d, 2-ml; e, 2-ml; f, 2-ml; and g, bottom 1-ml fraction). These fractions were diluted in cold PBS and centrifuged at 100,000g at 4 °C for 70 min. Sucrose gradient fraction pellets were resuspended in 200 µl of cold PBS.

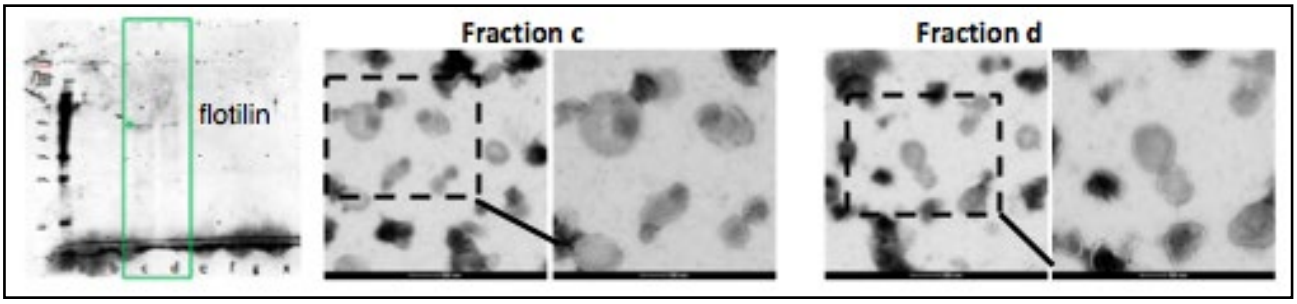


Figure 2.3 EM images of 2 fractions (c,d) containing exosomes *Fractions were isolated following sucrose gradient ultracentrifugation which verifies that frozen brain can be used for the isolation of intact exosomes (scale bar: 500nm).*

2.18 Stereotaxic injections

Male or female 2 months old transgenic mice (overexpressing A53T SNCA) were anesthetized with isoflurane (Abbott, B506) and stereotaxically injected with the viral vectors into the right SN. Control animals received GFP AAV. 2µl of a working dilution of 6 x 10^9 vp/µl of each virus was administered. A single needle insertion (co-ordinates: +0.2 mm relative to Bregma, +2.0 mm from midline) into the right forebrain was used to target the inoculum to the dorsal neostriatum (+2.6 mm beneath the dura, Figure 2.4). Injections were performed using a 10 µL syringe (Hamilton, NV) at a rate of 0.1 µL per min (2.5 µL total per site) with the needle in place for > 5 min at each target. Animals were monitored regularly following recovery from surgery.

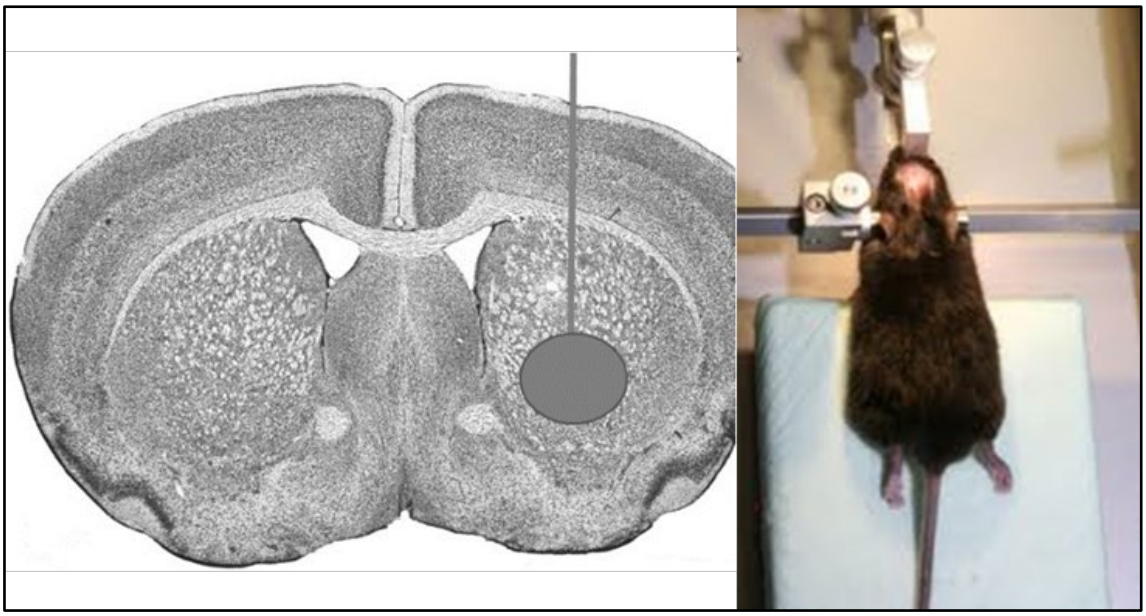


Figure 2.4 Stereotactical injection of AAVs in the dorsal neostriatum

2.19 Tissue Staining and Fluorescence measurement

Mice were perfused intracardially with PBS followed by paraformaldehyde 4%. Brains were dissected and post-fixed in 4% paraformaldehyde. They were then left in 30% sucrose overnight followed by 15% sucrose and stored in -80 ^{0}C. Tissue sections (35 μm) were cryostat-cut at the level of the striatum and stored in -20 ^{0}C. On the day of the experiment, sections were incubated (at room temperature) in PBS (3 x 5 mins) and then in blocking solution (10% NGS, 0.1% Triton in PBS for 1h). Sections were then incubated in the primary antibody (LC3 1:1000, p62 1:1000 [MBL Life Sciences], TUJ1 1:500 [BioLegend, Inc] in blocking solution) at 4 ^{0}C for 48 h. Following, sections were washed in PBS (3 x 15 mins) at room temperature and incubated for another 2 h at room temperature in secondary antibody (1:2500) and DAPI (1:2000, Sigma Aldrich) in blocking solution. Lastly, sections were washed in PBS (3 x 10 mins) before transferred on glass slides. Images were obtained using the Leica TCS SP5 (Wetzlar, Germany). The intensity of fluorescense signal of LC3 and p62 was measured with Imaris software (v7.7.2, Bitplane AG) as previously described (142). A set of parameters was set in order to identify neuronal cells, using TUJ1 as a filter (masking channel). Then, the quantification of immunofluorescent puncta of p62 or LC3 on TUJ1 positive cells was performed

automatically by the spot counter plugin of the software. The individual value of puncta for each subject is the ratio of ipsilateral side puncta to contralateral side puncta. Representative images were created using Fiji-ImageJ software.

3. Results

3.1 Adenovirus titration

To evaluate the efficiency of the adenoviruses and to determine the titer of each stock we used the Adeno-X Rapid Titer kit (Clontech, Figure 3.1). Briefly, each stock was diluted stepwise in 10^{-6} to 10^{-10} dilutions and added in duplicate in HEK293A cells. After two hours of incubation media were removed and a hexon-specific antibody, which is used to label infected cells, was added. Hexon protein is encoded by the adenoviral genome and is an essential component of the adenoviral capsid required for adenoviral replication. Its expression depends on the E1 gene product, found in cells such as HEK293 cells. Thus, only infected cells will produce the hexon protein. The following titers were obtained, expressed as viral particles (vp)/µl: $2{,}31 \times 10^8$ for rAd-WT GBA, $1{,}39 \times 10^8$ for rAd-D409V GBA, $5{,}9 \times 10^8$ for rAd-N370S GBA and 1.51×10^8 for rAd-GFP.

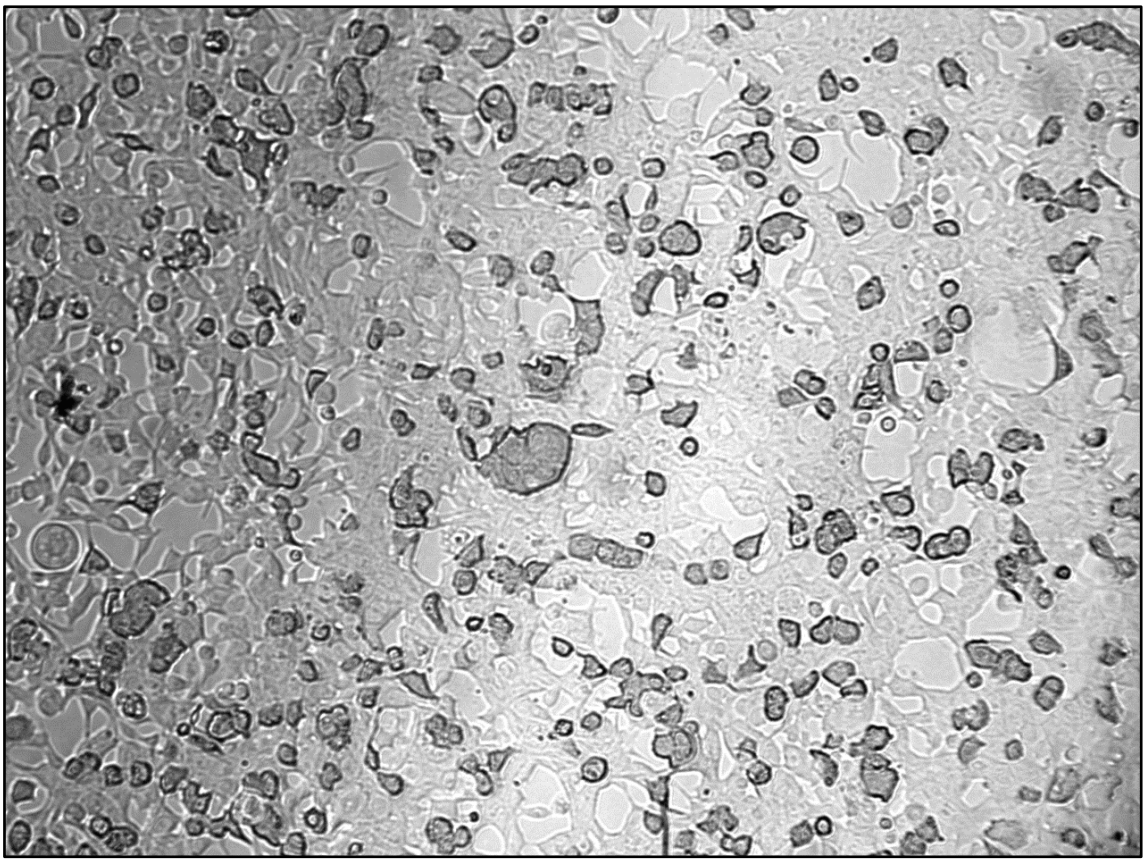


Figure 3.1 Infected HEK293 cells using Adeno-X Rapid Titer kit dye *Infected cells are labeled by the hexon-specific antibody. Counting the plaques, a titre of 1 x 10^{10} is obtained when approximately 1 plaque is found in the 10^{-10} dilution well, 10 in the 10^{-9} well, etc.*

3.2 Induction of adenoviruses in HEK293 cells resulted in GCase overexpression

To confirm the expression of the GBA1 genes after viral induction we used HEK293 cells. A GFP expressing AV was used as a positive control. Successful infection was confirmed by western blotting using a GBA polyclonal antibody as described in Materials and Methods (Figure 3.2). A lower than expected D409V GBA expression was observed owing to an underestimation of the D409V AV titer. The titer was reevaluated as described above using the Adeno-X Rapid Titer kit.

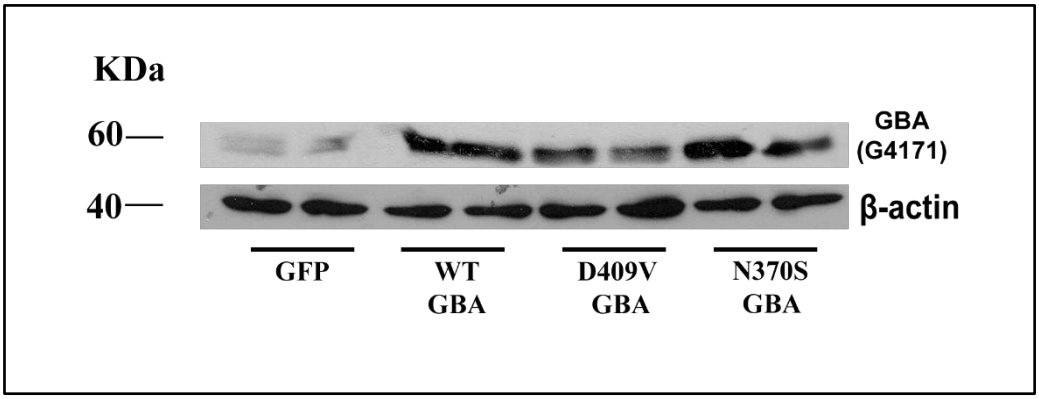


Figure 3.2 GCase overexpression by viral induction confirmed by western blotting in HEK293 cells *β-actin was used as a loading control.*

3.3 Adeno virus-mediated expression of wt and mutant glucocerebrosidase did not affect survival in primary mouse cortical neurons

To evaluate the toxicity of the adenoviruses cells were infected and collected in two different time points. Cells were lysed in a nuclei preserving buffer and living cells were calculated using a Neubauer chamber. There was no significant difference in survival between the non treated cells, the cells treated with the GBA1 expressing viruses and the cells treated with the GFP expressing virus (Figure 3.3).

