Glucocerebrosidase Mutations and the Pathogenesis of Parkinson's Disease

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A thesis submitted for the degree of Doctor of Philosophy

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2015

Declaration

I, Michelle Beavan, confirm that the work presented in this thesis is my own. Where information has been derived from other sources, I confirm that this has been indicated in the thesis.

Acknowledgement

I would like to thank my supervisor Professor Anthony Schapira for giving me the opportunity to study in the Department of Clinical Neurosciences part of the Institute of Neurology of University College London. Thank you for your guidance and support. I would also like to express my thanks and appreciation to Dr. David Chau for his commitment and dedication to my work. He has been a hands-on secondary supervisor and extremely encouraging and supportive.

During my PhD, I was responsible for the follow-up of a clinical cohort of individuals at risk for Parkinson's disease (PD). Thank you to Dr. Alisdair McNeill who recruited this cohort in 2010. I was further responsible for obtaining skin biopsies from subjects within the clinical cohort and I cultured the skin fibroblasts using a protocol developed by Dr. Jan-Willem Taanmen. Dr. Shi-Yu Yang used the subcutaneous fat from each skin biopsy to culture human adipose neural crest stem cells (NCSC). Thanks to Dr. Shi-Yu Yang for the extensive characterisation of this NCSC model and to Dr. Zhi Yao at Professor Michael Duchen's laboratory for performing the functional analysis of the differentiated neuronal cells. I used a protocol developed by Dr. Shi-Yu Yang to differentiate the NCSC into dopaminergic neurons. Using the above cell models, I went on to perform the laboratory studies as detailed in my thesis.

Thank you to my colleagues at the Department of Clinical Neurosciences, including Shi-Yu Yang, Alisdair McNeill, Revital Golan, Joana Magalhaes, Matthew Gegg, Christina Gewinner, Jan-Willem Taanmen, Christos Proukakis, Kenneth Pryde, and Stephen Mullin. I would like to express a special thank you to Revital Golan for her

technical support. I would also like to give special thanks to Shi-Yu Yang, an experienced senior scientist, who has passed on his wisdom and experience, and with whom I have formed a valuable collaboration.

I am grateful to Dr. Christos Proukakis, who has encouraged me to keep up my clinical skills, and inspired me to pursue the specialty of Movement Disorders.

Thank you to my parents, who have supported and encouraged me at all times. Thank you to my partner Ash Oswal who has also completed a PhD. We have shared the highs and lows of research, and provided mutual support.

Abstract

To date, a mutation of the glucocerebrosidase gene (*GBA*) is the strongest genetic risk factor associated to Parkinson's disease (PD). This leads to my prospective cohort study of a *GBA* mutation positive cohort for early features of PD. This study indicates that as a group, *GBA* mutation positive individuals show deterioration in clinical markers consistent with the prodrome of PD.

I have generated cell culture models from individuals within the clinical cohort studied, in order to delineate the molecular mechanism of mutant *GBA* to the pathogenesis of PD.

My results on skin fibroblast cultures reproduce the glucocerebrosidase enzyme (GCase) enhancement seen from previous studies following treatment with pharmacological chaperone (PC) molecules. These data further provide support for a link between *GBA* mutations and changes in the autophagic/lysosomal system, which could predispose to neurodegeneration.

Due to the limitation of fibroblasts as a model for interrogating the complete pathway in PD, I studied human adipose neural crest stem cell (NCSC) derived dopaminergic (DA) neurons. This model recapitulated the defects identified in the fibroblast model including: reductions in GCase activity and protein level, and lysosomal abnormalities including impairments of autophagy. In addition, reduced GCase was associated with increased α-synuclein (SNCA). PC treatment restored GCase function, upregulated macroautophagy and lead to a reduction in SNCA levels.

PC therapy could represent a novel therapeutic approach for PD.

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iv. Abbreviations

GD	Gaucher Disease
GBA	Glucocerebrosidase gene
GBA	Glucocerebrosidase protein
GCase	Glucocerebrosidase enzyme
PD	Parkinson's disease
IPD	Idiopathic Parkinson's disease
DLB	Dementia with Lewy bodies
SNCA	α-synuclein
LB	Lewy body
SNpc	Substantia nigra pars compacta
ERT	Enzyme Replacement Therapy
SRT	Substrate Reduction Therapy
PC	Pharmacological Chaperone
ABX	Ambroxol
IFG	Isofagomine
ER	Endoplasmic Reticulum
UPS	Ubiquitin-Proteasome Pathway
CMA	Chaperone Mediated Autophagy
UPSIT	University of Pennsylvania Smell Identification Test
RBD	Rapid Eye Movement Sleep Behaviour Disorder
BDI	Beck's Depression Inventory
UMSARS	Unified Multiple System Atrophy Rating Scale
MOCA	Montreal Cognitive Assessment
MMSE	Mini-Mental State Examintation
UPDRS	Unified Parkinson's Disease Rating Scale
NMS	Non-motor symptoms
ET	Essential tremor
NCSC	Neural crest stem cells
iPSC	Induced pluripotent stem cells
DA	Dopaminergic

Chapter 1: The Association Between Glucocerebrosidase Mutations and Parkinson's Disease

1.1 Gaucher disease

Named after the French physician Philippe Gaucher, who originally described the clinical features in 1882, Gaucher disease (GD) is the most common lysosomal storage disorder (Grabowski, 2008) and is more frequent in the Ashkenazi Jewish population. It follows an autosomal recessive mode of inheritance and is caused by mutations in both copies (homozygous or compound heterozygous) of the glucocerebrosidase (*GBA*) gene. *GBA* encodes a lysosomal enzyme (GCase), which cleaves the β-glucosyl linkage of glucosylceramide (GC) and glucosylsphingosine (GS). GD patients have reduced GCase activity, while heterozygote carriers have varying degrees of enzyme activity ranging from 50% to normal levels (Raghavan et al., 1980). However, although mutations in *GBA* are usually associated with a reduction in GCase activity, the exact mechanism that mediates the pathogenesis of GD is unknown. The effects of reduced GCase and lysosomal activity, mis-trafficking of glucocerebrosidase protein (GBA), and endoplasmic reticulum (ER) stress are all thought to be contributory. GD is characterized by widespread accumulation of substrates GC and GS within the lysosomes of several cell types, in particular cells of mononuclear phagocyte origin, and in several tissues, including brain. Macrophages with GC-laden lysosomes, or 'Gaucher cells', are a classic cellular

hallmark of GD and infiltrate the liver, spleen, and bone marrow. Common manifestations of the disease include anaemia, thrombocytopenia, hepatosplenomegaly, and bone disease (Beutler and Grabowski, 2001).

GD has historically been classified into three main clinical subtypes based on age of onset, clinical presentation, and nervous system involvement (Cox and Schofield, 1997). Type 1 GD is the most common phenotype and can manifest at any age. Although pan-ethnic, it is more frequent among persons of Ashkenazi Jewish heritage (Zimran et al., 1991). The neuronopathic forms of the disease, type 2 and type 3, usually appear in infancy or adolescence, respectively, and typically present with a more severe clinical phenotype. The wide spectrum of clinical manifestations seen in patients with type 1 GD has led many to argue, however, that this classification system is too simplistic (Sidransky, n.d.). Indeed, the observation of parkinsonism, dementia, and subclinical peripheral neuropathy in patients with type 1 GD has challenged its more traditional definition as non-neuronopathic.

Clinically, GD is highly variable, and genotype-phenotype correlations are limited. Although the spectrum of disease is thought to correlate in part with residual enzyme activity (Cox and Schofield, 1997), there is substantial clinical heterogeneity, even among siblings and identical twins (Lachmann et al., 2004; Biegstraaten et al., 2011).

Current treatment for patients with GD include enzyme replacement therapy (ERT) (but this does not cross the blood-brain barrier and is very expensive) and alternatives such as substrate reduction therapy (SRT) or the use of small molecule enzyme-chaperone therapy (Beutler and Grabowski, 2001). The last mentioned has the added benefit of crossing the blood–brain barrier, providing an opportunity to

treat additional features of the disease, e.g. the neurological manifestations of GD. Pharmacological chaperones, e.g. ambroxol and isofagomine, have been shown to bind mutant misfolded GCase stalled in the ER, assisting in the refolding and passage to the lysosome, where the chaperone-enzyme complex dissociates in the low pH and the residual activity of the enzyme can hydrolyse substrate (Sun et al., 2012; Bendikov-Bar et al., 2013).

1.2 Glucocerebrosidase gene (*GBA*)

The gene encoding *GBA* is located on chromosome 1q21 and comprises 11 exons and 10 introns, spanning 7.6 kb of sequence. Located 16 kb downstream and 2 kb shorter is a highly homologous pseudogene (*GPAP*), which has > 96% identity to the coding regions of the functional gene. This complicates sequencing, as many mutations encountered in GD patients are identical to sequences ordinarily found only in the pseudogene. Thus, for diagnostic purposes it is essential to differentiate between sequences from the gene and the pseudogene. So far 300 pathogenic mutations throughout the *GBA* gene have been identified (Hruska et al., 2008), including point mutations, insertions, deletions, frameshift mutations, splice-site alterations, and recombinant alleles that encompass segments of the pseudogene sequence. The c.1226A > G (N370S) mutation is the commonest *GBA* mutation in the literature followed by c.1448T > C (L444P) (Beutler and Grabowski, 2001). The frequency of *GBA* mutations, however, varies according to the different ethnicity. For instance, in the Ashkenazi Jewish population, 1 in 14–18 carries a *GBA* mutation. Therefore, screening for *GBA* mutations common in Ashkenazi Jews could be cost-effective but is not a reliable strategy for other ethnic groups, in

which *GBA* mutations are found in less than 1% and a large number of different mutations are seen. Moreover, the N370S variant, although frequent among those of European, American, or Middle Eastern origin, is not typically seen in Chinese or Japanese populations (Mitsui et al., 2009; Huang et al., 2011).

1.3 The link between *GBA* mutations and Parkinson's disease

Among the neurological complications of type 1 GD is parkinsonism. This has challenged the traditional classification of type 1 GD as non-neuropathic. The possibility of a link between the two diseases was first suggested in the clinic setting where a small number of case reports described parkinsonian features in GD patients (Van Bogaert, 1939; Sack, 1980; Turpin JC, Dubois G, Brice A, Masson M, Nadaud MC, 1987). Larger studies have since been published which have confirmed these findings (Bembi et al., 2003; Tayebi et al., 2003). Heterozygote carriers of mutations in the *GBA* gene also have an increased frequency of PD (Halperin et al., n.d.; Goker-Alpan et al., 2004), and approximately 5–10% of PD patients have *GBA* mutations (Mata et al., 2008; Sidransky et al., 2009a), confirming mutations of this gene as numerically the most important genetic predisposing risk factor for PD identified to date.