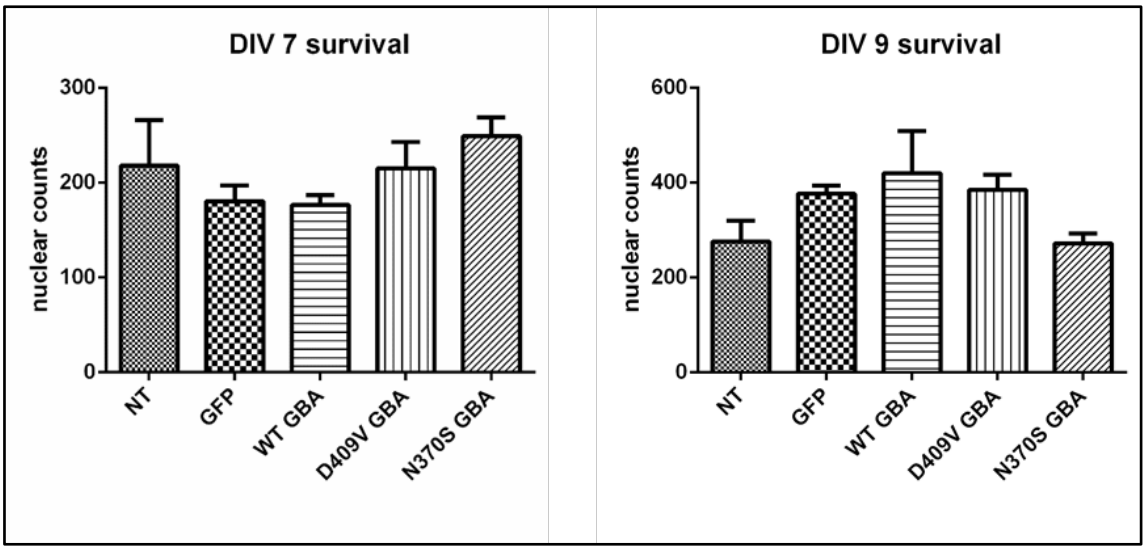


Figure 3.3 Primary mouse cortical neurons' survival after viral induction *Nuclei of living cells were estimated. There were no differences between groups. NT: non treated.*

3.4 Expression of wild type and mutant GCase does not affect the intracellular α-synuclein levels in primary mouse cortical neurons

It has been previously shown that the D409V and N370S GBA mutants can cause α-synuclein aggregation, both in animal models and iPSC dopamine neurons derived from GBA mutation carriers (87, 143, 144). Based on these observations, D409V and N370S GBA mutants were chosen for the in vitro experiments. Using these GBA variants we expected to see α-synuclein aggregation and hopefully increased α-synuclein secretion. Adenoviral vectors (AV) expressing wild type (WT) and mutant forms (D409V, N370S) of GBA were used to transduce primary mouse cortical neurons. A GFP-expressing-AV was used as a control for the viral transduction. GCase levels were measured by Western blotting using a polyclonal GBA antibody. Compared to the GFP transduced cells, WT GBA AV and D409V GBA AV showed a statistically significant increase in GCase levels (WT GBA: [4.80 ± 0.95]-fold increase vs GFP: [1.09± 0.25]-fold increase compared to non-transduced-cells, D409V GBA: [4.27 ± 1.14]-fold increase vs GFP: [1.09± 0.25]-fold increase of non-

transduced-cells); an increase in GCase levels was observed in the N370S mutant AV infected cells compared to the GFP transduced cells, however this increase was not statistically significant (N370S GBA: [3.00 ± 0.69]-fold increase vs GFP: [1.09± 0.25]-fold increase of non-transduced-cells, Figure 3.4).

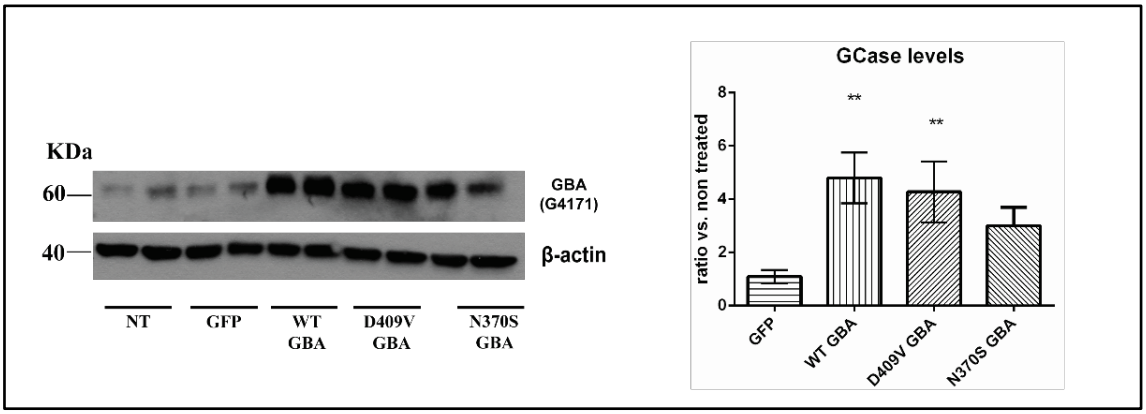


Figure 3.4 GCase overexpression in mouse cortical neurons *Mouse cortical neurons in 5th DIV were infected with control or WT GBA, D409V GBA and N370S GBA expressing AV viruses. Following 2 days of infection samples were analysed for the levels of GCase All GBA AV-transduced mouse cortical neurons showed an increase in GCase protein levels compared to neurons treated with the GFP AV control virus (n=6-8/group). The increase in GCase levels in WT GBA and D409V GBA infected neurons was statistically significant. β-actin was used as a loading control. Data represent mean values ±SEM. Differences were estimated using one-way ANOVA followed by Fischer's LSD test. p=0.0150. NT: non treated.*

To investigate whether α-synuclein alterations could be GCase-activity dependent, GCase activity assay of the cell pellets was estimated. WT GBA infected cells had 64% higher activity compared to that of the non transduced cells (WT GBA: 11.21 ± 0.54 vs GFP: 5.84± 0.97 RFU/μg of protein) while the cells infected with the mutant forms of GBA were found to have no difference in the GCase activity assay, when compared to GFP control (D409V GBA: 6.41 ± 1.45 vs GFP: 5.84± 0.97 RFU/μg of protein, N370S GBA: 5.26 ± 0.56 vs GFP: 5.84± 0.97 RFU/μg of protein, Figure 3.5). This suggests that the mutant forms expressed a non-active form of GCase which did not significantly affect the endogenous activity levels, in agreement with literature (68).

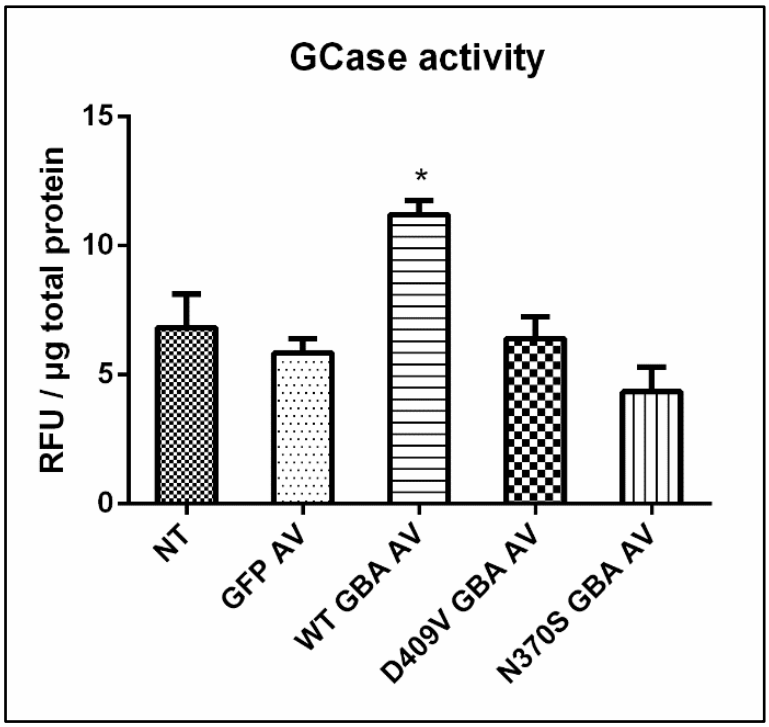


Figure 3.5 GCase activity increase in WT GBA1 AV transduced cells *Neurons treated with the WT GBA also showed a significant increase in GCase activity (n=3/group). Differences were estimated using one-way ANOVA followed by Fischer's LSD test. p= 0,0029. RFU: Relative fluorescent units. NT: non treated.*

α-synuclein levels were measured both by western blotting and by sandwich ELISA. A trend of increase in α-synuclein levels was observed by western blotting in all transduced cells, none of which was however statistically significant (Figure 3.6). This did not contradict previous results from our lab (84).

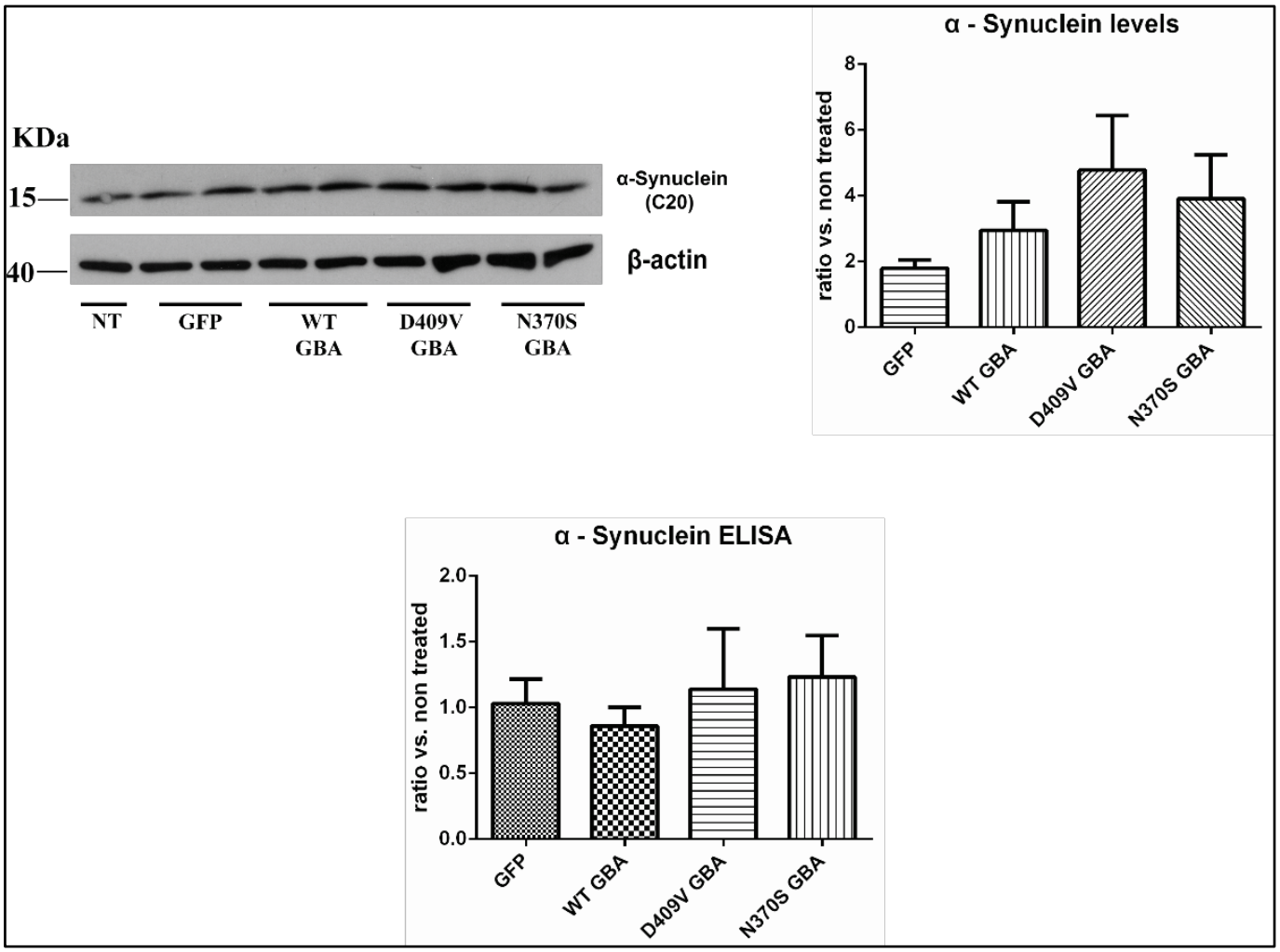


Figure 3.6 Effects of GBA expression on α-synuclein levels in mouse cortical neurons *α-synuclein levels in infected neurons were measured, both by Western Blotting and ELISA. No significant differences in α-synuclein levels between the different groups (n=6-8/group) were observed. β-actin was used as a loading control. Data represent mean values ±SEM. NT: non treated.*

3.5 GBA overexpression did not affect α-synuclein secretion in primary mouse cortical neurons

After completing the intracellular α-synuclein measurements, we proceeded to test secretion alterations in α-synuclein. Similar results were obtained for secreted levels measured by ELISA, as shown in Figure 3.7. A trend of increase that was not significant was observed in all mutant GBA-treated cells compared to control. However, taking the trend of increase into account, we were encouraged to test our interventions into longer timeframes. A constant GCase

activity inhibition could indeed result in α-synuclein secretion alterations and an in vivo setting was our next target.