Studies describing the penetrance and lifetime relative risk (RR) of PD in patients with GD and heterozygote *GBA* mutation carriers are summarized in **Table 1.1**. Data are conflicting, with some studies reporting a slightly higher estimated penetrance and lifetime RR of PD in heterozygous *GBA* mutation carriers compared to GD patients. This may reflect differences in the size and ethnicity of the cohorts that

were evaluated, rather than a significant difference in the overall risk of PD between the two groups.

Studies have investigated the frequency of *GBA* mutations in patients with sporadic PD and have shown that the odds ratio for a known patient with idiopathic PD to harbour one *GBA* mutation is 5.4 (Sidransky et al., 2009a). In other words, patients with PD are five times more likely to carry *GBA* mutations than healthy controls. Moreover, when compared to other PD-related genes, such as α-synuclein (SNCA) and *Parkin*, the frequency of *GBA* mutations is higher in individuals with parkinsonism (Lwin et al., 2004). Of further interest is a recent study that has identified a *GBA* mutation, E326K, that causes loss of GCase activity (McNeill et al., 2014) and can predispose to PD, but is a variant that does not, when homozygous, cause GD (Duran et al., 2013).

GBA mutations have been identified in other Lewy body disorders including dementia with Lewy bodies (DLB), Lewy body variant Alzheimer disease (LBV-AD), but not multiple-system atrophy (MSA). These disorders, known as synucleinopathies, are characterized by the deposition of fibrillated SNCA within brainstem or cortical inclusion bodies. In an autopsy study of 75 cases with pathologically confirmed Lewy body (LB) disorders, *GBA* mutations were identified in 23% of cases with DLB, 4% of those with PD, and none with MSA (Goker-Alpan et al., 2006). Furthermore, in a recent report, those with DLB were eight times more likely to carry a mutation in *GBA* than healthy controls, suggesting *GBA* mutations may play an even greater role in the genetic aetiology of DLB than in PD (Nalls et al., 2013). Conversely, studies in MSA patients, including 108 British pathologically confirmed cases (Segarane et al., 2009), have not identified significant differences in

the *GBA* mutations observed in cases and controls. This suggests a distinct pathogenesis for MSA, a disease in which SNCA is deposited in oligodendroglial cytoplasmic inclusions rather than neurons, that is not just related to PD but also shares traits of a demyelinating disorder. This also emphasizes the relationship between *GBA* mutations and SNCA neuronal aggregation in PD and DLB. Moreover, *GBA* mutations have not been identified in patients with progressive supranuclear palsy (PSP) or corticobasal degeneration (CBD) (Jamrozik et al., 2010).

Table 1.1 Age-specific penetrance rates and lifetime relative risk of Parkinson's disease in Gaucher disease patients and heterozygote *GBA* mutation carriers.

	GD patients		Reference	Heterozygote *GBA* mutation carriers	Reference
	Women	Men			
Penetrance of PD at 65 years, %	1.5	7.7	(Chetrit et al., 2013)	2.2	(Rana et al., 2013)
Penetrance of PD at 70 years, %	1.5	15.2	(Chetrit et al., 2013)	21.4	(Anheim et al., 2012)
	5.0	7.0	(Rosenbloom et al., 2011)		
Penetrance of PD at 75 years, %	-		-	6.8	(Rana et al., 2013)
Penetrance of PD at 80 years, %	9.0	12.0	(Rosenbloom et al., 2011)	29.7	(Anheim et al., 2012)
Penetrance of PD at 85 years, %	-		-	10.9	(Rana et al., 2013)
Overall lifetime RR of PD compared to that in the general population	21.4		(Bultron et al., 2010)	30.0	(McNeill et al., 2012a)

1.4 GBA-associated PD

GBA-related parkinsonism largely resembles sporadic PD, but it has been characterized by some subtle differences, for instance a younger age of onset (Neumann et al., 2009), a higher frequency of cognitive decline (Goker-Alpan et al., 2008; Neumann et al., 2009), bradykinesia (Gan-Or et al., 2009), olfactory dysfunction (Goker-Alpan et al., 2008), and a lower frequency of rigidity (Clark et al., 2007).

1.4.1 Clinical phenotype

Parkinsonism in GD patients and GBA heterozygotes

PD is typically characterized by the motor symptoms of resting tremor, bradykinesia, rigidity, and postural instability. Patients with GD who develop parkinsonism typically exhibit the symptoms of sporadic PD. The majority of patients with type 1 GD who developed PD therefore present with asymmetric onset, tremor, and bradykinesia (Goker-Alpan et al., 2008), the cardinal features of 'idiopathic' PD. **Table 1.2** summarizes all the published studies looking at the frequency and clinical features of PD patients with mutations in *GBA*. In patients with PD who had heterozygous mutations in *GBA*, Neumann et al. (Neumann et al., 2009) reported hallucinations in 45% and signs of cognitive decline or dementia in 48% by the time the patient died. A study of Spanish PD patients with heterozygous *GBA* mutations confirmed an increased frequency of dementia (Setó-Salvia et al., 2012). Furthermore, in a recent longitudinal (~71 months) community-based unselected PD cohort, there was a significantly greater risk for progression to dementia in heterozygote *GBA* mutation carriers (Winder-Rhodes et al., 2013).

Table 1.2 Frequency and Clinical Features of PD patients with *GBA* Mutations.

Sample Population, *n*		Frequency of *GBA* Mutations (%)		Mutations Sequenced	Most common variant (s)	Ethnicity	Mean Age of Onset of PD, yrs	Clinical Features	Ref.
PD cases	Control cases	PD cases	Control cases						
57	44	21.0	0.0	*GBA* exons	N370S	North American	5/12 before age 60.	In most, dementia and/or psychiatric symptoms developed.	(Lwin et al., 2004)
99	1543	31.3	6.2	N370S, L444P, c.84dupG, IVS2+A>G, V394L, R496H	N370S	Israeli Jewish	60.0	Typical features of PD.	(Aharon-Peretz et al., 2004)
88	122	5.7	0.8	N370S, L444P, IVS2+A>G, K198T, R329C, c.84dupG, Rec*Nci*1	Rec*Nci*1	Canadian	31-52	Typical features of PD.	(Sato et al., 2005)
311	474	2.3	1.7	N370S, L444P	N370S	Norwegian	56.9	Three developed cognitive decline.	(Toft et al., 2006)
33	31	12.0	3.2	*GBA* exons	RecNcil, L444P	Venezuelan	-	-	(Eblan et al., 2006)
331	347	2.4	0.0	L444P, N370S	N370S	Chinese	49.0	Typical features of PD.	(Tan et al., 2007)
92	92	4.3	1.1	*GBA* exons	L444P	Chinese	63.0	One developed dementia.	(Ziegler et al., 2007)
518	339	3.1	1.2	L444P, Rec*Nci*1, R120W	L444P, RecNcil	Taiwanese	50.6	Typical features of PD.	(Wu et al., 2007)
278	179	13.7	4.5	*GBA* exons	N370S, c.84dupG	New York (64% Jewish)	1.7 yrs earlier	Presence of dyskinesia greater.	(Clark et al., 2007)
395	483	2.8	0.2	N370S, L444P	L444P	Italian	55.7	Typical features of PD.	(De Marco et al., 2008)
65	267	3.0	0.0	N370S, L444P, G377S	L444P	Brazilian	44.0	Typical features of PD.	(Spitz et al., 2008)
721	554	2.9	0.4	N370S, L444P	N370S, L444P	American	56.5	5/21 developed cognitive impairment.	(Mata et al., 2008)
420	333	17.9	4.2	N370S, R496H, L444P,		Israeli Jewish	57.2	Higher frequency of weakness and	(Gan-Or et al., 2008)

				c.84dupG, IVS2+1, V394L, D409H	N370S			lower frequency of rigidity.	
230	430	6.1	0.7	*GBA* exons	N370S, N396T	Portuguese	-	-	(Bras and Singleton, 2009)
172	132	4.7	0.8	*GBA* exons	H255Q, L444P	Greek	-	-	(Kalinderi et al., 2009)
1325	359	12.6	5.3	N370S, T369M, L444P, IVS6, IVS10, E326K, K303K, R262H	E326K, T369M, N370S, L444P	North American (10% Jewish)	56.8	Typical features of PD.	(Nichols et al., 2009)
790	257	4.2	1.2	*GBA* exons	N370S, L444P	British	52.7	Hallucinations in 45% and cognitive decline or dementia in 48%.	(Neumann et al., 2009)
534	544	9.4	0.4	*GBA* exons	R120W, Rec*Nci*1	Japanese	52.5	-	(Mitsui et al., 2009)
330	240	2.7	0.4	N370S, L444P	N370S	Russian	-	-	(Emelyanov et al., 2012)
250	-	12.8	-	N370S, L444P, 84GG, IVS2+1G>A, V394L, del55bp, D409H, R496H	N370S	New York Jewish	56.5	Olfaction impaired in 2/4 and neuro-psychological features in 3/4.	(Saunders-Pullman et al., 2010)
616	411	3.2	0.2	L444P	L444P	Chinese	54.9	Typical features of PD.	(Mao et al., 2010)
402	413	2.7	0.0	L444P, F213I, R353W, N370S	L444P	Chinese	54.6	Typical features of PD.	(Sun et al., 2010)
328	300	1.8	0.7	N370S	N370S	Chinese	54.3	Typical features of PD.	(Hu et al., 2010)
194	117	4.6	0.5	*GBA* exons, *LRRK2* (G2019S)	N370S, L444P, Rec*Nci*1	North African	49.8	Typical features of PD.	(Lesage et al., 2011b)
212	189	3.8	0.5	*GBA* exons	L444P, p.L236F, p.S378L, p.W417G	French-Canadian	-	-	(Noreau et al., 2011)
1130	391	6.7	1.0	*GBA* exons	N370S	European	51.0	Bradykinesia at onset in 73% and levodopa-induced dyskinesias in 62%.	(Lesage et al., 2011a)
967	780	3.7	0.3	*GBA* exons	L444P	Chinese	49.7	Cognitive decline in 32.3%.	(Huang et al., 2011)
277	291	3.2	0.0	*GBA* exons	N188S, P201H, R257Q, S271G,	Korean	48.6	-	(Choi et al., 2012)