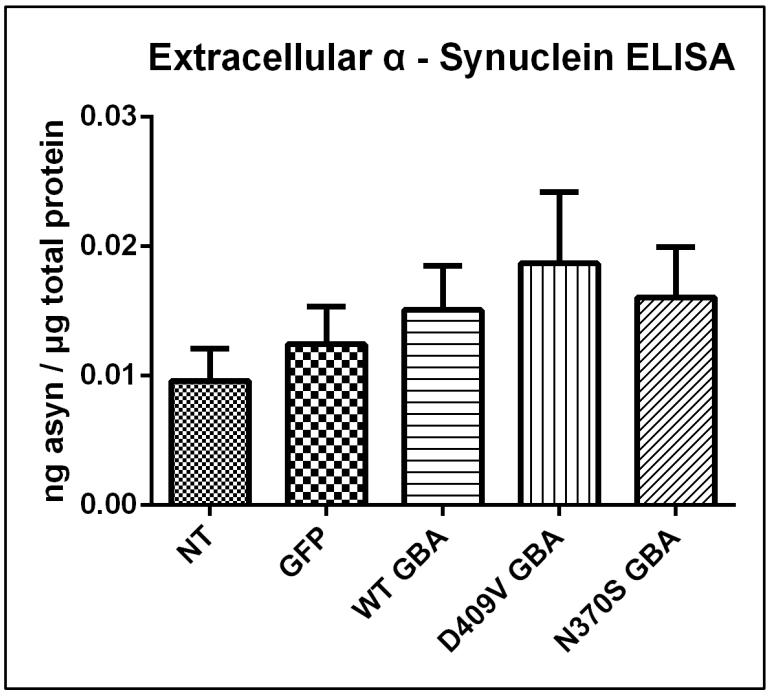


Figure 3.7 Extracellular levels of α-synuclein in GBA overexpressing mouse cortical neurons *The CM of AV-transduced mouse cortical neurons were used to measure secreted α-synuclein levels by sandwich ELISA. No significant changes were observed (n=7-10/group) in secreted total α-synuclein levels. Data represent mean values ±SEM. NT: non treated.*

3.6 Overexpression of GBA does not affect exosome associated α-synuclein levels while wild type GBA overexpression reduces exosome release in primary mouse cortical neurons

Secreted α-synuclein has been shown to be associated with exosomes (26, 91, 110). We sought to investigate whether overexpression of GBA pathological forms could affect the levels of exosomes. To this end, exosomes from the CM were isolated and used for an AChE activity assay. The activity of AChE, an enzyme specific to exosomes and not other EVs, offers an estimation of the exosome numbers of the fraction collected from the centrifugation. As shown in Figure 3.8 the cells infected with the WT GBA AV were found to have 37% less

exosomes secreted compared to the non transduced cells (WT GBA: 0.34±0.05 vs non transduced: 0.54±0.02 ng AChE/ µg of protein) as judged by the AChE assay.

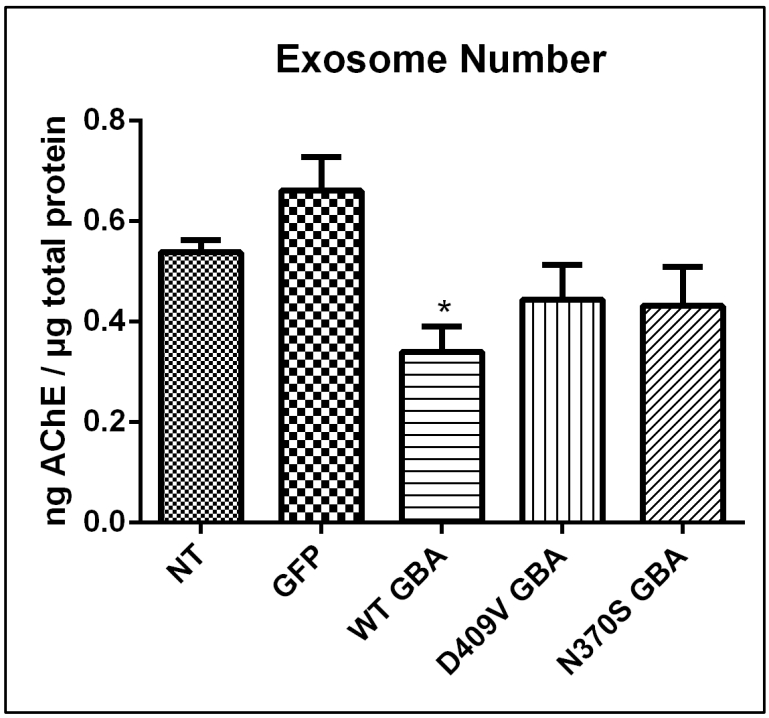


Figure 3.8 Wild type GBA overexpression reduces exosome secretion in mouse cortical neurons *Exosomes from the CM were isolated and their levels were estimated using an AChE assay. Cells treated with WT GBA1 AV, secreted significantly less exosomes compared to the GFP AV control treated cells (n=4-5/group). Data represent mean values ±SEM. Differences were estimated using one-way ANOVA followed by Tukey's post-hoc test. p=0.0197. NT: non treated.*

In order to investigate whether exosome-associated α-synuclein was different between groups, α-synuclein levels of the exosome fractions were measured by ELISA. Results were normalized to the total exosome protein measured by Bradford Protein Assay. As depicted in Figure 3.9, total exosome associated α-synuclein levels showed a trend of decrease in the WT GBA overexpressing cells (WT GBA: 0.17 ± 0.05 vs non transduced: 0.22 ± 0.05 ng α-synuclein per µg exosome protein). A normalization of exosome fraction α-synuclein levels with the total exosome fraction AChE levels offered an estimate of the "α-synuclein cargo per exosome". Again, no differences were found between

groups (Figure 3.9). In total, we were optimistic of testing similar interventions in an in vivo setting. An alteration in α-synuclein secretion seemed plausible if our model allowed GCase activity knockdown to have a longer effect on exosome secretion and thus, exosome associated α-synuclein.

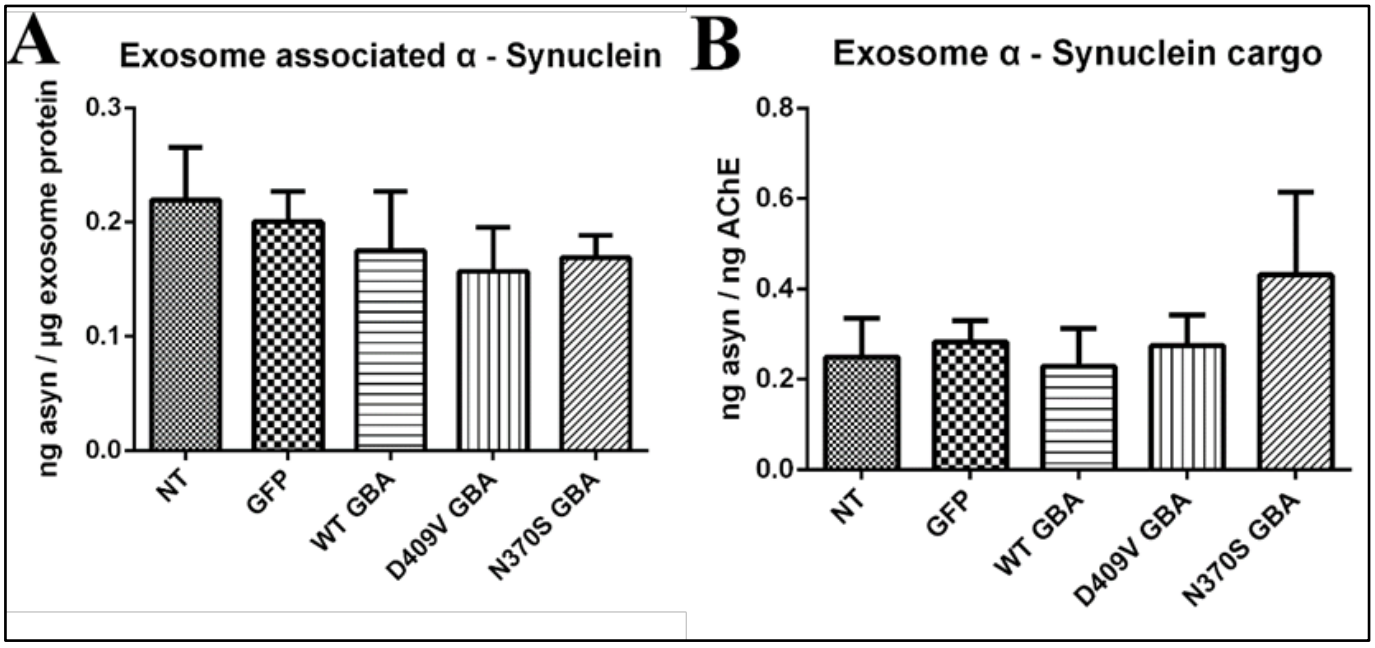


Figure 3.9 GBA effect on exosome associated α – Synuclein *Exosome α-synuclein levels were measured by ELISA. No changes were observed in total exosome associated α-synuclein levels (A). Similarly, no changes were noted in the "per exosome" α-synuclein cargo, when exosome α-synuclein levels were normalized to the total exosome AChE levels (B) (n=3-6/group). NT: non treated.*

3.7 Pharmacological inhibition of GCase activity in the presence of AV-induced α-synuclein overexpression does not significantly affect the intracellular and secreted α-synuclein levels in primary mouse cortical neurons

We further tested the effect of GCase activity alterations on α-synuclein by using a pharmacological compound in vitro. We have previously reported that GCase inhibition does not affect endogenous intracellular α-synuclein levels in cultured rat cortical neurons (84). Effects on secreted α-synuclein however have not been reported. We sought to investigate whether GCase inhibition in the presence of α-synuclein overexpression could alter intracellular and

extracellular α-synuclein levels. To this end, WT α-synuclein or GFP were overexpressed using AVs in primary mouse cortical neurons. GCase activity was inhibited in one group by adding CBE. The enzyme activity was measured through a fluorescence assay as described in Materials and Methods. α-synuclein overexpression reduced GCase activity significantly compared to GFP (ASYN AV: 15.68 ± 0.70 vs GFP AV: 20.40 ± 3.01 RFU/µg of protein, Figure 3.10), in agreement with previous observations in cortical lysates of A53T mice (145).

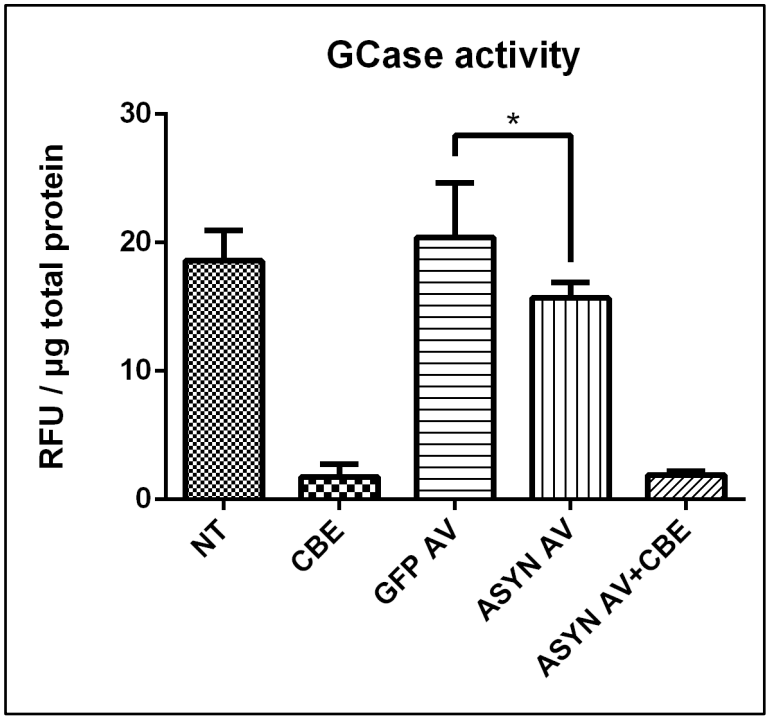


Figure 3.10 α – Synuclein overexpression reduces GCase activity in primary mouse cortical neurons *Mouse cortical neurons in 5th DIV were infected with control or WT α-synuclein expressing AV virus. On the 6th day CBE was added in some groups. On the 9th day in vitro cells were collected. Neurons treated with α-synuclein AV showed a significant decrease in GCase activity compared to GFP treated neurons (a) (n=3/group). p=0.036, NT: non-treated, CBE: CBE-treated.*

CBE treatment did not influence GCase levels, intracellular Triton-x soluble α-synuclein levels as measured by Western Blotting (CBE+α-synuclein AV: 120.50 ± 6.88, α-synuclein AV: 114.50 ± 12.18 levels normalized to β-actin, Figure 3.11 A). The SDS-soluble α-synuclein fraction showed no differences

between the two groups (CBE+α-synuclein AV: 1.93 ± 0.55, α-synuclein AV: 1.52 ± 0.47 levels normalized to flotillin-1, Figure 3.11 B).

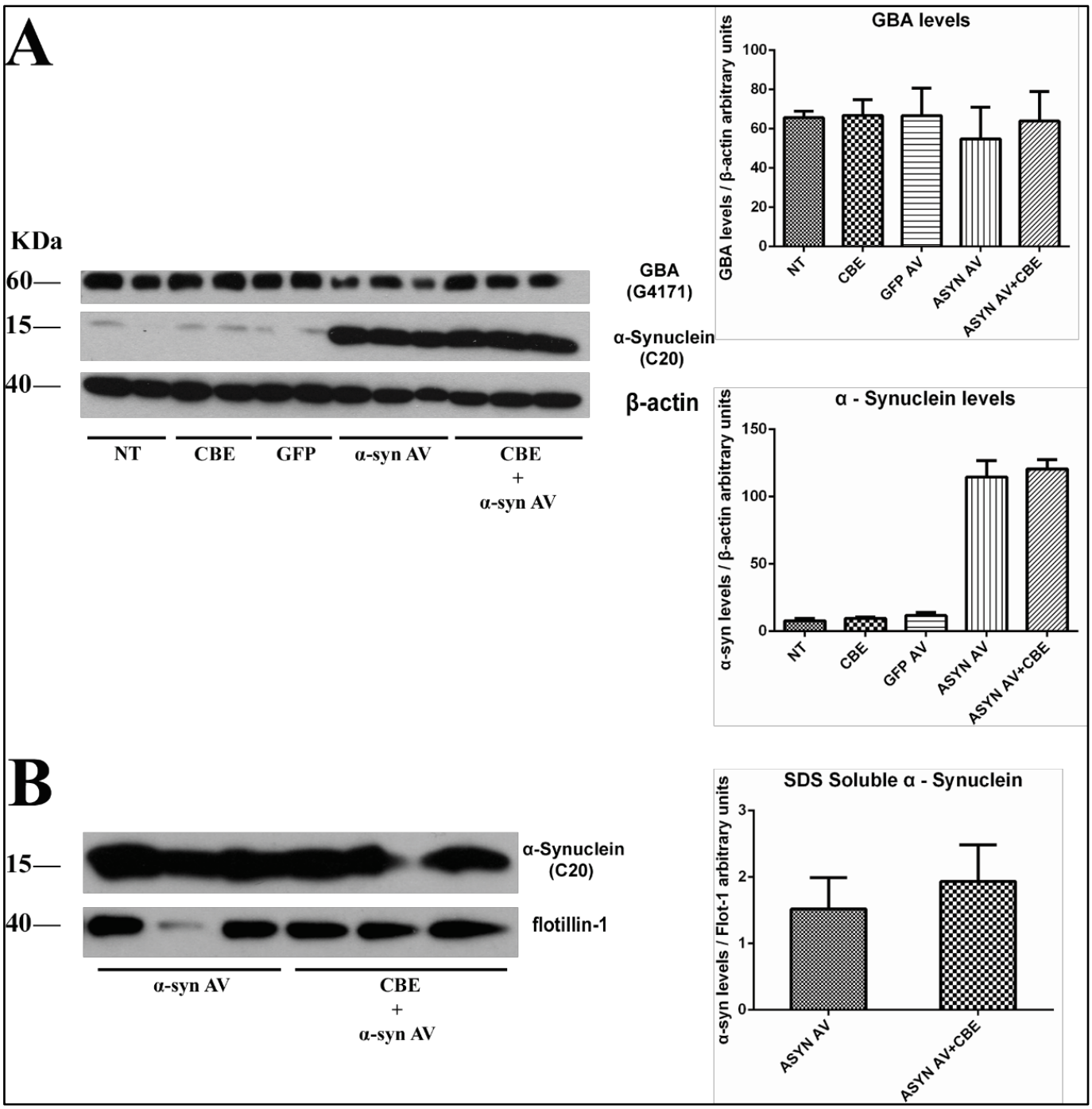


Figure 3.11 Effects of GCase inhibition on α-synuclein levels in mouse cortical neurons *(A) GCase levels were measured by Western blotting. No differences were observed between the groups. α-synuclein levels were measured by Western blotting.*