					L444P				
195	443	3.1	0.0	N370S L444P R120W	L444P	Chinese	62.0	Typical features of PD.	(Zhang et al., 2012)
225	186	9.8	0.5	*GBA* exons	N370S L444P	Spanish	54.2	Significant risk of dementia during the clinical course.	(Setó-Salvia et al., 2012)
360	348	5.8	1.4	*GBA* exons	N370S	Serbian	49.7	Higher frequency of rigidity and postural instability.	(Kumar et al., 2013)
208	298	3.4	0.3	N370S L444P R120W	L444P	Chinese	56.1	-	(Wang et al., 2012)
259	-	3.5	-	*GBA* exons	N370S, L444P	British	57.1	Greater risk for progression to dementia and Hoehn and Yahr stage 3.	(Winder-Rhodes et al., 2013)
28	252	5.5	0.0	N370S, L444P	L444P	Mexican	37.7	Psychiatric symptoms in 85.7% and cognitive decline in 57%.	(González-Del Rincón et al., 2013)

1.4.2 Response to treatment

Reports on the efficacy of L-dopa treatment in patients with PD and mutations in *GBA* have been conflicting. Most studies report a good or excellent response to L-dopa treatment in heterozygote *GBA* mutation carriers (Ziegler et al., 2007; Neumann et al., 2009), as would be expected in the presynaptic dopamine deficiency seen in PD with and without *GBA* mutations, but some report a less favourable response (Tayebi et al., 2003).

1.4.3 Cognitive impairment and neuropathological findings

The pathological hallmarks of PD include a loss of dopaminergic neurons from the substantia nigra pars compacta (SNpc) and the deposition of Lewy bodies (LBs) in

the remaining nerve cells of affected brain regions. Neuropathological findings demonstrate that GBA-PD brains show the typical hallmarks of sporadic PD, such as brainstem and cortical LBs (Wong et al., 2004; Neumann et al., 2009). GBA is an important component of SNCA-positive pathological inclusions (Goker-Alpan et al., 2010). Immunohistochemistry of seven brain samples from subjects carrying *GBA* mutations (three individuals with GD and four with heterozygous mutations in *GBA*) and with pathological diagnoses of PD and/or DLB demonstrated a co-localization of mutant GBA and SNCA in protein aggregates and LBs. In contrast, in samples from those without *GBA* mutations, < 10% of LBs were GBA-positive (Goker-Alpan et al., 2010).

There are several reports now demonstrating more cognitive impairment in GBA-PD (Sidransky et al., 2009a; Brockmann et al., 2011; Alcalay et al., 2012). It is interesting to speculate that the cognitive impairment in GBA-related parkinsonism results from a higher burden of LB disease than sporadic PD. Two autopsy studies demonstrated that, in addition to the classic PD pathology, in PD patients with heterozygous *GBA* mutations, SNCA inclusions were detectable in cortical areas corresponding to Braak stages 5 to 6 suggesting *GBA* mutation carriers may have more extensive neuropathological disease (Clark et al., 2009; Neumann et al., 2009). However, in a later study on the same autopsies, authors did not identify increased LB pathology in PD associated with *GBA* mutations (Parkkinen et al., 2011). The findings from both cellular and animal studies suggest that mutant *GBA* can promote the accumulation of SNCA (Sardi et al., 2011) and led authors to propose that any subsequent formation of SNCA oligomers could kill the cell prior to LB formation (Parkkinen et al., 2011).

1.4.4 Non-motor symptoms

By the time the classic motor problems of PD develop, it is estimated that over 50% of dopaminergic neurons in the substantia nigra (SN) have degenerated (Lees et al., 2009). There is now evidence that the PD neurodegeneration process begins long before the onset of the motor involvement (Savica et al., 2010; Schapira and Tolosa, 2010). During this period, other neurotransmitter systems may also degenerate and are associated with non-motor symptoms (NMS), including olfactory and autonomic dysfunction, sleep disorders, mood changes, and cognitive decline. The recognition that the pathological process may start outside of the SN (Braak et al., n.d.) explains why some of these NMS manifest before motor problems emerge, and can therefore precede the diagnosis of PD. Moreover, NMS occur more frequently in GBA-PD (McNeill et al., 2012a). Therefore, further screening for non-motor manifestations is needed in *GBA* mutation-positive individuals prior to conversion to PD, to develop an early biomarker set to predict individual pre-symptomatic risk of conversion to clinical PD.

1.4.5 Neuroimaging

Dopamine imaging by single photon emission tomography (SPECT) of the dopamine transporter and positron emission tomography (PET) are abnormal in patients with PD and *GBA* mutations, in a pattern that is similar to sporadic PD (McNeill et al., 2013a). In a PET study to investigate underlying dopaminergic dysfunction in *GBA* mutation carriers with and without parkinsonism, Kono et al. demonstrated

presynaptic dopaminergic dysfunction characteristic of sporadic PD in both a patient with GD and PD, and three subjects with heterozygous *GBA* mutations and PD (Böttcher et al., 2013). In another study, homozygous and compound heterozygous *GBA* mutation carriers identified through screening of 250 Ashkenazi Jewish parkinsonian individuals were assessed with 18F-fluorodopa PET and showed bilateral reductions in striatal F-dopa uptake, similar to idiopathic PD (Saunders-Pullman et al., 2010). However, findings from a larger study showed that although the pattern of dopamine loss is similar in patients with GD and PD to that seen in patients with sporadic PD, $H_2$15O PET demonstrates reduced resting regional cerebral blood flow in additional areas (precuneus and lateral parietal-occipital association cortex bilaterally) in patients with PD and *GBA* mutations, in a pattern characteristic of diffuse LB disease (Kono et al., 2010).

In idiopathic PD, transcranial sonography (TCS) detects SN hyper-echogenicity in 90% of patients, which likely reflects increases in local iron content and microglial activation. However, there are concerns regarding the specificity and sensitivity of this technique in PD. SN echogenicity is a finding that may also change over time in a normal individual (Hagenah et al., 2010). Furthermore, there is no relationship of the TCS abnormalities to PD stage or progression. Nevertheless, SN hyper-echogenicity has also been a reported feature of PD associated with *GBA* mutations, and there is a comparable degree of SN hyper-echogenicity and common features regarding SN pathology (Berg et al., 2008). Recent evidence suggests, however, that this is a frequent finding in GD patients, irrespective of whether they had developed PD or not, and could be related to disturbances of iron metabolism in GD (Goker-Alpan et al., 2012).

1.4.6 Cerebrospinal fluid findings

In patients with PD and *GBA* mutations there are altered cerebrospinal fluid (CSF) fatty acid levels compared with patients with idiopathic PD (Schmid et al., 2012). In a study comparing CSF fatty acid concentrations in 41 patients with idiopathic PD and 8 patients with PD associated with heterozygous *GBA* mutations, results demonstrated specific abnormalities of fatty acid metabolism in those with *GBA* mutations, which authors propose may be involved in the pathogenesis of PD (Schmid et al., 2012). Larger studies would be needed to confirm this. Of further interest is a 12-year follow-up study of a case of PD in a patient who later developed GD (Machaczka et al., 2012). The cognitive decline and cortical atrophy seen in this patient were not paralleled by any change of dementia markers in the CSF. Concentrations of tau and β-amyloid (proteins elevated in Alzheimer disease) remained normal, while concentrations of monoamine metabolites were low. This could suggest the cognitive decline observed in this patient with *GBA* mutations involved pathogenetic pathways other than those typically found in Alzheimer disease.

1.5 Proposed mechanisms linking *GBA* and Parkinson's disease pathogenesis

The mechanisms by which *GBA* mutations result in PD are not yet understood (**Figure 7.1**). Most autosomal recessive forms of PD, such as PINK1, DJ-1, and parkin, are associated with loss-of-function mutations and early onset disease. In contrast, autosomal dominant forms are attributed to a gain-of-function mutation. GD

is an autosomal recessive disorder. However, the inheritance of PD caused by *GBA* mutations does not follow a strict Mendelian pattern. Therefore, in GBA-PD, both gain-of-function and enzymatic loss-of-function models have been put forward (Sidransky and Lopez, 2012) to explain how mutations in *GBA* contribute to the pathogenesis of PD. Mutations in *GBA* could result in a mutant misfolded protein. Misfolded GBA could lead to the development of PD by promoting SNCA accumulation or preventing its degradation. Alternatively, the misfolded protein could cause lysosomal dysfunction by overwhelming the ubiquitin-proteasome pathway (UPS) or by impairment of autophagy. Most mutations in *GBA* are missense mutations that give rise to a misfolded protein, and therefore this favours a gain-of-function model. However, some studies have implicated *GBA* mutations that do not encode the glucocerebrosidase protein, e.g. 84GG null allele, in patients who also go on to develop PD (Gan-Or et al., 2008). Albeit rare, these mutations could argue against an exclusively gain-of-function mechanism. Alternatively, parkinsonism could arise from a deficiency/reduction of wild-type (WT) GCase resulting from a loss-of-function mutation, with subsequent substrate accumulation and altered lipid homeostasis, or lysosomal dysfunction. Neither model, however, explains why only a minority of patients with mutations in *GBA* go on to develop PD.

1.5.1 A Synucleinopathy

The synucleinopathies are all characterized by the presence of aggregated insoluble SNCA, in the form of amyloid fibrils, within LBs of the central nervous system. The presence of mutant GBA in SNCA-positive LBs, as demonstrated by immunohistochemistry studies (Goker-Alpan et al., 2010), has suggested a

relationship could exist between the two proteins. GCase deficiency, overexpression of mutant *GBA*, or inhibition of GCase by condruitol β-epoxide (CBE) can interfere with the clearance of or promote the aggregation of SNCA (Manning-Boğ et al., 2009; Cullen et al., 2011; Mazzulli et al., 2011; Xu et al., 2011; Gegg et al., 2012; Cleeter et al., 2013; Osellame et al., 2013). Furthermore, augmenting GCase activity can reduce SNCA levels (Sardi et al., 2011). Albeit, a recent report did not find any significant alteration in SNCA levels following GCase inhibition with CBE (Dermentzaki et al., 2013), but this probably reflected too short a period of toxin exposure (Cleeter et al., 2013).