A modest, non-significant increase was observed between the cells overexpressing α-synuclein in the presence of GCase inhibition compared to the group overexpressing α-synuclein in the presence of normal endogenous GCase activity. (B) No differences in α-synuclein levels were observed in the SDS-soluble fraction. NT: non-treated, CBE: CBE-treated.

Extracellular α-synuclein levels showed a trend for increase that was not statistically significant, as measured by ELISA (CBE+α-synuclein AV: 1.71 ± 0.45, α-synuclein AV: 1.06 ± 0.36 ng α-synuclein/µg total protein, Figure 3.12).

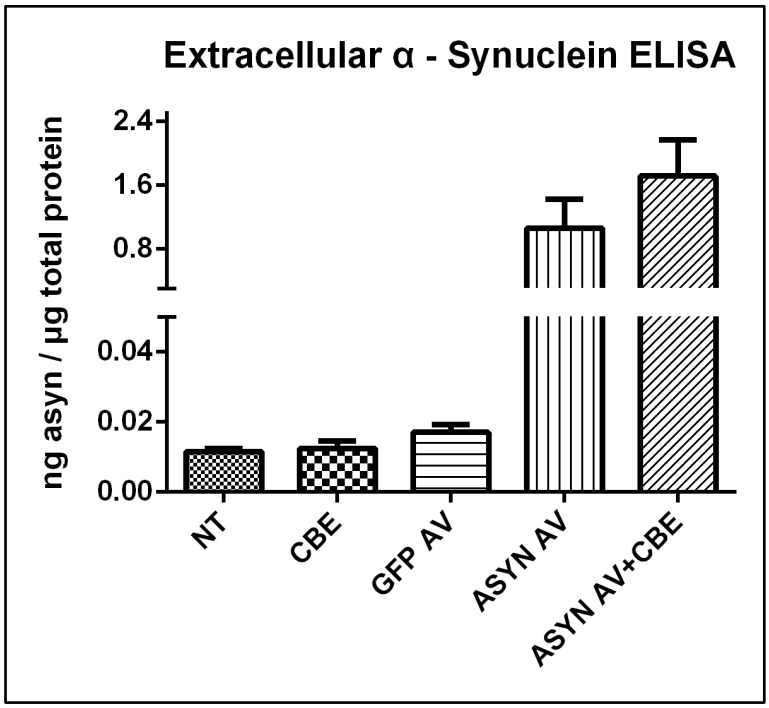


Figure 3.12 Effects of GCase inhibition on secreted α-synuclein levels in mouse cortical neurons *Extracellular α-synuclein levels were also measured by ELISA. Cells overexpressing α-synuclein in the presence of GCase inhibition showed a non-significant increase in α-synuclein levels compared to the group overexpressing α-synuclein in the presence of normal endogenous GCase activity. NT: non-treated, CBE: CBE-treated.*

3.8 Pharmacological inhibition of GCase activity does not affect exosome number and exosome-associated α-synuclein in primary mouse cortical neurons

We next sought to investigate whether GCase inhibition could affect exosome release in mouse cortical neurons. CBE was used to inhibit GCase as described previously. As shown in Figure 3.13, exosome release in cells treated with CBE was not different compared to non-treated cells (CBE: 0.25 ± 0.01 vs non treated: 0.25 ± 0.05 ng AChE/ µg of protein). Similarly, total exosome associated α-synuclein levels were not different between groups (CBE: 0.29 ± 0.03 vs non-treated: 0.29 ± 0.03 ng α-synuclein per µg exosome protein). No differences were found in the "α-synuclein cargo per exosome" between groups (CBE: 1.15 ± 0.14 vs non-treated: 1.24 ± 0.24 ng α-synuclein per ng AChE). Together with the previous results, we reasoned that apart from a longer time frame, a pathogenic α-synuclein background could be crucial for a GCase effect on α-synuclein secretion. It was time to move to an in vivo model that could provide these parameters.

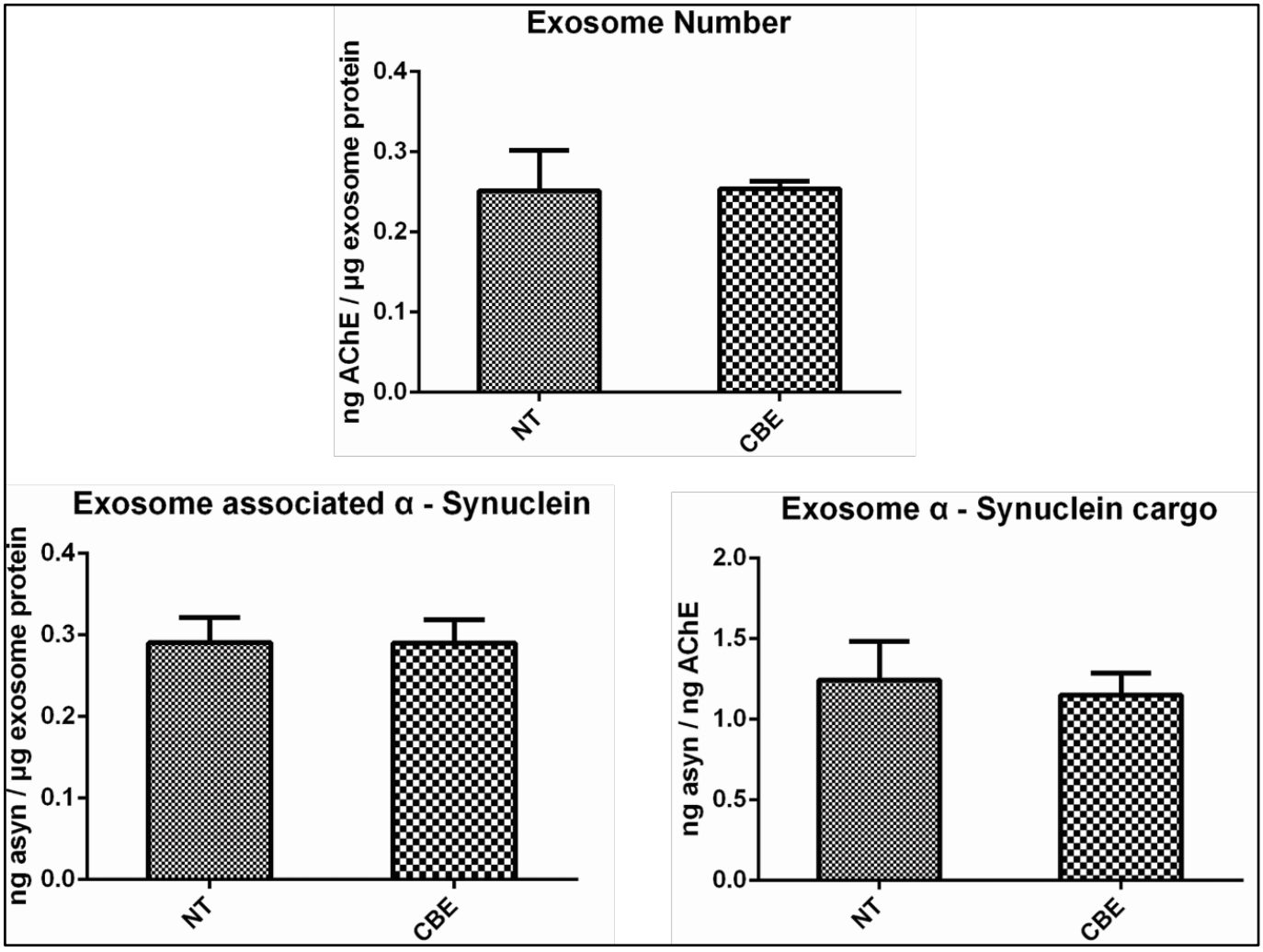


Figure 3.13 Effects of GCase inhibition on exosome – associated α-synuclein levels in mouse cortical neurons *GCase inhibition did not affect the number of exosomes secreted. Exosome – associated α-synuclein levels were similar between the groups, both the total and the "per exosome" fractions. NT: non treated.*

3.9 Pharmacological inhibition of GCase activity increases oligomeric α-synuclein levels in select brain regions in vivo

To further elucidate a role of GBA in α-synuclein levels in vivo we pharmacologically inhibited GCase in mice overexpressing A53T α-synuclein with chronic (8 weeks) exposure to CBE. Previous studies indicated that an administration interval of 48 to 72 hrs was optimal to achieve a residual brain GCase activity close to 50% (135). The effect of GCase inhibition via intra peritoneal injections was confirmed by measuring the GCase activity in the

brain of the treated mice. Cortex or striatal tissue was collected 48 to 96 hours after the last injection and the enzyme activity was measured through a fluorescence assay as described in Materials and Methods. Mice treated with CBE were found to have a 20% reduction in enzyme activity compared to the saline treated mice (Figure 3.14 A). This partial reduction was satisfactory to provide a model that mimics the enzyme deficiency of GD patients (134).
It has previously been reported that in mice lacking GBA proteasomal activity is compromised (86). To investigate whether the GCase activity inhibition would have an effect in proteasomal activity in our model, proteasomal activity was also measured in a similar manner. As shown in Figure 3.14 B there was no difference found between the CBE and the saline treated groups (CBE: 155.50 ± 40.03 vs saline: 203.30 ± 23.36 RFU/ μg total protein).