Increased SNCA results in a decrease in GBA protein and GCase activity in cells. SNCA can also affect the transport of GBA from the ER/Golgi apparatus to the lysosome (Mazzulli et al., 2011; Gegg et al., 2012). Recent evidence has suggested a self-propagating feedback loop in GD and synucleinopathies. A deficiency of GCase may lead to an accumulation of substrate, which could in turn influence the amyloid formation of SNCA, and the resulting toxic build-up of insoluble SNCA within lysosomes could compromise lysosomal protein degradation. SNCA could then impair the trafficking of GBA, exacerbating substrate accumulation and SNCA aggregation (Mazzulli et al., 2011). The further finding that GCase is reduced in the SN in PD brains with or without *GBA* mutations (Gegg et al., 2012) suggests that SNCA could cause GCase deficiency directly, even in the absence of mutant enzyme, and this could be exacerbated in GBA-PD and hold significance for the wider PD population.

Experimental evidence now suggests that GBA interacts directly with SNCA at membranes, within lysosomes (Yap et al., 2013). The interaction site has been

mapped to the C-terminus of SNCA. It has also been suggested that the binding between the WT GBA and SNCA, under lysosomal conditions, could have a beneficial effect by either promoting SNCA degradation by the lysosome and/or preventing aggregation (Yap et al., 2011). The authors propose another self-perpetuating model. Mutant GBA or reduced levels of WT GBA reaching the lysosome could weaken the beneficial interaction between SNCA and GBA, reduce lysosomal protein degradation, and contribute to the SNCA aggregation characteristic of PD pathology. The resultant accumulation of substrate and SNCA would then mediate the interaction of the SNCA-GBA complex on intralysosomal vesicles, leading to a further loss of GCase activity (Yap et al., 2013).

Recent studies therefore demonstrate a reciprocal relationship between GCase and SNCA (Mazzulli et al., 2011; Gegg et al., 2012), which not only contribute to our understanding of the pathogenesis of GBA-PD and idiopathic PD but also highlight an important therapeutic target. Modulating GCase activity or improved targeting of GBA to lysosomes could be an important therapeutic approach for PD (Sidransky and Lopez, 2012; Schapira and Gegg, 2013).

This gain-of-function model, however, does not explain why only a minority of GD patients or carriers of *GBA* mutations ever develop PD. Other pathological factors may be at work and help determine whether a positive feedback loop can surpass the cellular homeostatic mechanisms to an extent that the levels of SNCA become toxic. It could be that *GBA* contributes to SNCA pathology, but only in the presence of other insults, e.g. increasing age, where the number of lysosomes and lysosome function decreases and cellular SNCA concentrations increase. Another possibility is

that *GBA* may act as a 'second hit' in some individuals who are genetically predisposed to develop PD (Sidransky and Lopez, 2012).

1.5.2 Autophagic dysfunction

In PD, the accumulation of SNCA and other ubiquitinated proteins within LB inclusions has implicated protein mishandling in the pathogenesis of the disease. Abnormal protein accumulation appears in both PD and GD and may influence the severity of disease. Proteins are degraded via the lysosome or the proteasome. The lysosome is the major cellular compartment for protein degradation (De Duve and Wattiaux, 1966), and within the lysosome the degradation of proteins occurs through three distinct pathways: macroautophagy, microautophagy, and chaperone-mediated autophagy (CMA). The lysosomal autophagy process destroys misfolded, long-lived or aggregate-prone proteins in the cell and, in doing so, confers a cytoprotective role (García-Arencibia et al., 2010). Defective autophagy and/or depletion of lysosomes have been implicated in PD pathogenesis (Cuervo et al., 2004; Alvarez-Erviti et al., 2010; Dehay et al., 2010; Wong and Cuervo, 2010) and in a Gaucher mouse model (Cullen et al., 2011).

Evidence for the role of the lysosome under conditions of protein burden came from a study looking at the overexpression of SNCA in transgenic mice (Mak et al., 2010). Overexpression led to its subsequent enhanced clearance by the lysosomal CMA pathway. This was demonstrated by an up-regulation of lysosome-associated membrane protein type 2A (LAMP-2A) and lysosomal heat shock cognate protein of 70 kDa (hsc70), two mediators of CMA. An important point to note, however, is the compensatory action of macroautophagy on specifically blocking CMA (Alvarez-Erviti

et al., 2010). Rapamycin, an inducer of macroautophagy, reverses the SNCA accumulation caused by the overexpression of D409V glucocerebrosidase (Goker-Alpan et al., 2012). Despite this, any up-regulation of macroautophagy may be unable to compensate for all functions of CMA (Massey et al., 2006).

SNCA is degraded by proteasomal pathways, but to a greater extent through CMA (Cuervo et al., 2004). It has been suggested that the efficacy of CMA declines with age (Kiffin et al., 2007), with decreasing lysosomal numbers and function. Of particular interest is the evidence of defective CMA in the PD brain. In a post-mortem study of seven PD brains, there was a significant reduction of CMA markers within the SNpc and amygdala compared to controls, and autophagy-related proteins were shown to accumulate in LBs (Alvarez-Erviti et al., 2010). Moreover, gene replacement of beclin 1, which activates autophagy, has been shown to ameliorate the neurodegenerative pathology in animal models of PD and DLB (Spencer et al., 2009).

The mechanism by which mutant GBA causes lysosomal dysfunction is not yet understood. The decrement in lysosomal function that would result from mutant GBA or decreased amounts of WT GBA reaching the lysosome, could in turn reduce SNCA turnover through CMA and lead to its accumulation and aggregation. The suggested beneficial interaction between SNCA and WT GBA proposed to occur under lysosomal conditions (Yap et al., 2011) may suggest that techniques to increase GCase activity and improve its delivery to the lysosome could promote degradation through the autophagy pathway and reduce SNCA concentrations.

1.5.3 Endoplasmic reticulum stress and disruption of the ubiquitin-proteasome pathway

The ER operates a stringent quality control system to ensure proper protein folding, trafficking, and degradation. Misfolded proteins are degraded via the UPS. Some studies have demonstrated defects of the UPS in sporadic PD (Furukawa et al., 2002; McNaught et al., 2003). Despite this, the application of proteasome inhibitors to induce PD in animal models has produced conflicting results (Beal and Lang, 2006).

Mutations in *GBA* can cause GCase to fold abnormally, whereupon it is arrested in the ER, targeted by endoplasmic reticulum-associated degradation (ERAD), and translocated to the cytoplasm for degradation by the UPS. This results in decreased amounts of GBA protein present in the lysosome, and the degree of ER retention and proteasomal degradation is thought to correlate with the severity of GD (Ron and Horowitz, 2005).

The unfolded protein response (UPR) is activated in response to ER stress, aids the elimination of misfolded proteins, and is neuroprotective (Wang et al., 2010). Misfolded GCase arrested in the ER can cause ER stress, activate the UPR, and affect the UPS degradation of other proteins, e.g. SNCA, thereby allowing them to accumulate. Markers of the UPR are increased in both GBA-PD and sporadic PD brains and may play a role in reducing protein levels (Gegg et al., 2012).

However, only one study has shown a direct relationship between PD and *GBA* which involves ERAD (Ron et al., 2010). Parkin and mutations in its encoding gene (PARK2) cause early-onset PD. Parkin is an E3 ubiquitin lipase,

ubiquitinating proteins for destruction by the lysosome or the proteasome. A loss of normal parkin leads to impaired degradation of some of its substrates, including SNCA, and to their accumulation. Parkin also forms part of the UPR, enhancing removal of misfolded proteins from the ER (Imai et al., 2000). One theory proposed is that mutant GBA could interact with parkin to block its interaction with other substrates, impairing their degradation via the UPS and exacerbating ER stress, leading to neuronal death with the development of PD (Ron et al., 2010). This theory has attracted some criticism. The role of parkin is not restricted to the UPS and the targeting of proteins for degradation, and evidence from a recent report in fibroblasts suggests parkin is not a critical ubiquitin ligase for GBA (McNeill et al., 2013b).

1.5.4 Dysfunctional mitophagy

The turnover of mitochondria by mitophagy, a form of autophagy, is essential for the protection of neurons. If damaged mitochondria are not removed, reactive oxygen species and free radicals are allowed to accumulate, causing damage to neurons and eventual cell death. Therefore, mitochondrial dysfunction and oxidative stress are thought to be important in PD pathogenesis (Schapira, 2008). Several studies have shown that PD causative genes are implicated in mitochondrial morphology and function (Schapira and Gegg, 2011). For instance, PINK1 and parkin play a pivotal role in mitochondrial quality control (Poole et al., 2008). Parkin binds to the mitochondrial membrane in response to a fall in membrane potential (Narendra et al., 2008), and the selective recruitment of parkin to damaged mitochondria is mediated by PINK1 through direct phosphorylation (Kim et al., 2008).

Despite this, the evidence in the literature for a role of mitochondrial dysfunction or mitophagy in PD patients with *GBA* mutations remains limited. Of recent interest was a study suggesting that inhibition of GCase activity could induce the abnormalities in mitochondrial function and oxidative stress previously only seen in sporadic or familial PD. Authors showed that inhibition of GCase in cell culture leads to decreased ADP phosphorylation, reduced mitochondrial membrane potential and increased free radical damage, together with accumulation of SNCA (Cleeter et al., 2013). This implies that mutations in *GBA* could increase the risk of PD through abnormalities in mitochondrial function. However, the mechanism for this remains uncertain. Of further relevance is a recent mouse model of GD where there was an accumulation of dysfunctional mitochondria as a result of impaired autophagy and proteasomal pathways. Both lysosomes and mitochondria have a role in autophagy and organelle turnover, and authors suggest that the pathogenesis of neurodegeneration seen in both GD and in PD could result from global defects in proteostasis (Osellame et al., 2013).

1.5.5 Altered lipid metabolism

Deficiencies in *GBA* can cause lipids to accumulate. GCase normally degrades glucosylceramide to glucose and ceramide. It has been proposed that changes in ceramide metabolism are associated with LB formation, and ceramide-related genes have been implicated in PD. For instance, a number of genes have been identified which interfere with ceramide metabolism associated with LB pathology (Bras et al., 2008). It is also thought that focal accumulation of lipids in specific brain regions

could impair protein degradation through the lysosomal autophagy pathway, and SNCA could accumulate.

SNCA binds to lipids in the plasma membrane and synaptic vesicles (Jo et al., 2000). The helical binding of SNCA to lipid membranes is thought to prevent the formation of its fibrillar forms. This property of SNCA to form amyloid fibrils is thought critical for the development of neurotoxicity (Mazzulli et al., 2011). The lipids which accumulate as a result of deficiencies in GCase could alter the sphingolipid composition of membranes and prevent the membrane binding of SNCA, thereby enhancing its aggregation in the cytoplasm (Piccinini et al., 2010).

Despite this, there are limitations to the hypothesis that *GBA* mutations in patients with PD cause alterations in lipid metabolism due to a loss of functional GCase activity. Ceramide metabolism is thought to be a tightly regulated process, and ceramide can be derived from several different degradative and synthetic pathways. In addition, it is not clear how a partial deficiency in GCase seen in heterozygotes, insufficient to cause substrate accumulation, would impact lipid metabolism significantly. Furthermore, there is no evidence that ceramide is deficient in those with GD, even those with severe forms of the disease (Velayati et al., 2010).

1.6 The future: a role for modulating GCase activity?

GBA has now been identified as numerically the most important genetic risk factor for the development of PD, and, unlike other PD-related genes, considerably more is known about this protein, its processing, and how it functions (Sidransky and Lopez, 2012). Although the mechanisms underlying the association between *GBA* mutations

and PD remain unclear, further investigation and understanding of the involvement of this protein as a susceptibility factor for PD and other synucleinopathies is likely to prove valuable. So far to date, the delineation of the different molecular pathways, which underlie the pathogenesis of GBA-PD, has shown there is considerable connection between them.

Recent findings suggest that SNCA and *GBA* are implicated in a common cellular pathway and are of great significance in understanding the pathogenesis of GBA-PD as well as sporadic PD. SNCA propagation by templating could be promoted by GCase deficiency, e.g. increased SNCA levels, promotion of fibril formation, and increased SNCA endosomal release through lysosomal inhibition. Furthermore, Sardi et al. demonstrate that augmenting GCase activity can modulate SNCA aggregation and could represent a novel therapeutic strategy for PD (Sardi et al., 2013; Schapira and Gegg, 2013). In a mouse model of GD (D409V/D409V genotype), there was a progressive accumulation of SNCA, tau, and ubiquitin aggregates in hippocampal neurons and a measurable memory deficit. Increasing GCase activity in this mouse model, through adeno-associated viral vector delivery (AAV) of the recombinant enzyme into the brain, modulated the accumulation of protein aggregates such as SNCA and improved memory defects. It is notable that the memory improvement observed by Sardi and colleagues, in response to GCase administration into the CNS, was seen in both pre-symptomatic (Sardi et al., 2011) and symptomatic mouse models (Sardi et al., 2013), and before any significant protein accumulation had established itself. This has implications for the asymptomatic population at risk of development of PD, e.g. *GBA* mutation-positive carriers, where modulating GCase activity could be a potential strategy to prevent the onset/conversion to clinical PD. Moreover, the clinical evaluation

of *GBA* mutation-positive individuals for pre-motor signs of PD could be important not only in developing a clinical biomarker set to predict individual pre-symptomatic risk of conversion to clinical PD, but this cohort would also be invaluable to the assessment of any future therapy to manipulate GCase activity and slow PD progression. Strategies to modulate GCase activity include the use of pharmacological chaperones such as ambroxol or isofagomine, histone deacetylase inhibitors which reduce the recognition of misfolded GBA by the chaperone protein Hsp90β, and increasing *GBA* expression, e.g. using gene therapy (Schapira and Gegg, 2013). Enhancing GCase activity in this way and improving lysosomal function could also hold relevance for those with GD, where there is currently no treatment that can cross the blood–brain barrier and treat the neurological forms of the disease.

Why a global genetic defect, such as that which is seen in patients with GBA-PD, shows selective neuronal pathology, including LB formation and loss of dopaminergic neurons, remains unclear. Defects in autophagy and the UPS have been implicated in neurodegenerative disease (Korolchuk et al., 2009), and it has been proposed that lysosomal storage disorders could be diseases of autophagy (Settembre et al., 2008). The level of intracellular SNCA is critical for the onset of neurodegeneration with LBs and dependent, to a large extent, on degradation via the lysosomal autophagy pathway. Therefore, impairment of lysosomal pathways is increasingly viewed as a major pathogenic event and unifying theme in PD (Dehay et al., 2013). Depletion of GCase may result in the toxic accumulation of insoluble SNCA within lysosomes, compromise lysosomal protein degradation, and promote a bidirectional feedback loop in which SNCA accumulates and causes a self-propagating disease (Mazzulli et al., 2011; Gegg et al., 2012).

Recent developments in this field have put forward an interesting concept. The suggested interaction between SNCA and GCase and the ability to manipulate this, using strategies to enhance GCase activity and in turn modulate SNCA aggregation, could provide a novel therapeutic approach for the treatment of PD (**Figure 7.2**) (Schapira and Gegg, 2013). This could be significant for those with GBA-PD, asymptomatic *GBA* mutation positive carriers at risk of conversion to clinical PD, or even those with sporadic PD, where reductions in GCase have also been noted and may hold significance (Gegg et al., 2012).

1.7 Aims of this PhD project

PD is the second most common neurodegenerative disease after Alzheimer's disease with a lifetime risk in the UK population of 5%. There is currently no therapy to slow progression in PD. Apart from aging, the most important risk factor known for the development of PD is certain mutations of *GBA*. This knowledge has led to my hypothesis that *GBA* mutations are pathogenic in PD.

I began to continue the clinical evaluation of individuals carrying the *GBA* mutation for prodromal features of PD. This unique cohort (n=135) has proved of great value in identifying risk factors for individuals carrying the *GBA* mutation prior to developing PD, and is being used to develop an early biomarker set to predict individual pre-symptomatic *GBA* mutation carrier risk of conversion to clinical PD. This cohort and these results will be invaluable to the assessment of any future therapy to manipulate mutated GBA and thereby restore enzymatic activity (GCase) in view of delaying the development of PD. To date, the pharmacological chaperones (PC) that I have chosen to study are considered the most promising approach.

My laboratory studies involve generating cell culture models from the individuals within the cohort studied, in order to delineate the molecular mechanism of mutant *GBA* to the pathogenesis of PD. In addition, these cell models support my investigation for assessing the efficacy of PC treatment in restoring GCase function, and reversing the underlying molecular events. My goal is to generate solid data from the cell models to support the use of PC therapy in PD.

Chapter 2: Predicting Parkinson's Disease

2.1 The clinical prodrome of Parkinson's disease

PD is a very common disorder with a lifetime risk in the UK population of 4% over the age of 80. It is the second most common neurodegenerative disease worldwide after Alzheimer's disease and the incidence is rising with an aging population (Pringsheim et al., 2014). There is currently no therapy to slow disease progression in PD, current treatment is aimed at improving symptoms, but increasing efforts are being made to trial neuroprotective drugs that potentially slow or prevent the development of symptoms (Lang et al., 2013). It is thought that these drugs may be more advantageous if used early in the disease process, before substantial neuronal loss has occurred (Bar-Or et al., 2011; Lang et al., 2013).

It is now acknowledged that PD can have a prodromal period, during which neurodegeneration has begun, but disease is not yet clinically evident. Therefore, the PD neurodegeneration process begins long before the onset of the motor involvement (Savica et al., 2010; Schapira and Tolosa, 2010). The rational for this prodrome is based on the staging system of Braak, which suggested that the pathological process may not start in the SNpc (Braak et al.). Examination of SNCA deposition patterns shows that the deposition of SNCA is not restricted to the brain, with deposits found in the olfactory bulb, peripheral nervous system, enteric nervous

system (ENS), cardiac, and pelvic plexuses, etc (Wakabayashi et al., 2010). Braak proposed that the first stage of PD involves deposition in the anterior olfactory nucleus and dorsal motor nucleus of the vagus. Stage 2 consists of pontomedullary involvement (lower raphe, reticular formation, coeruleus/subcoeruleus complex), Stage 3 affects the midbrain (including SNpc), and at Stages 4 to 6, cortical structures are affected. Investigators in other groups have also confirmed these findings (Halliday et al., 2008; Zaccai et al., 2008). However, there are some limitations to this model. The brains selected for analysis included those with SNCA deposition in the dorsal motor vagus, a potentially important selection bias. It is unlikely that the pattern of pathology is universal; the dorsal motor nucleus of the vagus is not the obligatory trigger site of PD (Kalaitzakis et al., 2008). There are variable progression patterns and disease can begin in the SNpc. It is also worth noting that SNCA deposition does not necessarily correlate with neurodegeneration; advanced stages of SNCA deposition can be present without clinical parkinsonism or dementia (Parkkinen et al., 2008). The speed of progression through the early stages remains unclear. If the premotor interval is short, this could limit the effectiveness of predictive markers. In spite of these limitations, the recognition that initial pathology of PD may occur outside the SNpc suggests that screening for non-motor manifestations may detect earlier stages of PD.

The early non-motor manifestations of prodromal PD may precede the onset of more typical motor symptoms by several years (Braak et al., n.d.). During this period, other neurotransmitter systems may also degenerate and are associated with non-motor symptoms (NMS) including olfactory and autonomic dysfunction, sleep disorders, mood changes, and cognitive decline. This implies the potential to predict PD by detecting prediagnostic markers several years before the diagnosis. When in the

disease course each of these non-motor symptoms first occurs is unknown (Hawkes et al., 2010). Insight into the first clinical presentations of these prediagnostic features would help to delineate the pathophysiology of early PD progression and to identify people at increased risk of development of overt PD, who would be eligible for inclusion in clinical trials of neuroprotective strategies. The ultimate goal in neurodegeneration is a neuroprotective agent to slow or stop the underlying degenerative process. If a neuroprotective treatment becomes available, it will become essential to identify patients as early as possible. An agent that is partially effective in established disease could slow or halt the onset of clinical disease if given in preclinical stages. Therefore, every effort to design and validate predictive markers should be made to plan for future neuroprotective therapy.

2.2 Biomarkers for Parkinson's disease

Candidate biomarkers have been proposed, and may be useful objective measures for the early detection of PD (Postuma et al., 2012), (Schapira, 2013). A biomarker is defined as a characteristic that can be objectively measured and evaluated as an indicator of normal biological processes, pathogenetic processes or pharmacologic responses to a therapeutic intervention (Anon, 2001). There are a considerable number of potential markers for prediction of PD including clinical, biochemical and imaging markers.