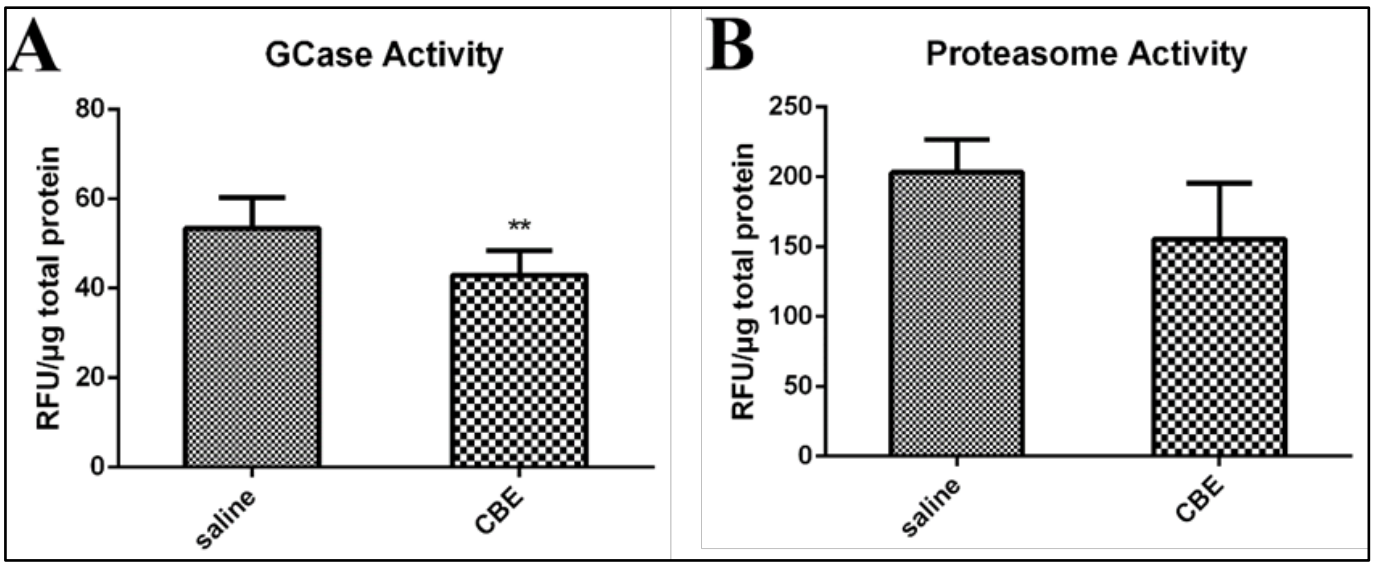
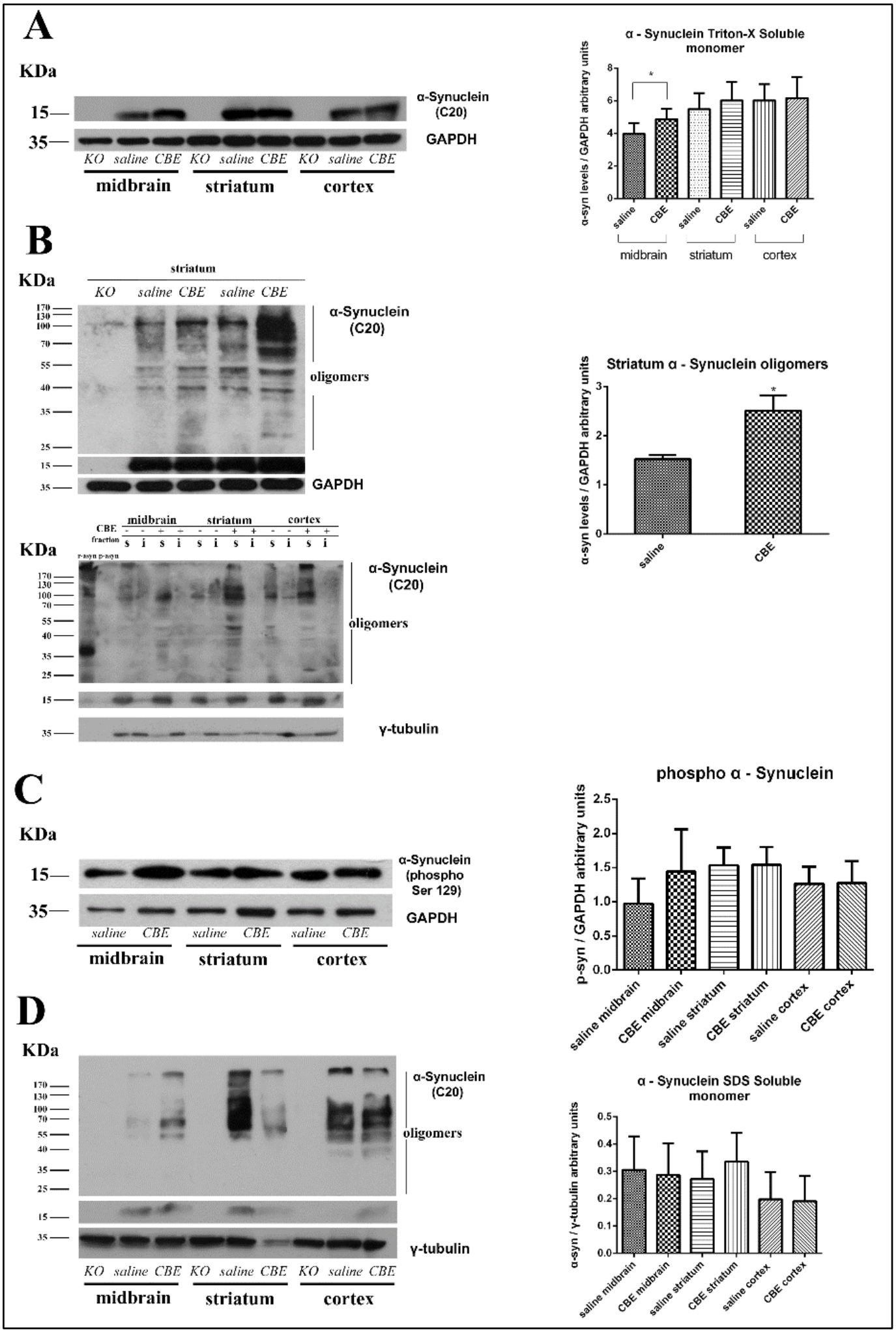


Figure 3.14 Pharmacological inhibition of GCase did not affect proteasomal activity *Cortical and striatal brain tissue of IP injected A53T SNCA overexpressing mice was used to measure GCase and proteasome activity. (A) Tissue from the CBE treated mice showed a small but significant decrease of GCase activity (n=6/group). (B) No differences were observed in proteasomal activity. Data represent mean values ±SEM. Differences were estimated using paired Student's t-test. A: p=0.019. CBE: CBE-treated, Saline: N/S 0,9% treated.*

Brain α-synuclein levels and phospho α-synuclein levels were measured by western blotting. In animals treated with CBE a ~23% increase of total

α-synuclein levels in the midbrain of the Triton-x soluble fraction was observed, compared to the control, saline treated animals (4.86 ±0.66 vs 3.96 ± 0.66 levels normalized to GAPDH). The levels of monomeric α-synuclein in the striatum and the cortex were not different between the two groups (Figure 3.15 A). Western blot analysis of midbrain, striatum and cortex brain tissue using C20 and syn1 antibodies revealed the presence of abundant Triton-x soluble α-synuclein oligomers in the CBE treated animals compared to the saline treated. Oligomers were found to be statistically increased in CBE treated mouse striata (2.51 ± 0.31 vs 1.52 ± 0.08 levels normalized to GAPDH), whereas no differences were found in cortex and midbrain regions (Figure 3.15 B). To investigate whether CBE treatment affected the levels of phosphorylated α-synuclein we analyzed both the Triton-x and SDS fractions of the three isolated brain regions using the specific phospho α-synuclein antibody. No differences were noted between the two groups (Figure 3.15 C). α-synuclein levels in the SDS soluble fractions of all three brain regions examined were not different between the two animal groups (Figure 3.15 D). As expected, no oligomeric α-synuclein species were detected by C20 in the SNCA KO mice that were used as a negative control (Figure 3.15 B). Intrigued by obtaining results that seemed in line with the hypotheses made after the in vitro data, we were eager to further examine GCase activity inhibition effects on secretion in vivo.


A
KDa
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35
α-Synuclein (C20)
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saline CBE saline CBE saline CBE
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striatum
cortex
phospho α - Synuclein
p-syn / GAPDH arbitrary units
saline midbrain
CBE midbrain
saline striatum
CBE striatum
saline cortex
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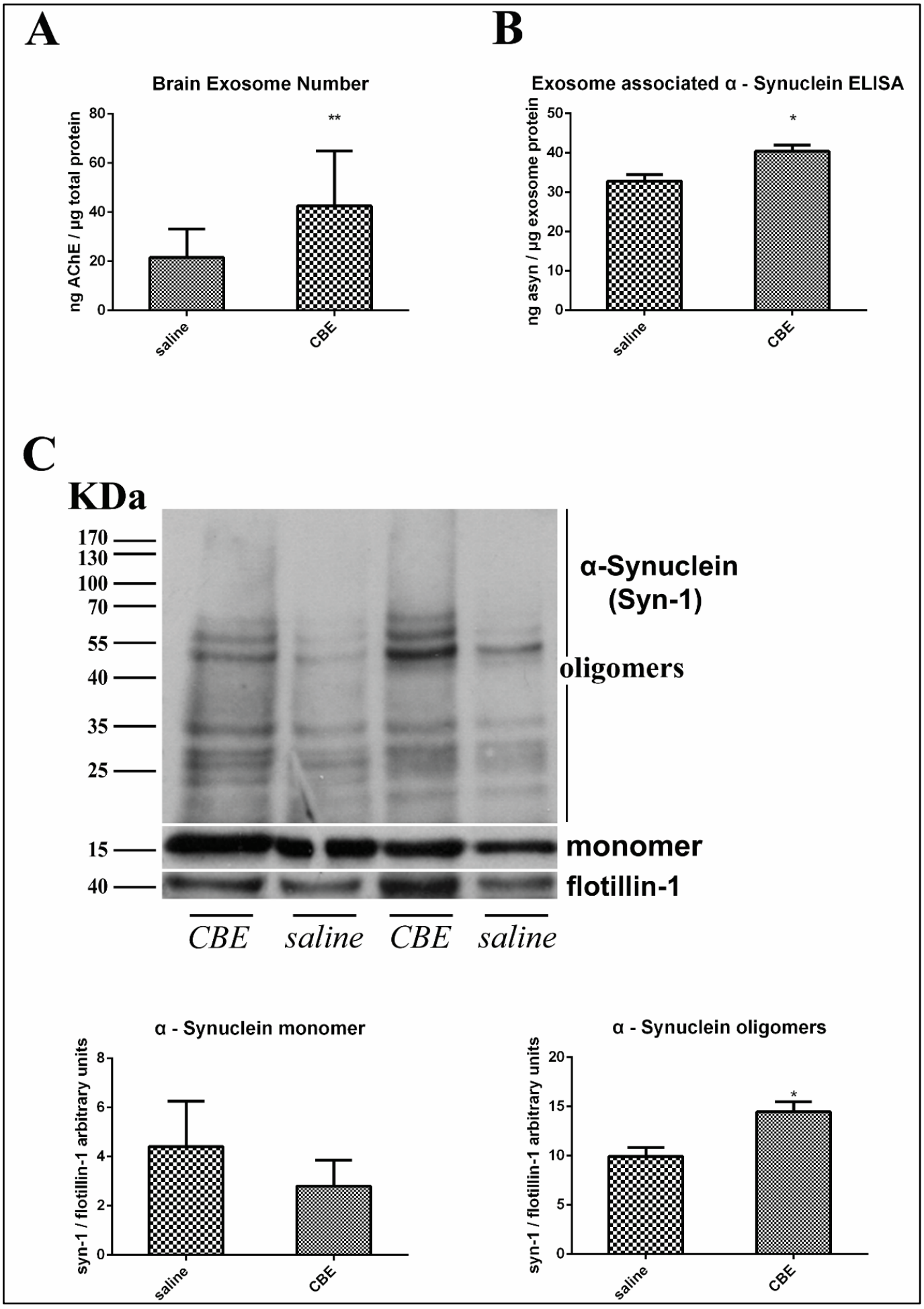
midbrain
striatum
cortex
α - Synuclein SDS Soluble monomer
α-syn / γ-tubulin arbitrary units

Figure 3.15 Increase of α-synuclein levels in CBE injected animals *The levels of α-synuclein in the midbrain, striatal and cortical brain tissue of CBE treated animals were analysed using the C20 antibody. Midbrain Triton-X soluble α-synuclein of CBE injected animals showed a significant increase compared to control. Striatal and cortical Triton-X soluble samples showed no difference between the groups (A) (n=5/group). Western blotting revealed the presence of Triton-X soluble α-synuclein oligomers. Striatal α-synuclein oligomers were found to be significantly increased in the CBE-treated group (B) (n=3/group). No differences were observed in phosphorylated α-synuclein levels between the groups in midbrain, striatum and cortex areas (C) (n=5/group). SDS soluble monomeric and oligomeric α-synuclein levels were found to have no differences in midbrain, striatum and cortex of the saline or CBE treated animals (D) (n=4-6/group). Recombinant α-synuclein (r-asyn) and recombinant phosphorylated α-synuclein (p-asyn) were used as controls for the antibodies. Data represent mean values ±SEM. Differences were estimated using paired Student's t-test. a. p=0.019 b. p=0.0368 (KO: SNCA knockout, CBE: CBE-treated, Saline: N/S 0,9% treated, s: Triton-x soluble fraction, i: Triton-x insoluble fraction). GAPDH and γ-tubulin were used as loading controls.*

3.10 Inhibition of GCase activity increases brain exosome levels

Exosome associated α-synuclein has been shown to be released in the medium of neuronal cells in vitro (26, 91). In this study, we observed exosome alterations in the presence of GCase overexpression/ activity increase in vitro. In order to investigate the effect of GCase inhibition on the numbers of exosomes released in vivo, brain tissue of the A53T mice was dissected and papain treated. The homogenized brain tissue was sequentially filtered and centrifuged and exosome pellets were collected and assessed as described above. Remarkably, the tissue from CBE treated mice showed an almost twofold increase of exosome number compared to the saline treated mice (CBE: 42.56 ±22.31 vs saline: 21.60 ±11. 59 ng AChE/ μg of protein, Figure 3.16 A). The exosome pellets were subsequently sonicated in order to analyze the α-synuclein contained in exosomes by sandwich ELISA and Western blotting. α-synuclein ELISA measurements showed a ~22.9% increase in levels in the CBE treated brain compared to the saline treated control (CBE: 40.40 ±

1.61 vs saline: 32.18 ±1.62 ng α-synuclein/ µg exosome protein, Figure 3.16 B). Western Blot analysis of CBE treated mice tissue showed an increase in α-synuclein oligomers which was statistically significant (CBE: 14.46 ±1.06 vs saline: 9.93± 0.89 levels normalized to flotillin-1). There were no differences in monomeric α-synuclein levels between the saline and CBE-treated groups (Figure 3.16 C). While an increase of α-synuclein oligomers has been found in a GD mouse model (78), an increase of exosome associated α-synuclein oligomers in a GBA PD mouse model was a first.


A
Brain Exosome Number
ng AChE / µg total protein
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20
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saline
CBE
B
Exosome associated α - Synuclein ELISA
ng asyn / µg exosome protein
50
40
30
20
10
0
*
saline
CBE
C
KDa
170
130
100
70
55
40
35
25
15
40
α-Synuclein
(Syn-1)
oligomers
monomer
flotillin-1
CBE
saline
CBE
saline
α - Synuclein monomer
syn-1 / flotillin-1 arbitrary units
8
6
4
2
0
saline
CBE
α - Synuclein oligomers
syn-1 / flotillin-1 arbitrary units
20
15
10
5
0
*
saline
CBE

Figure 3.16 Inhibition of GCase activity increases both brain exosome number and exosome associated α-synuclein *Whole brain tissue from the two groups was used to measure exosome number. The CBE-treated mouse brain tissue was found to have a statistically significant higher exosome number compared to the saline-treated group (A) (n=4/group). Exosome fractions were used to measure α-synuclein levels by ELISA. α-synuclein levels were found to be significantly increased in the CBE treated group (B) (n=4/group). Exosome associated α-synuclein levels of the two different groups were measured by Western Blotting. Triton-X soluble α-synuclein oligomers were significantly increased in the CBE treated group (C) (n=3-4/group). Data represent mean values ±SEM. Differences were estimated using ratio paired Student's t-test and Student's t-test. A. p=0.001 B. p=0.030 C. p=0.026 CBE: CBE-treated, Saline: N/S 0,9% treated.*

Further investigation of exosome associated phosphorylated α-synuclein levels by western blotting showed no differences between the groups (Figure 3.17).