Despite rigorous research, patient management and clinical research are still hindered by suboptimal methods for diagnosis, refining prognosis, predicting individual response to therapeutic interventions, and tracking disease progression. A

biomarker for PD may be useful to aid in the diagnosis of PD or the early prodromal premotor period, follow disease progression, test the efficacy of potentially neuroprotective interventions, and risk-stratify asymptomatic individuals in order to assess potential preventive approaches. The application of a single marker alone may be insufficient to fulfil all of these criteria in the majority of cases. However, when used in combination, it is reasonable to think that a combination of biomarkers might achieve some.

2.2.1 Clinical markers

PD is diagnosed when typical motor features occur but numerous motor and non-motor features can occur before diagnosis, early in the disease process.

Motor prodrome

The diagnosis of PD is clinical and dependent on identification of the classical motor symptoms, which are integral to the clinical diagnostic criteria (Hughes et al., 1992). This has led several researchers to use quantitative motor tests to detect subtle motor changes. Potential tests include the alternate tap test, Purdue PegBoard, precision grip and lift task, alternate finger tap, and Timed up and Go (Postuma et al., 2006). However, by the time the classic motor problems of PD develop, it is estimated that over 50% of dopaminergic neurons in the SNpc have degenerated, so they are not useful as an early marker of disease (Lees et al., 2009). Furthermore, the rate at which motor features progress is variable in different individuals. Another potential limitation to quantitative motor testing is that up to 40% of elderly persons

will show subtle motor slowing (Bennett et al., 1996) suggesting suboptimal specificity.

Non-motor prodrome

Several non-motor symptoms occur more frequently in patients with PD before the onset of typical motor symptoms, including hyposmia, rapid eye movement sleep behaviour disorder (RBD), depression, autonomic dysfunction and constipation. The sensitivity, specificity, positive and negative predicted values of these clinical features have recently been reviewed (Chahine and Stern, 2011).

Olfaction

Olfactory abnormalities are found in those with PD with mutations in *GBA* (Saunders-Pullman et al., 2010), but are not a reported feature of GD or its treatment. It has been proposed that the earliest SNCA changes occur in the dorsal motor nucleus of the vagus and olfactory bulb (Braak et al., n.d.), and evidence suggests that smell impairment is not simply a consequence of aging but rather is a prodromal phenomenon that may predict PD (Hawkes et al., 2010). Accordingly, the large majority of PD patients have severe olfactory loss at disease onset (Hawkes, 2003). Olfactory loss may also be an important preclinical marker of dementia, especially DLB (Olichney et al., 2005) and Alzheimer's disease. A major advantage of olfactory tests is that they are inexpensive and non-invasive. The commonest test includes the University of Pennsylvania Smell Identification Test (Doty et al., 1984).

One of the drawbacks of olfactory loss as a predictor of PD is the interval between detection of the olfactory abnormality and clinical disease may be short. In the

Honolulu study, olfactory loss did not predict PD when assessed >4 years before disease onset (Ross et al., 2008). Olfactory impairment can be a sensitive marker for an impending diagnosis of PD. However, it is non-specific, because olfaction is impaired in many disorders. Of all those in the lowest quartile of the Honolulu study who had severe hyposmia, only 2% developed PD (Ross et al., 2008). This suggests that olfactory testing alone will be insufficient to indicate the need for neuroprotective therapy. Finally, olfactory loss may also screen the preclinical stages of dementia, implying that olfactory testing would be a more effective screen for a neuroprotective therapy that has a non-disease-specific action.

Rapid eye movement sleep behaviour disorder

Rapid eye movement sleep behaviour disorder (RBD) is characterised by a loss of the normal atonia of REM sleep. Affected patients typically thrash out in association with dream content (Gagnon et al., 2006). RBD occurs in approximately a third of patients with PD. Due to its high specificity and long latency to clinical disease, RBD is one of the strongest clinical predictors of neurodegenerative disease, and a potential prodromal marker for preventative therapy (Postuma et al., 2012). It has a strong male predominance. RBD is predominantly linked with brainstem lesions, especially those involving the pons (Boeve et al., 2007). These pontine areas correspond most closely to Braak stage 2. In those with idiopathic RBD, the risk of developing a neurodegenerative disease ranges from 19 to 38% at 5 years of follow-up, and from 40 to 65% after 10 years. Approximately half develop PD, and half develop dementia (most, if not all, of these are DLB). This high risk and long latency to PD makes RBD an ideal biomarker. RBD carries a high sensitivity, and in those

without existing neurological or sleep disorders, a high specificity, and therefore, represents a good tool to detect subjects with RBD (Stiasny-Kolster et al., 2007).

However, some caveats must be noted. Firstly, the majority of RBD patients do not present to physicians. This presents a challenge to those who want to identify RBD patients for neuroprotective therapy. Second, RBD diagnosis is not simple and the definitive diagnosis requires polysomnography. Third, as recognition of RBD improves, it is likely that milder cases will come to medical attention and therefore, disease risk may not be the same in these cases.

Despite these limitations, RBD has considerable potential as a predictive marker. The high conversion rate to disease implies a marker with immediate clinical application. If a safe and effective neuroprotective agent were developed tomorrow, RBD patients may have to consider taking it. Also, patients with RBD may be the ideal candidates for clinical trials of neuroprotective agents, since their earlier stage of neurodegeneration provides an additional window of opportunity.

Autonomic dysfunction

The incidence of autonomic dysfunction is higher in people who go on to develop PD compared with controls (Schrag et al., 2014). In particular, constipation is more common in patients than in controls 10 years before diagnosis (Schrag et al., 2014). According to Braak's staging, peripheral PD pathology, including SNCA neuropathology in the enteric nervous system, might precede the classic changes in the midbrain and limbic areas. Moreover, the recent detection of SNCA pathology in colon biopsies obtained from individuals without parkinsonian symptoms who later

developed PD is promising (Lebouvier et al., 2010). In the Honolulu–Asia Aging Study, less than one bowel movement per day was associated with a more than three times increased risk of PD (Ross et al., 2012). However, this symptom is common in the general population and therefore, its specificity in identifying preclinical PD is low. Other autonomic features with which individuals present with are dizziness, urinary dysfunction, hypotension, and erectile dysfunction (in men), which are also pre-diagnostic features of PD (Schrag et al., 2014). Erectile dysfunction has also been associated with a diagnosis of PD more than 10 years later (Gao et al., 2007). However, erectile dysfunction is under-reported and/or patients do not complain about this symptom in the face of other, more troublesome symptoms.

Assessment of the autonomic nervous system using MIBG, which is taken up by postganglionic adrenergic neurons like norepinephrine, demonstrates a significant reduction of MIBG uptake in cardiac sympathetic efferents irrespective of disease severity, disease duration, treatment, and pre-existing dysautonomic signs (Mitsui et al., 2006). Other studies indicate that a large majority of PD patients have abnormalities on MIBG scintigraphy, even early in disease. However, it remains unclear if MIBG scintigraphy can identify prodromal PD.

In summary, the evidence that autonomic dysfunction predicts disease is less strong than it is for olfaction. Symptoms and signs of autonomic dysfunction are not universally present early in disease and frequently progress, which may indicate a lower sensitivity. As a further limitation, specificity of autonomic dysfunction is probably low, as other medical conditions (e.g. diabetes, drug treatment) can also

lead to autonomic dysfunction. Finally, a marker such as MIBG scintigraphy, which is potentially more specific, is time-consuming and expensive.

Cognitive impairment

Mild cognitive impairment (MCI) can occur as a prodrome to parkinsonism (Dalrymple-Alford et al., 2010) or DLB (Williams et al., 2009). MCI in PD indicates a shorter time to dementia and represents a poor prognostic marker (Aarsland et al., 2009). Whether cognitive impairment without dementia is a possible target for treatment is still an unresolved question. In PD patients without dementia, clinical trials might explore two concepts: (i) whether drugs modifying impaired cognition produce an improvement in either practical function or quality of life of PD patients; (ii) if disease modifying agents may prevent the progression from MCI to dementia.

Depression

Depression can precede the onset of the motor symptoms of PD and is a presenting complaint in 12-22% of patients (O'Sullivan et al., 2008). Depression in PD has been explained by multiple neurotransmitter dysfunctions, including dopamine (SNpc), serotonin (raphe nuclei), and noradrenaline (locus coeruleus). At Braak stage 2, the involvement of both raphe nuclei and locus coeruleus might indicate depression as a prodromal symptom of PD. Additionally, personality changes are associated with PD (Arabia et al., 2010), although whether these represent a lifelong personality trait or a marker of preclinical PD is unclear. The eventual use of depression as a disease predictor will likely be limited by low specificity since the large majority of persons with depression will never develop PD.

2.2.2 Biochemical markers

Biochemical markers have been developed based on the understanding of the pathogenesis of disease. There are several pathogenetic pathways which are considered relevant including mitochondrial dysfunction, oxidative stress, inflammation and protein accumulation, aggregation and propagation (Obeso et al., 2010; Schapira, 2012). Advances in the genetics of PD have also aided the identification of potential markers, for example the use of SNCA or DJ-1.

Alpha synuclein

The rationale for SNCA as a biomarker of PD stems from the key role of this protein in the pathogenesis of PD. SNCA is the main protein contained in the defining neuropathological lesions (LBs) of PD. Initially thought to be a strictly intracellular protein, SNCA has also been detected in plasma, saliva and cerebrospinal fluid (CSF). Efforts to study CSF SNCA have showed some promise with reductions in SNCA noted in the CSF of PD and other parkinsonian syndromes (Devic et al., 2011; Mollenhauer et al., 2011). Lower levels of SNCA in PD may reflect increased clearance, or alterations in transcription, splicing and processing. Recent studies suggest that data should be expressed as the ratio of oligomeric to total SNCA. In one study, this ratio demonstrated 89.3% sensitivity and 90.6% specificity for the diagnosis of PD (Tokuda et al., 2010). Two additional studies have supported the application of a oligomer:total SNCA ratio (Park et al., 2011; Sierks et al., 2011). The relationship between SNCA levels and the stage of disease and/or its evolution over time is unknown.

A blood based SNCA biomarker has not fared as well. There have been attempts to measure oligomeric species of SNCA (El-Agnaf et al., 2006), total SNCA (Tinsley et al., 2010), and phosphorylated SNCA (Foulds et al., 2011) in plasma. In a study comparing oligomeric SNCA levels in 34 PD patients with age-matched controls, a high signal occurred in approximately 3.5 times more PD patients compared to controls (El-Agnaf et al., 2006). Another study by the same group comparing levels of different forms of SNCA in 32 PD patients and 30 controls was not able to reproduce this (Foulds et al., 2011). Various technical difficulties such as high abundance of SNCA within red blood cells as well as confounding variables, such as other comorbidities, could account for this. Subsequent studies have reported both increases (Lee et al., 2006) and decreases (Li et al., 2007) in plasma SNCA in PD patients compared to controls.

DJ-1

DJ-1 emerged as a candidate biomarker in PD after mutations in the gene encoding this protein were identified in familial PD (Bonifati et al., 2003). The function of DJ-1 is still unknown; however, it is associated with various cellular processes, including response to oxidative stress, and signalling with mitochondria. The CSF and plasma levels of DJ-1 have been found to be unaffected, elevated or reduced compared with controls (Hong et al., 2010; Shi et al., 2011).

Others

Other potential protein markers investigated in PD include urate, β-amyloid, tau, neurofilaments, interleukins, osteopontin and hypocretin. However, results have been negative or inconclusive.

The association between PD and uric acid levels has gained interest in recent years. Studies have shown that lower levels of the antioxidant urate are associated with an increased risk of PD (Cipriani et al., 2010; Chen et al., 2012), and the levels are lower in patients with PD (Schwarzschild et al., 2011). However, patients with PD and their drug treatment as well as lifestyle factors such as dietary habits and physical exercise, might influence the metabolism of uric acid.

Levels of CSF β-amyloid (1–42) and tau have been used as a marker of neurodegenerative disease, in particular dementia, but also to predict cognitive decline in PD. In PD, tau levels are unchanged and there is only a small decrease in β-amyloid (Přikrylová Vranová et al., 2012).

Low levels of plasma ApoA1 have also been proposed as a significant risk factor for PD, and the concentrations also correlate with putaminal loss on Dopamine Transporter (DaT) scans (Qiang et al., 2013). Lower ApoA1 levels are associated with more severe DaT deficit, even after adjusting for age and gender. In addition, ApoA1 levels are modifiable with statin medications, which increase HDL, and statins are associated with a reduced risk for PD (Gao, 2012).

2.2.3 Imaging markers

Single-photon emission tomography (SPECT), positron emission tomography (PET), magnetic resonance imaging (MRI) or transcranial sonography (TCS) can provide useful information on the loss of nigrostriatal dopamine neurons as well as information about the time frame of neuron loss. They represent a non-invasive approach and scans may be abnormal before motor signs appear and can progress

as neurodegeneration continues. However, with the exception of TCS, they are expensive and do not always correlate with the clinical findings, especially in the early stages of the disease (Vogt et al., 2011), limiting their usefulness in standard diagnostic situations. Furthermore, these imaging modalities do not make the distinction between different parkinsonian syndromes, and they can be affected by dopaminergic medication. For instance, L-dopa causes a decline in both PET and SPECT. Thus, they are limited as a biomarker, but are a useful diagnostic tool in certain situations.

In idiopathic PD, TCS detects SN hyper-echogenicity in 90% of patients, which likely reflects increases in local iron content and microglial activation. However, there are concerns regarding the specificity and sensitivity of this technique in PD. TCS is very dependent on operator skill, and requires an adequate temporal acoustic bone window for good imaging. SN echogenicity is a finding that may also change over time in a normal individual (Hagenah et al., 2010). Furthermore, there is no relationship of the TCS abnormalities to PD stage or progression.

Evolving neuroimaging techniques that visualise nigrostriatal structural changes might prove helpful to improve diagnostic accuracy. MRI with diffusion weighted imaging could help to distinguish patients with progressive supranuclear palsy or multiple system atrophy from those with PD. Findings from these techniques include changes in diffusivity or fractional anisotropy in the SN and the striatum on diffusion-tensor MRI (Scherfler et al., 2006) and changes in the SN morphology on high-field MRI (Cho et al., 2011; Kwon et al., 2012). Reductions seen in fractional anisotropy in

the SN correlate with motor severity and therefore, may correlate with disease progression.

2.2.4 Pathological markers

Post-mortem studies of those with incidental LB pathology suggest that SNCA pathology may begin in the tissues of the gastrointestinal (GI) tract, salivary glands, and olfactory system (Braak et al., 2006; Beach et al., 2010). Recent data demonstrates the presence of SNCA and LBs in the gut of patients with PD in colon biopsies 2-5 years before PD onset (Shannon et al., 2012). This suggests that colon biopsies could serve as a biomarker for premotor PD and/or as a marker for the efficacy of drugs designed to reduce SNCA levels that penetrate systemic tissues.

2.2.5 Conclusion

Although, there is considerable promise for predictive markers of PD, It is unlikely that one single measure will be able to predict the disease with good reliability and sensitivity. It is much more likely that a combination of markers will be required and this may include clinical, biochemical and imaging markers to define at-risk individuals. When planning for the eventual use of these markers, it may become necessary to design a simple non-invasive screen which could be applied to the general population, followed by more specific confirmatory tests for those who screen positive. The key feature of any biomarker is not just to diagnose PD early, but to further reflect the pathogenesis and progression of the disease against which a disease-modifying strategy could be judged.

2.3 A unique cohort of at-risk individuals

In an age of neuroprotection, it is important to identify those at high disease risk for developing PD who will be motivated to undergo even relatively invasive procedures to determine the need for therapy. However, high-risk populations are not easy to find. Defined causative gene mutations are identifiable in some patients, and these gene carriers are ideal candidates for studies of predictive markers. Many environmental risk factors (e.g. pesticide use, non-smoking) can also increase the risk of PD.

If there are no methods to reliably identify persons at substantial risk of disease, screening may need to be population-wide. This adds considerable additional challenges. In addition to being sensitive and specific, screens must now also be non-invasive and inexpensive. Invasive or expensive tests may eventually be used predominantly as secondary confirmation of a positive screen or primary screens for the few who are definable as high-risk.

To investigate potential early markers of PD, I studied asymptomatic individuals from the Royal Free London and Addenbrooke's Hospital Cambridge who had mutations in the *GBA* gene. These mutations increase the risk of developing PD by 20-30 times and are present in around one in ten PD patients. A range of baseline clinical assessments were carried out on the participants, including motor function and cognition, and a number of subtle abnormalities were identified in the participants that were significant in comparison to matched controls. This supported the hypothesis that a proportion of the individuals would develop PD based on the knowledge that the presence of the *GBA* mutation is a significant risk factor. Two years later I repeated the assessments and for a proportion of them the

abnormalities present at baseline had progressed and 1 individual has developed PD.

I am now attempting to distinguish a pattern of abnormalities that determine in individuals with a *GBA* mutation whether they are at a greater risk of developing PD before deterioration in function indicates the start of disease. The idea is that by following the evolution of individuals with *GBA* mutations, it will be possible to see if they exhibit abnormalities that might be detected in the general PD population. The hope is that should a therapy become available that can slow the disease down, then this would be targeted at those individuals in particular. What my results support in principle is that it may be feasible to tell individuals that they are at low risk of developing PD, which is a great benefit to them, or, alternatively, at higher risk, which could mean in the future we are able to offer them treatments that will slow the disease down and may even prevent their PD.

2.4 Assessing prodromal clinical markers of Parkinson's disease in a *glucocerebrosidase* mutation positive cohort

I began my PhD by re-examining a *GBA* mutation positive cohort at high risk for the development of PD. The aim was to identify biomarkers or symptoms indicating progression to early PD. The first clinical evaluation of this cohort has been published previously (McNeill et al., 2012b) and the results presented here represent the two year follow up.

This cohort of *GBA*-positive individuals has been established in collaboration with the Lysosomal Storage Disease Units at the Royal Free and Addenbrooke's Hospitals. Initial examinations were performed in 2010 and prodromal features were identified (McNeill et al., 2012b). Assessments included tests for motor function, olfaction, cognition, sleep, and depression.

My results have identified individuals who are exhibiting signs of an evolving clinical prodrome of PD (but do not yet have PD). The aim is to provide a clinical road map of the pre-motor, pre-diagnostic prodrome of PD to enable biomarker development and early diagnostic markers in this population.

OBJECTIVE

To continue the clinical evaluation of *GBA* mutation positive individuals for prodromal features of PD. Clinical assessment will include tests for non-motor symptoms, including olfactory and autonomic dysfunction, sleep behaviour disorder, mood changes and cognitive impairment.

2.4.1 Introduction

Homozygous *GBA* mutations cause GD, a lysosomal storage disorder. It is presently estimated that homozygous or heterozygous *GBA* mutations confer an increased risk for PD of 20-30 fold (Bultron et al., 2010; McNeill et al., 2012a) and at least 7% of PD patients have *GBA* mutations (McNeill et al., 2012a)'(Sidransky et al., 2009a), and this is higher in the Ashkenazi Jewish population (Zimran et al., 1991). The penetrance of *GBA* mutation carriers to develop PD has been estimated as 13·7% at age 60 years and 29·7% at age 80 years (Anheim et al., 2012), and so a method to determine individual risk for PD expression in this population would be very valuable. In addition, those with dementia with Lewy bodies (DLB) are 8 times more likely to carry a mutation in *GBA* than healthy controls, suggesting a role for *GBA* mutations in other Lewy body disorders (Nalls et al., 2013).

For any neuroprotective treatment or disease modifying therapy to be most effective, PD should be detected at as early a stage as possible. The recognition that the pathological process may start outside of the substantia nigra (Braak et al., n.d.) explains why non-motor symptoms (NMS) manifest before motor problems emerge, and can therefore precede the diagnosis of PD. Moreover, NMS occur more frequently in GBA-PD (McNeill et al., 2012a). Therefore, further screening for non-motor manifestations is needed in *GBA* mutation-positive individuals prior to conversion to PD, to develop an early biomarker set to predict individual pre-symptomatic risk of conversion to clinical PD. In this study, I have used early clinical markers to quantify non-motor symptoms such as hyposmia, rapid eye movement sleep behaviour disorder (RBD), depression, cognition, and autonomic dysfunction.

The aim of this study was to provide longitudinal data on a *GBA* mutation positive cohort at high risk for the development of PD, and to identify biomarkers or symptoms indicating progression to early PD.