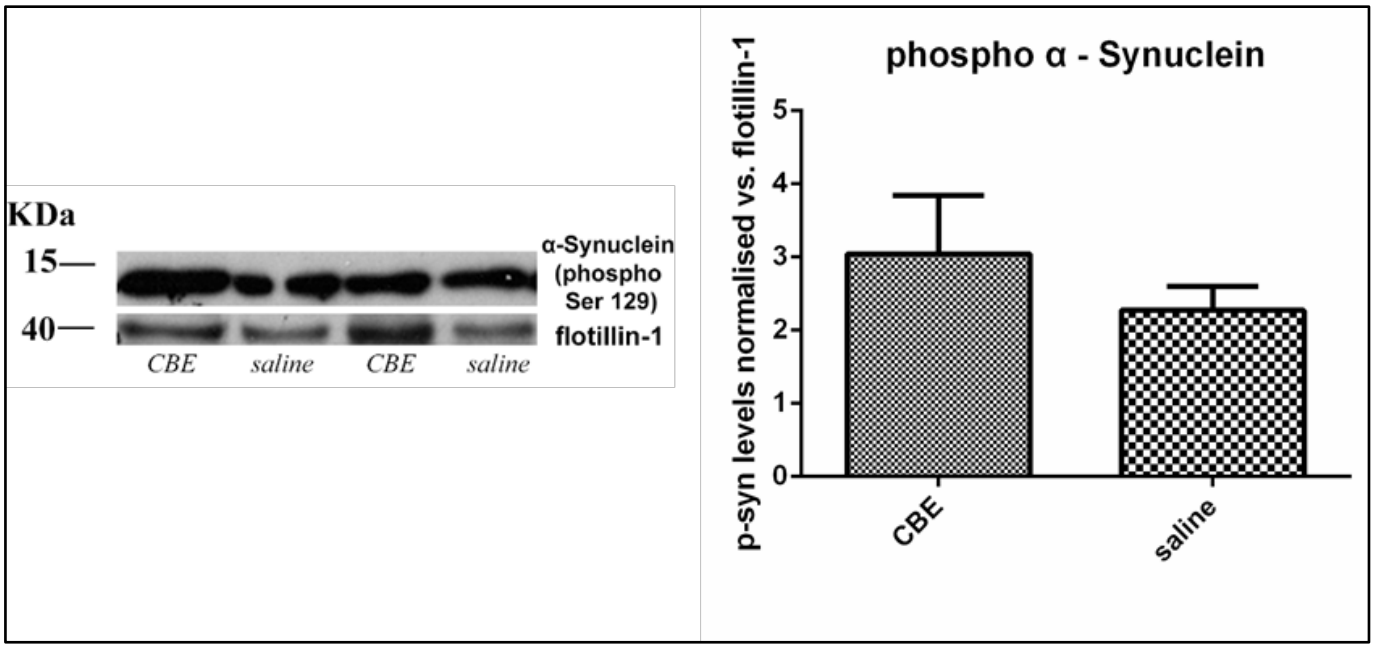

Figure 3.17 GCase inhibition did not alter brain exosome – associated phosphorylated α-synuclein *Whole brain tissue from the two groups was used to measure brain exosome – associated phosphorylated α-synuclein by western blotting. There were no differences between groups. CBE: CBE-treated, Saline: N/S 0,9% treated.*

3.11 GCase inhibition does not affect free form α-synuclein secretion in vivo

We next sought to investigate if GCase inhibition affects the release of free, non-exosome associated α-synuclein. Striatal microdialysates from Tg A53T mice treated for 8 weeks with CBE were collected and analysed with ELISA. No differences between the two groups were detected suggesting that the observed drop of GCase activity in the CBE treated group was not enough to elevate the levels of free α-synuclein in the interstitial fluid (ISF, Figure 3.18).

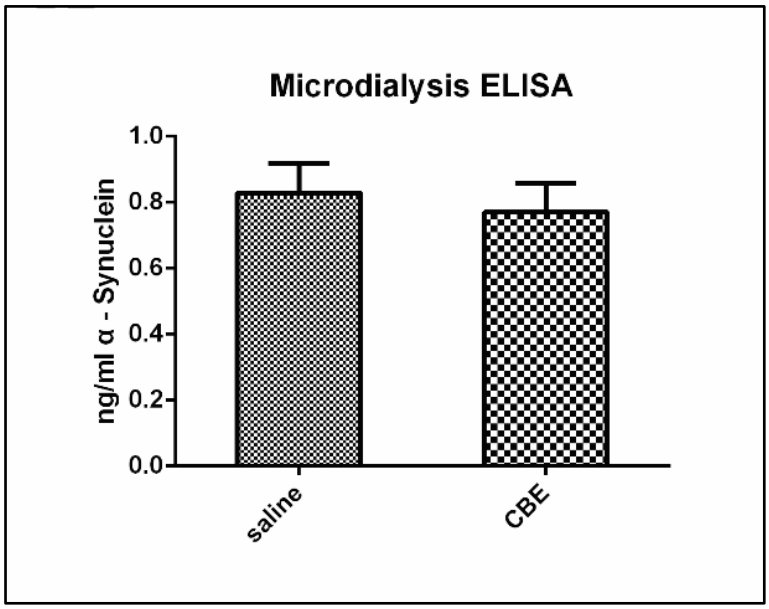

Figure 3.18 GCase inhibition does not affect striatal ISF α-synuclein levels in vivo *GCase inhibition in the CBE injected animals did not affect striatal α-synuclein secretion, as measured in striatal microdialysates by ELISA (n=5–6/group). CBE: CBE-treated, Saline: N/S 0,9% treated.*

3.12 Overexpression of mutant N370S GCase increases α-synuclein secretion in vivo

Then, we sought to examine whether overexpression of either WT or mutant GBA affects α-synuclein secretion in vivo, an effect that mimicked a gain of function model of disease. Two months old Tg A53T mice received a stereotactic injection of AAVs overexpressing GFP, WT GBA or N370S GBA in

the striatum and protein levels were verified with western blotting and immunohistochemistry (Figure 3.19 A, B).

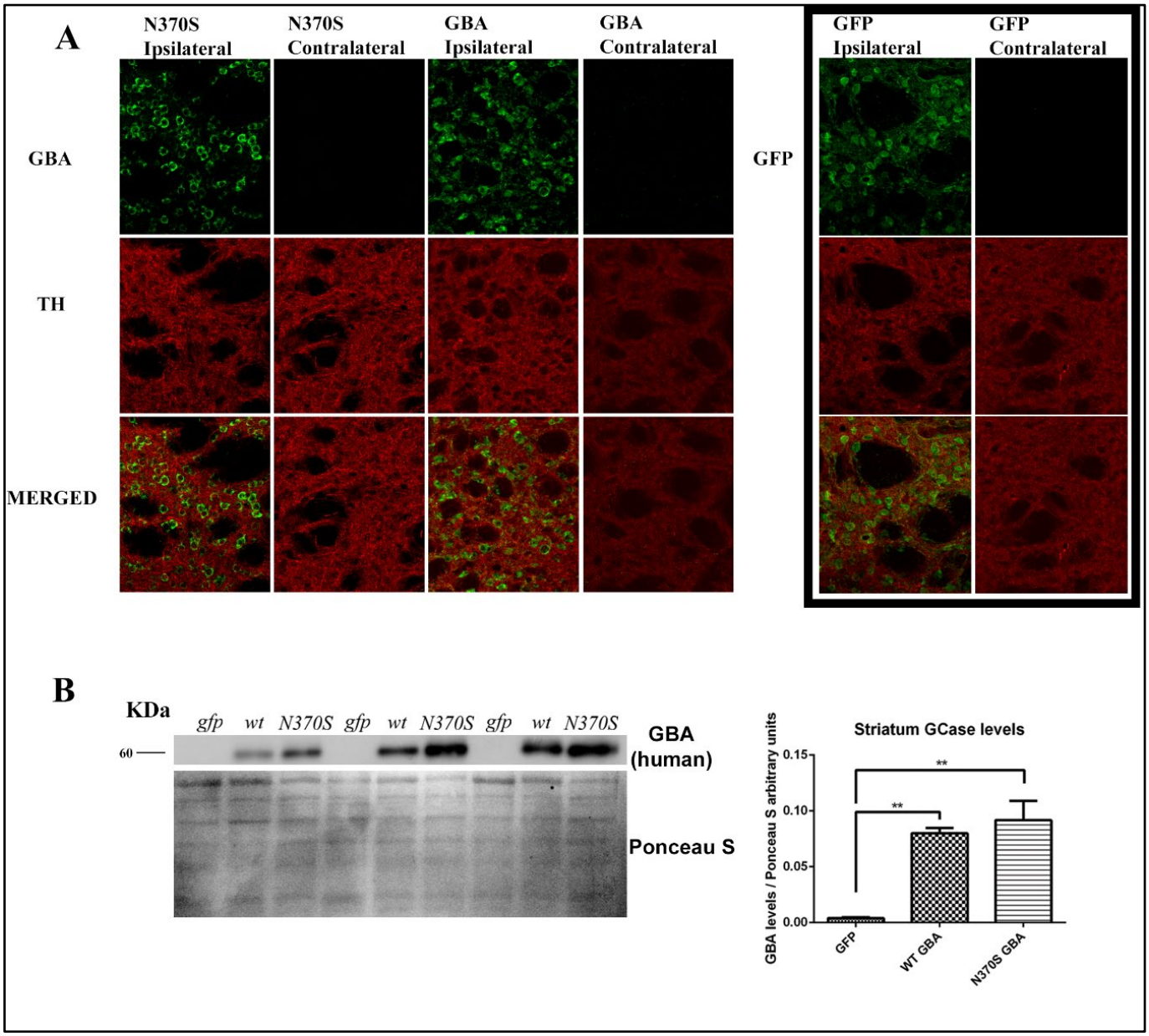

Figure 3.19 Stereotactic injections of GBA AAVs resulted in profound striatal GCase overexpression *AAVs expression was estimated via immunohistochemistry of striatum sections (A). Expression levels of WT and N370S GBA were measured by western blotting using a monoclonal anti-human GBA antibody. GCase levels were significantly increased in both wild type and N370S GBA overexpressing groups, and no differences were observed between the GBA overexpressing groups (B) (n=3/group). Green: human GBA/ GFP, Red: TH. Ponceau – S was used as a loading control (B). Data represent mean values ±SEM. Differences were estimated using one-way ANOVA followed by Tukey's post-hoc test. B. p=0.0019.*

2 months later ISF samples were collected by microdialysis and analyzed by ELISA. As shown in Figure 3.20 , the α-synuclein levels of the AAV-N370S GBA injected mice were twofold higher compared to the AAV-GFP group (N370S GBA: 0.87 ± 0.16 vs GFP: 0.36 ± 0.08 ng/ml α-synuclein). AAV-WT GBA-injected mice showed unaltered α-synuclein secretion compared to GFP.

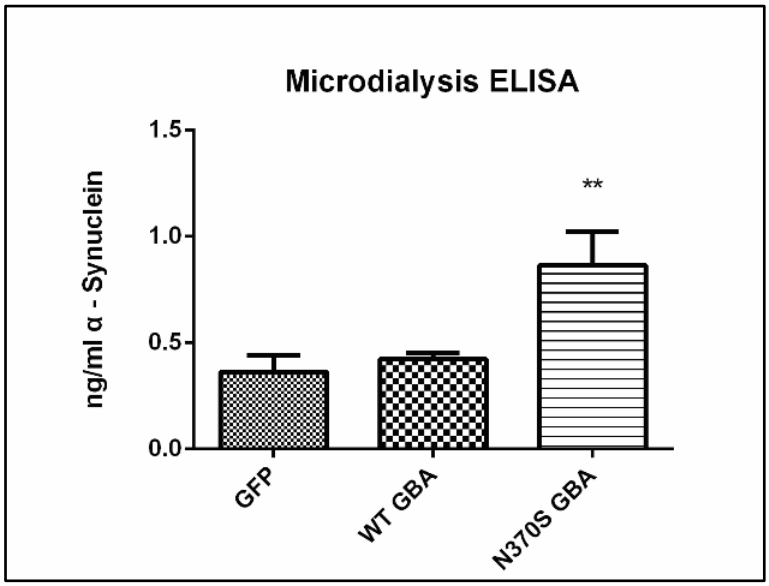

Figure 3.20 N370S GBA overexpression increases secreted α-synuclein levels in vivo *A53T mice overexpressing N370S GBA by AAV injections were found to have a profound increase of α-synuclein levels in the striatum compared to mice overexpressing GFP or WT GBA (n=4–6/group). Data represent mean values ±SEM. Differences were estimated using one-way ANOVA followed by Tukey's post hoc test. p=0.0037.*

3.13 N370S mutant GBA significantly increases LC3 levels in vivo

To investigate whether the abundant increase in α-synuclein secretion in N370S GBA overexpressing mice was due to a lysosomal or proteasomal dysfunction, we performed immunohistochemistry for LC3 and p62, and western blotting for polyubiquinated proteins. LC3 fluorescence intensity was significantly increased in the N370S GBA overexpressing group compared to both the WT GBA and the GFP overexpressing group (N370S: 1.27 ± 0.13, WT GBA: 0.80 ± 0.15, GFP: 0.77 ± 0.07 ratio of the ipsilateral side puncta to the

contralateral side puncta, Figure 3.21 A, B). This was in line with previously published data that showed autophagy defects in GBA-PD patient derived neurons (87). p62 staining showed no differences between the two groups (Figure 3.21 C, D).

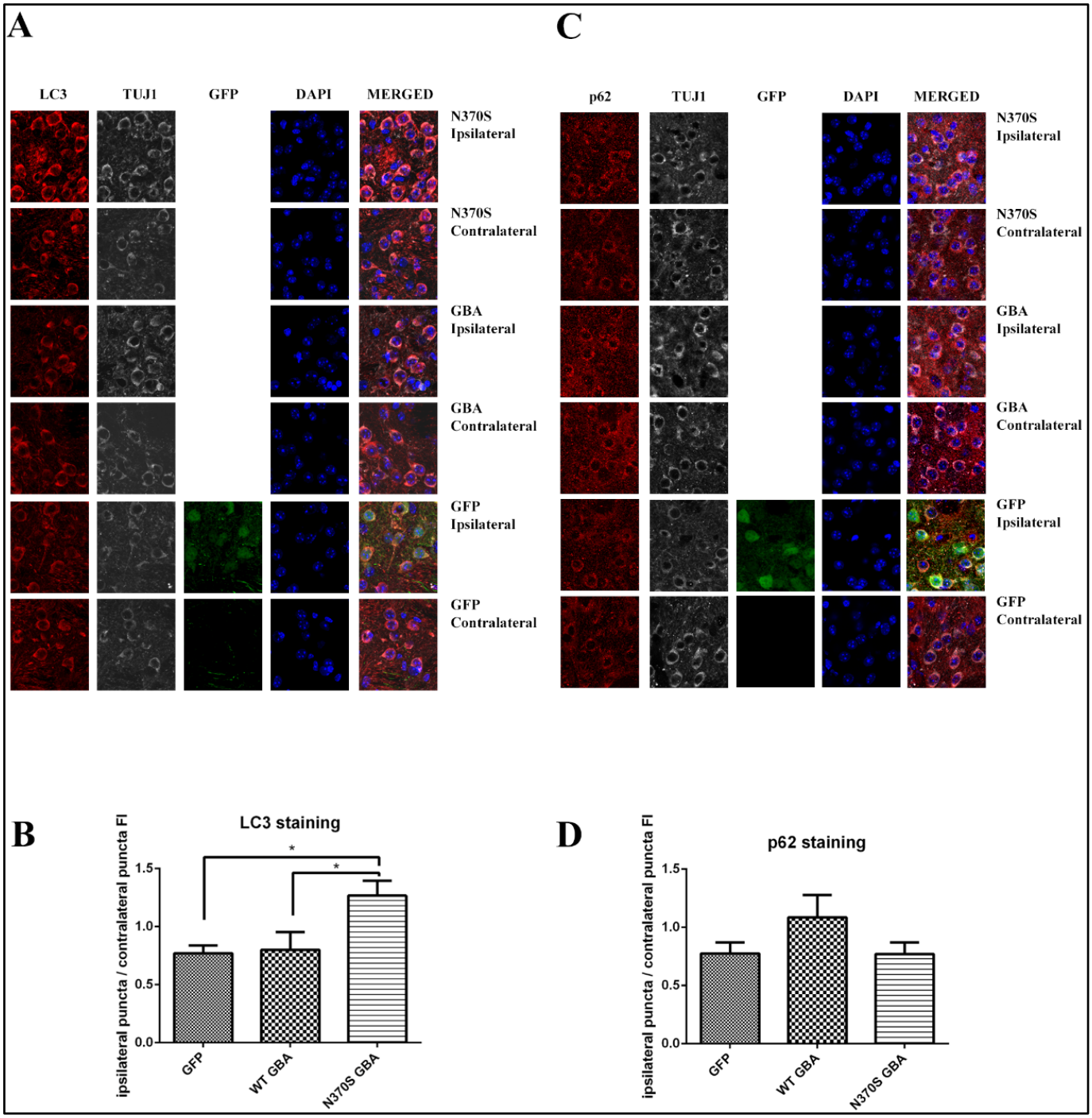

Figure 3.21 N370S GBA overexpression increases LC3 levels in vivo *Mice received striatal injections of one of the following AAVs; GFP, N370S GBA, WT GBA. Eight weeks later, mice were perfused and sections were stained for LC3, TUJ1, and p62. The intensity of fluorescense signal of LC3 and p62 in TUJ1-positive neurons was*

measured and quantified in ipsilateral (injected) vs contralateral (non-injected) side. LC3 fluorescence intensity was significantly increased in the N370S GBA overexpressing group compared to GFP and WT GBA overexpressing groups (A, B, n=6/group, A: p=0.017, B: p=0.023 respectively) There were no differences in the P62 staining fluorescence intensity between the groups (C,D). Data represent mean values ±SEM. Differences were estimated using one-way ANOVA followed by Tukey's post-hoc test.

None of the expressed GBA forms appeared to saturate the UPS as verified by examining the levels of polyubiquitin proteins (Figure 3.22).

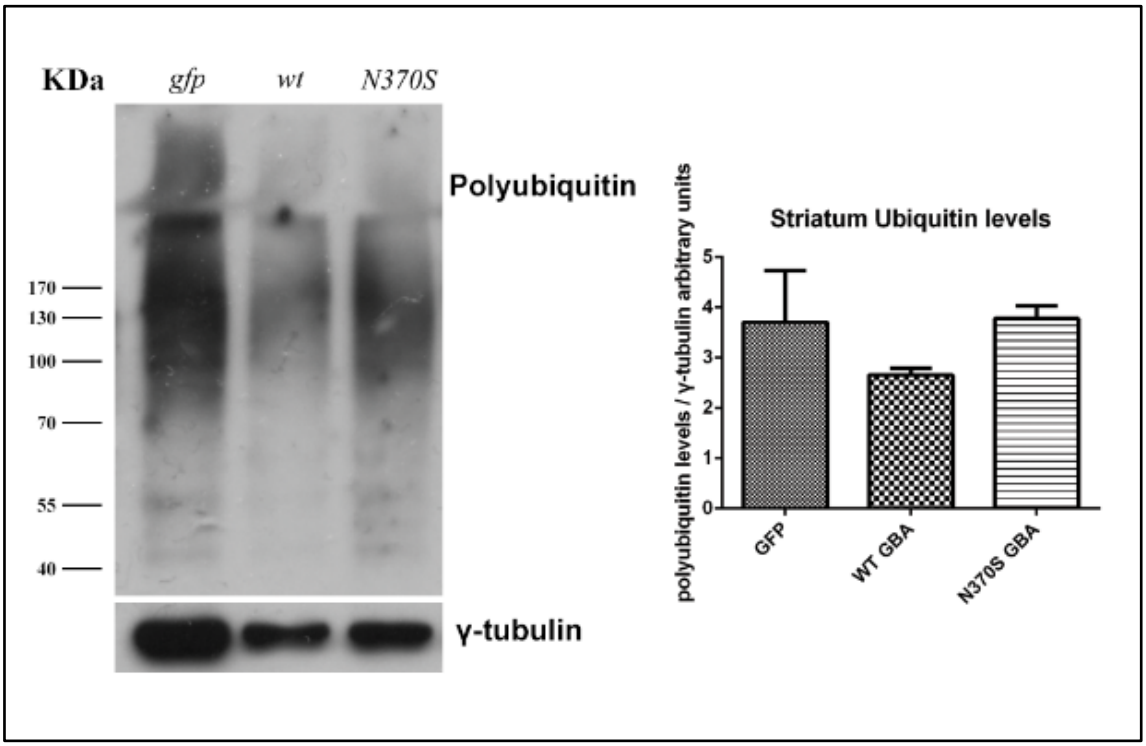

Figure 3.22 GCase overexpression does not affect polyubiquitin levels in mouse striatum *Mouse striatal levels of ubiquitin were measured by western blotting. There were no differences of ubiquitin levels between the GFP, wild-type GBA and N370S GBA overexpressing groups (n=3/group).*

3.14 Inhibition of GCase activity does not change the LC3I, LC3II and P62 levels in vivo

We finally investigated whether GCase inhibition and its effect on α-synuclein levels and secretion affected autophagy and proteasome pathways. We measured LC3I, LC3II and p62 levels in the Triton-x soluble fraction of midbrain and striatal tissue by western blotting. There were no differences between the Triton-x soluble fractions between the two groups in the P62 (Midbrain: 0.52 ± 0.06 vs 0.52 ± 0.16, Striatum: 0.32 ± 0.02 vs 0.52 ± 0.07 levels normalized to β-actin), LC3I (Midbrain: 2.09 ± 0.57 vs 2.46 ± 0.73, Striatum: 1.60 ± 0.32 vs 1.59 ± 0.34 levels normalized to β-actin) or LC3II (Midbrain: 1.38 ± 0.44 vs Saline: 1.29 ± 0.39, Striatum: 1.16 ± 0.21 vs 0.96 ± 0.18 levels normalized to β-actin, Figure 3.23 A). Similarly, there were no differences in the SDS soluble fractions of the saline and CBE-injected groups in the p62 (Midbrain: 1.20 ± 0.02 vs 1.24 ± 0.28, Striatum: 1.06 ± 0.12 vs 1.19 ± 0.04 levels normalized to γ-tubulin), LC3I (Midbrain: 1.56 ± 0.35 vs 1.74 ± 0.13, Striatum: 0.92 ± 0.31 vs 1.23 ± 0.15 levels normalized to γ-tubulin) or LC3II (Midbrain: 0.71 ± 0.09 vs 0.70 ± 0.10, Striatum: 0.35 ± 0.18 vs 0.41 ± 0.05 levels normalized to γ-tubulin, Figure 3.23 B).

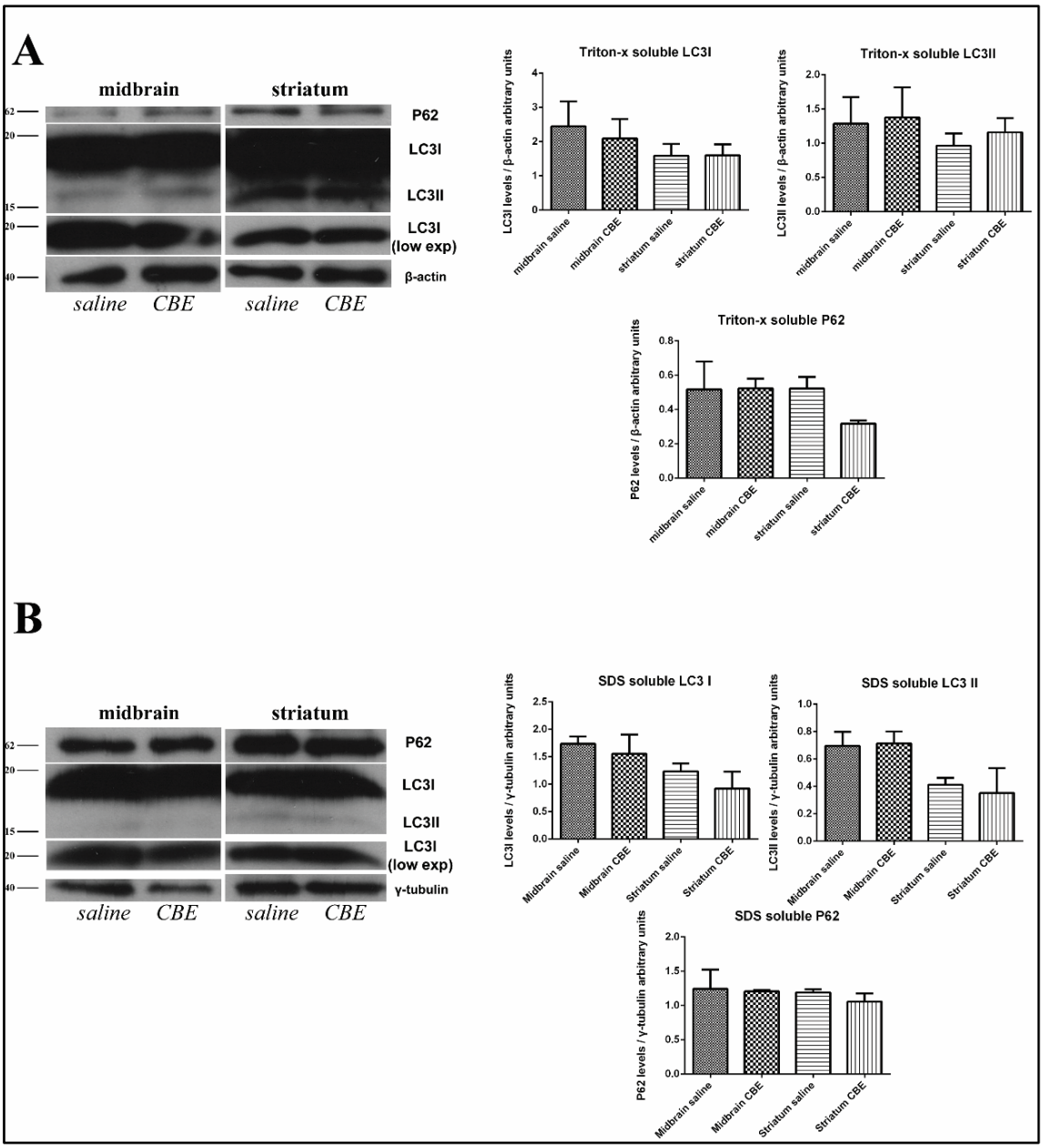

Figure 3.23 Inhibition of GCase activity does not affect LC3I, LC3II and P62 levels *Mibrain and striatal brain tissue of IP (saline or CBE)- injected A53T SNCA overexpressing mice were used to measure LC3I, LC3II and P62 levels by Western Blotting. As shown in (A), Triton-x soluble samples showed no differences between the groups (n=3/group). Similarly, SDS-soluble samples showed no differences (n=3/group) (B). β-actin (A) and γ-tubulin (B) were used as respective loading controls, and levels of LC3I, LC3II and p62 were normalized to these. CBE: CBE-treated, Saline: N/S 0,9% treated.*

4. Discussion

The dysregulation of extracellular α-synuclein as a contributor to PD initiation and progression has been the basis for the hypothesis of a prion-like mechanism of PD pathology (114). Recent studies have also used this hypothesis in order to evaluate α-synuclein and α-synuclein aggregates as a potential biomarker for PD (146). Indeed, pathogenic species of α-synuclein can aid toxic seed formation and potentially act as a prion protein when located extracellularly while pathogenic α-synuclein has also been found in exosomes (26, 147). It has previously been reported that prion proteins can be associated with exosomes (148, 149). Taken together, the above data point to the suggestion that α-synuclein can act as a prion-like protein through exosome-mediated propagation.

Meanwhile, the increased incidence of reduced GCase activity in sporadic PD patients and the fact that GBA is the most common genetic risk factor for PD point to a pathogenic feedback loop between GCase and α-synuclein (78). As already mentioned, this bidirectional effect between α-synuclein and GCase has been approached by both loss of function and gain of toxic function models of pathology. It is plausible that a turning point exists when a pathogenic threshold is crossed and neurodegeneration occurs. More specifically, an effect of GCase in the dysregulation of extracellular α-synuclein and exosome associated α-synuclein could serve as this turning point and has only been recently examined in vitro (91).

To investigate this intriguing hypothesis we set out to examine how GCase modulation could affect free α-synuclein secretion, exosome secretion and exosome associated α-synuclein, both in vitro and in vivo.

In line with our previous results (84), GCase inhibition was not sufficient to influence α-synuclein in neuronal cell cultures, neither intracellular nor secreted. Moreover, exosomes were also not affected by profound GCase inhibition. WT GBA overexpression in neuronal cell cultures, while leading to a

reduction of exosome release, did not significantly reduce exosome-associated α-synuclein. In total, both WT and mutant GBA overexpression or GCase inhibition did not influence intracellular or secreted α-synuclein significantly. However, our model indicated that GCase activity might have an effect on exosome secretion. These results could be interpreted as demonstrating a lack of interaction between GCase and α-synuclein at the biochemical level. However, they could also be explained by the major handicap of this primary cellular model. In both experiments, the time frame employed was short; although GBA PD is known to have an earlier age of onset than sporadic PD, it is a neurodegenerative disorder that appears in middle aged or, more commonly, elderly patients. It was thus conceivable that the intrinsic limitations in the time frame of our cell cultures did not allow for a long time course that is needed for pathology to develop. Furthermore, none of our in vitro models had a pathogenic α-synuclein form that would resemble that found in a PD patient. The GCase inhibition model had a WT α-synuclein overexpression and the GBA overexpression model only had an endogenous WT α-synuclein expression. We reasoned that an in vivo setting which could provide a longer time frame for other gene modifiers to interact with a pathogenic α-synuclein background could be crucial to demonstrate effects on α-synuclein aggregation and secretion. This prompted us to move on to an in vivo model that would provide such a setting. As suggested by previous studies, lysosome dysfunction (110) or protein aggregation (150) can increase exosome release. In our cell model, GBA overexpression could have reversed either of the above pathways reducing exosome number.

Based on these observations and on the fact that certain non-significant trends of increase of α-synuclein were observed in the mutant GBA overexpressing cells as well as upon GCase inhibition, we moved on to evaluate the effects of chronic GCase inhibition on intracellular and extracellular α-synuclein species in vivo. Our aim was to replicate previously published data that showed an increase of α-synuclein oligomers in a GD mouse model (78) and investigate whether this GCase inhibition effect altered α-synuclein secretion. α-synuclein levels were measured in the midbrain, striatum and cortex of A53T-SNCA transgenic mice following treatment with saline or CBE. A modest, but

significant, increase of α-synuclein monomer was found specifically in the midbrain and, more interestingly, α-synuclein oligomers were found to be significantly increased in the striatum, suggesting specific effects on the nigrostriatal axis. A number of studies have reported specific nigrostriatal pathway abnormalities in mice treated with CBE including synaptic dysfunction and increased inflammation (83, 135). Such alterations may aid the increase of α-synuclein oligomeric species, as observed in the striatum. These results, combined with the preliminary in vitro results, allowed us to confidently test our hypothesis of an altered α-synuclein secretion in the presence of GCase activity inhibition. Interestingly, animals overexpressing A53T α-synuclein in the presence of reduced GCase activity had a two-fold increase of exosome number and exosome associated α-synuclein. More importantly, this increase was found to be due specifically to an increased presence of α-synuclein oligomers in the brain of the same group. This confirms previous findings of increased transmission of α-synuclein aggregates in GCase deficient cells (92) and indicates that this increased transmission could be exosome-mediated. In contrast to these profound effects on exosomal α-synuclein, chronic CBE treatment did not influence free ISF α-synuclein. To examine whether GCase inhibition affected α-synuclein by modifying the autophagy pathway, we measured brain P62 and LC3 levels by Western Blotting. Interestingly, there were no differences observed between the two groups. This suggests that chronic modest GCase loss of function affects α-synuclein species and exosomal secretion through an autophagy-independent mechanism.

Based on previous work from our lab that examined the effect of a GBA mutant in the deregulation of extracellular α-synuclein and exosome associated α-synuclein in human iPSC dopaminergic neurons (91), we redirected our efforts towards examining a possible gain of function effect of the N370S GBA mutant on extracellular α-synuclein levels of PD model mice. While the CBE experiments simulated a chronic loss of function of GCase, such as is expected in GBA-PD and likely sporadic PD, a gain of function effect of GBA mutations has also been proposed as a mechanism to explain the link of GBA mutations to PD (98). To examine the possible role of a gain of function pathology model in extracellular α-synuclein, we used stereotactically injected AAVs

overexpressing N370S GBA in the striatum of the same mouse model of α-synuclein overexpression. Through ISF microdialysis, mice overexpressing N370S GBA in the striatum were found to have a profound increase of secreted α-synuclein compared to both the GFP and the WT GBA overexpressing groups. These results indicate that gain of function effects of GBA mutations, likely through misprocessing of GCase, ER stress upregulation, and autophagic/lysosomal dysfunction, as demonstrated in our previous work (91), may be responsible for excessive release of α-synuclein. Interestingly, LC3 staining of brain sections from the two groups revealed a significant increase in the N370S GBA-overexpressing group as calculated by fluorescence intensity measurement. N370S GBA overexpression did not alter P62 levels. It has been suggested that misfolded GCase trapped in the ER leads to both an increase in the UPS and ER stress (91, 129). In our study, viral expression of either WT or N370S GBA did not seem to affect proteasomal activity as judged by the levels of polyubiquitinated proteins detected in the striatum of injected mice. Further experiments are needed to investigate the effect of mutant GBA on ER and the release of α-synuclein in our model. It remains to be seen whether this profound increase in α-synuclein secretion caused by mutant GCase can lead to oligomeric α-synuclein formation, thus effectively contributing to PD pathology. Due to experimental limitations, it was not possible to test whether mutant GCase overexpresion affects exosomal α-synuclein.

Thus, in a chronic in vivo setting, either gain of function effects of mutant GCase through autophagic/lysosomal dysfunction or loss of function of endogenous GCase lead to profound alterations in extracellular α-synuclein. In the former case, effects are observed on free ISF α-synuclein, whereas in the latter, on exosomal α-synuclein, and in particular oligomeric species (Figure 4.1). Hence, combined effects of loss of function and gain of function may lead to alteration of the homeostasis of α-synuclein secretion in PD patients with GBA mutations.

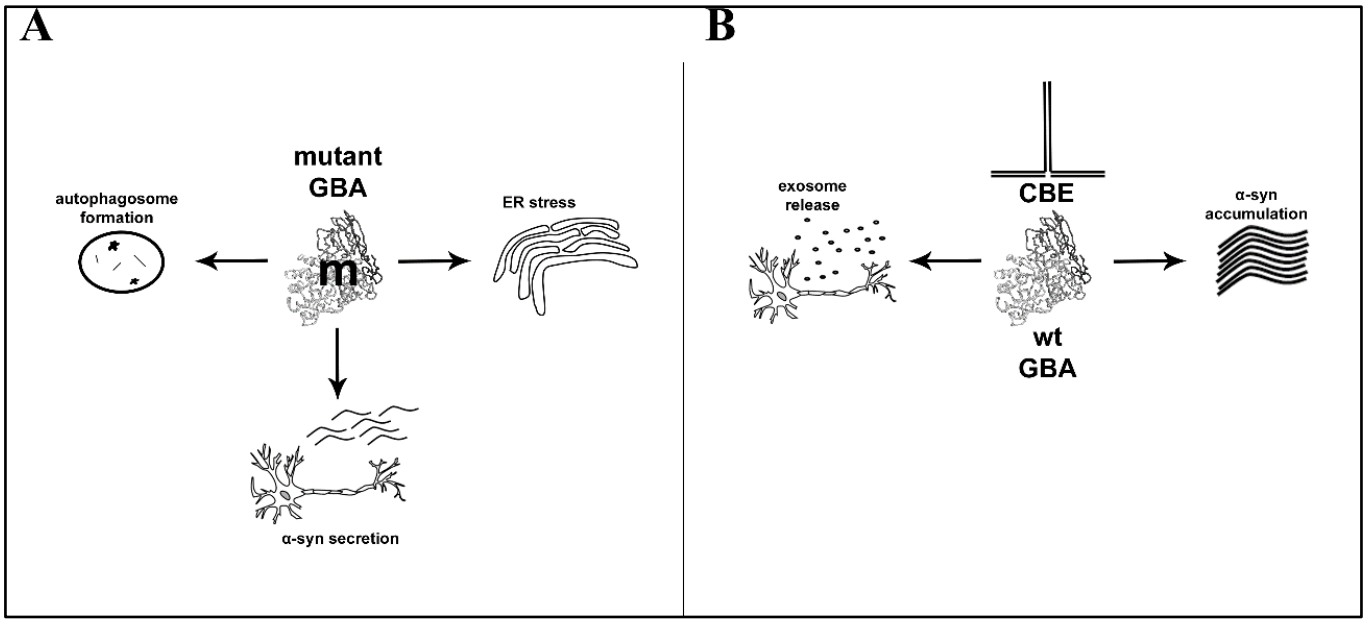

Figure 4.1 Proposed mechanisms of gain-of-function and loss-of-function effects of GCase on α-synuclein *(A) A gain-of-toxic-function mechanism was replicated by intrastriatal injections of N370S mutant GBA in mice overexpressing α-synuclein. An erratic GCase could lead to increased autophagosome formation as well as ER stress. Taken together, these two pathways could alter α-synuclein secretion. (B) A loss-of-function mechanism was achieved by inhibiting GCase through intraperitoneal injections of CBE in mice overexpressing α-synuclein. A decreased GCase activity led to increased exosome release as well as α-synuclein accumulation in the form of soluble oligomers. These changes in the intracellular and exosome-associated α-synuclein levels were mediated through an autophagy independent mechanism.*

Taken together, our results provide for the first time in vivo evidence that GCase has a role in extracellular α-synuclein homeostasis and that exosome associated pathogenic α-synuclein can be increased in the presence of GCase activity inhibition. Exosome-associated oligomeric α-synuclein, apart from a therapeutic target, may also prove to be a relevant biomarker for PD, and thus the study of its modifiers, such as GCase shown here, may prove to be clinically relevant.

Remarkably, the quest for a PD biomarker has been rather unsuccessful since the discovery of α-synuclein. Despite extensive research (151-153), studies

have failed to produce a consistent result that would allow for a broad screening of patients. Even though α-synuclein has proved to be the driving force for pathology and had been expected to appear in greater amounts in fluids of PD patients, it has mainly appeared to be decreased in amount (154).

This could be interpreted in many ways. A hypothesis of α-synuclein being transferred via a “Trojan horse” mechanism of externalized vesicles like exosomes seems the most appealing. Pathological α-synuclein could travel in disguise and avoid degradation by extracellular cleaving agents and thus, unprotected free form α-synuclein would appear normal or, as observed in literature, reduced. It seems as we might have been measuring a responsive collateral degradation of non-pathological α-synuclein all along.

At the same time, the fact that PD and synucleinopathies encompass a wide spectrum of pathology owing to different steps along the evolvement of a “Lewy Body disease” suggests that genotype-tailored biomarkers could be a step in the right direction.

With all these in mind, studying GD and GBA-PD patients while measuring serum or CSF exosome-associated α-synuclein can indeed offer useful new insights on PD screening. A reproducible blood test for PD could be a few steps away.

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Appendix

I. Glossary

i. α-Synuclein

The normal neuronal function of the 140 amino acid α-synuclein protein is not fully understood. It is known that it occurs in the cytosol, possibly also in mitochondria and the nucleus. It probably has a role in synaptic vesicle dynamics, mitochondrial function, intracellular trafficking and might be a potential chaperone. α-Synuclein acquires neurotoxic properties during a pathogenetic process in which soluble α-synuclein monomers initially form oligomers, then progressively combine to form small protofibrils and eventually large, insoluble α-synuclein fibrils (that is, the aggregates that make up Lewy pathology)

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ii. β-Glucocerebrosidase

The active protein transcribed by GBA, GCase, is a 497- amino acid (AA) lysosomal hydrolase. The main function of GCase is to degrade glucocerebroside into ceramide and glucose, but it also cleaves glucosylsphingosine and potentially other β-glucosides. During its transport to the lysosome, GCase undergoes several modifications. Having two functional

ATG initiation sites, GCase is transcribed as a 536 or 516 AA protein which is further processed into the functional 497 AA enzyme. By using cellular and animal models, it was shown that the lysosomal integral membrane protein type 2 (LIMP2/SCARB2) is a mannose-6-phosphate independent receptor which transfers GCase to the lysosome. Within the lysosome, GCase is peripherally associated to the inner membrane where it exerts its activity together with Saposin C and negatively charged lipids that are essential for its proper function.

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iii. Exosomes

Exosomes are small cell-derived vesicles of 30–120nm that are present in many and perhaps all biological fluids. Exosomes were first discovered in the mid-1980s by the Johnstone group, who found that, in maturing mammalian reticulocytes, the transferrin receptor and some other membrane associated elements are selectively released in multivesicular body- (MVB-) derived circulating vesicles, which they named exosomes. Exosomes have now been recognized as an important communication and signaling pathway in the body in both normal and disease settings.

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ORIGINAL ARTICLE

Modulation of β-glucocerebrosidase increases α-synuclein secretion and exosome release in mouse models of Parkinson's disease

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