2.4.2 Method

Participants

Type 1 GD patients were recruited from the Lysosomal Storage Disorder Unit at the Royal Free London NHS Foundation Trust in 2010. Potential heterozygous *GBA* mutation positive carrier relatives (parents: 78·6%; siblings: 10·7%; children >21 years: 10·7%) and genetically unrelated controls (spouses/partners) were identified by taking a detailed family history from each GD patient, and recruited with consent. Individuals were also recruited from the UK Gaucher Disease Association. In all, this unique cohort included one hundred and thirty-five participants. Among them, ninety participants have been followed longitudinally with target follow-up assessments at two year intervals beginning in 2012. For both GD and carrier subjects, exclusion criteria included a diagnosis of PD or dementia and for controls, any existing neurological disease. The diagnosis of PD was made according to the UK Parkinson's Disease Society Brain Bank Criteria (Hughes et al., 1992). Dementia was diagnosed according to DSM-IV criteria in patients with a Mini-Mental State Examination score of ≤24. The *GBA* mutation status in all participants was confirmed by Sanger sequencing of the *GBA* gene, as previously described (McNeill et al., 2012b). The senior researcher was blinded to genotype. The study was approved by the Hampstead Research Ethics Committee (reference number 10/H0720/21). All individuals provided written informed consent.

Follow-up evaluation

Of ninety individuals who were evaluated at baseline (2010-2011) (McNeill et al., 2012b), four participants (4·4%) were lost to follow-up because they either declined to participate (n=2) or were uncontactable (n=2). In addition, two deaths (2·2%) had occurred: one caused by pneumonia, and one by breast carcinoma. Therefore, eighty-four (93·3%) participants (thirty previously diagnosed Type 1 GD patients, twenty-eight heterozygous *GBA* mutation carriers, and twenty-six controls) completed the follow-up evaluation that comprised: a structured clinical work-up, a standardised clinical history, complete neurological assessment including the Unified Parkinson's Disease Rating Scale activities of daily living and motor subscale (UPDRS parts II and III), olfactory function using the University of Pennsylvania Smell Identification Test (UPSIT), cognitive function using the Mini-Mental State Examination (MMSE) and Montreal Cognitive assessment (MoCA), RBD with the RBD Questionnaire (RBDQ), depression using the Beck's Depression Inventory (BDI), and autonomic dysfunction using a subscale of the Unified Multiple System Atrophy Rating Scale (UMSARS). Anosmia was interpreted using age- and sex-adjusted normative scores (www.sensonics.com). All participants were examined independently by a movement disorders-trained physician (M.B.). All procedures were performed and scored identically at follow-up to those carried out at baseline. A senior neurologist expert on movement disorders (A.H.V.S.) evaluated individuals where there was a significant difference between UPDRS scores measured at follow-up and at baseline.

Statistical analysis

The data was analysed using IBM SPSS Statistics (version 21). To assess the differences between the group means across the two different time points, I performed a two-way ANCOVA with factors Group (e.g. Gaucher vs. Carrier vs. Control) and Time (Time 1 vs. Time 2). The covariates age, gender, education, and family relationship were added to the design matrix, in order to account for differences in these between the groups. Post-hoc tests were used to compare the groups at follow-up. Paired t-tests were used to compare the scores within each group before and after follow up. Differences in age, sex, and ethnicity between groups were checked using the One-way ANOVA and the Chi-squared test. I also accounted for performing multiple statistical tests across dependent variables (UPSIT, UMSARS, RBDQ, MMSE, MoCA, UPDRS II, UPDRS III, BDI) by defining a significance threshold for statistical tests of $P < \cdot 05$, and correcting this for multiple comparisons using the Benjamini-Hochberg FDR (False Discovery Rate). In brief, this procedure involves ordering all *P* values in ascending order and applying a sequential threshold.

2.4.3 Results

The eighty-four participants (40 men [47·6%]) had a mean follow-up duration of 1·9±0·2 years (range, 1·5-2·3 years). The demographic, clinical and genetic characteristics, with statistical comparisons, of the cohort are shown in **Table 2.1**. Participants with Type 1 GD did not differ significantly from heterozygous *GBA* positive carriers or controls in terms of age, sex, and ethnicity (One-way ANOVA and Chi-squared test, in all $P > \cdot 05$). Both Type 1 GD patients and heterozygous *GBA* mutation carriers were significantly more likely to have a family history of PD than

controls (*P* = ·03). As described previously (McNeill et al., 2012b), the most common genotype in GD patients was N370S/L444P (11/30; 36·7%). No GD patients had features of Type III disease such as generalized seizures or progressive myoclonic epilepsy. In carriers, the most common genotype was N370S (14/28; 50%).

Table 2.1. Demographic, clinical and genetic characteristics of the study cohort

Characteristic	Type 1 GD patients (n=30)	Heterozygous *GBA* mutation carriers (n=28)	Controls (n=26)	*P* value
Age, years	61·0 (2·1)	63·6 (2·0)	61·7 (2·2)	·19[a]
Gender (F/M)	16/14	16/12	12/14	·29[b]
Ethnicity (Ashkenazi/White British)	10/20	5/23	6/20	·38[b]
Family history of PD, (%)	16·7	7·1	0·0	·03[bc]
Most frequent genotype	N370S/L444P	N370S	-	-
GD treatment (ERT/SRT/none)	25/2/3	-	-	-

Results are presented as mean and SEM. Significance was taken at the 5% level.

Abbreviations: *ERT* Enzyme Replacement Therapy. *SRT* Substrate Reduction Therapy.

[a] One-way ANOVA.

[b] Chi-squared test.

[c] Significant difference.

***GBA* mutation positive individuals show significant deterioration in clinical markers**

The results of prodromal clinical features of PD at baseline and follow-up are reported in **Table 2.2** (see also **Figure 7.3** and **Figure 7.4**). Please refer to **Table 2.2** for the exact *P* values. There was a significant deterioration in RBDQ, UPDRS II and III scores for GD patients over the mean two years of follow-up. Over the same period, the *GBA* mutation carriers showed a significant deterioration in RBDQ, UPDRS II and III, and BDI scores. There was a marginal but significant deterioration only in the UPDRS II score in the matched controls. There was no difference between baseline and follow-up scores for all groups for assessments of olfaction, cognition and autonomic dysfunction.

At 2 years follow-up, GD patients showed a significant difference in mean UPSIT, MMSE, MoCA, UPDRS II and III scores when compared to controls. Similarly, at 2 years, *GBA* mutation carriers showed a significant difference in mean follow-up UPSIT, MMSE, and MoCA scores when compared to controls. When the GD patients and *GBA* mutation carriers were compared at baseline, there was a significant difference in the mean BDI score. At 2 years follow-up, GD patients demonstrated significantly worse mean BDI, UPDRS II and III scores compared to carriers. There was no significant difference between mean UPSIT, UMSARS, MMSE, MoCA, or RBDQ scores in GD patients and carriers at follow-up.

Table 2.2. Baseline and follow-up clinical markers in a group comparison between Type 1 GD patients, carriers and controls.

		Type 1 GD patients (n=30)	Heterozygous *GBA* mutation carriers (n=28)	Controls (n=26)	*P* (between)[b]		
					P^1	P^2	P^3
UPSIT	Baseline	32·57 (0·96)	31·11 (0·93)	35·32 (0·40)			
	Follow-up	31·21 (0·98)	30·22 (1·10)	33·95 (0·62)	·003[c]	·001[c]	·52
	P (within) [a]	·03	·29	·13			
UMSARS	Baseline	0·40 (0·15)	0·37 (0·15)	0·08 (0·06)			
	Follow-up	0·63 (0·16)	0·53 (0·16)	0·13 (0·07)	·004[c]	·02[c]	1·00
	P (within) [a]	·11	·59	·32			
RBDQ	Baseline	0·93 (0·31)	0·10 (0·10)	0·25 (0·14)			
	Follow-up	2·93 (0·55)	2·30 (0·40)	1·08 (0·30)	·04	1·00	·23
	P (within) [a]	<·001[c]	<·001[c]	·07			
MMSE	Baseline	29·23 (0·17)	29·23 (0·18)	29·28 (0·16)			
	Follow-up	28·40 (0·48)	28·63 (0·32)	29·50 (0·21)	·01[c]	·03[c]	1·00
	P (within) [a]	·08	·05	·30			

MoCA	Baseline	25·93 (0·53)	25·55 (0·58)	27·32 (0·23)			
	Follow-up	26·33 (0·75)	26·21 (0·57)	27·73 (0·26)	·001[c]	·001[c]	1·00
	P (within) [a]	·07	·38	·20			
UPDRS II	Baseline	1·45 (0·82)	0·33 (0·21)	0·00 (0·00)			
	Follow-up	2.72 (0·66)	1·33 (0·30)	0·58 (0·19)	<·003[c]	1·00	·009
	P (within) [a]	·003[c]	<·001[c]	·006[c]			
UPDRS III	Baseline	4·29 (1·45)	1·97 (0·65)	0·21 (0·17)			
	Follow-up	7·82 (1·91)	4·50 (0·75)	0·92 (0·37)	<·001[c]	·04	·006
	P (within) [a]	<·001[c]	<·001[c]	·06			
BDI	Baseline	2·68 (1·78)	0·65 (0·41)	0·33 (0·33)			
	Follow-up	5·84 (1·14)	2·88 (0·68)	0·58 (0·43)	·04	1·00	·03[c]
	P (within) [a]	·04	·01[c]	·11			

Abbreviations: *UPSIT* Smell Identification Test, *UMSARS* Unified Multiple System Atrophy Rating Scale, *MoCA* Montreal Cognitive assessment, *MMSE* Mini-Mental State Examination, *RBDQ* Rapid Eye Movement Sleep Behaviour Disorder Questionnaire, *UPDRS* Unified Parkinson's Disease Rating Scale, *BDI* Becks Depression Inventory.
Results are presented as mean and SEM. Significance was taken at the 5% level for all variables. Only values which survived multiple comparisons with the FDR procedure were denoted significant.

Reported *P* values compare the mean values for clinical markers within groups (baseline and follow-up) and between groups (Type 1 GD, carriers and controls) at follow-up.

[1] Controls versus Type 1 GD patients.
[2] Controls versus heterozygote *GBA* mutation carriers.
[3] Type 1 GD patients versus heterozygote *GBA* mutation carriers.
[a] Paired t-test.
[b] Two-way ANCOVA with Bonferroni correction.
[c] Statistically significant difference.

Pooled analysis

When the results from individuals with homozygous or heterozygous mutations in *GBA* were combined in a secondary, pooled analysis (see **Table 2.3**), there was a significant deterioration in mean RBDQ, BDI, UPDRS II and III scores in *GBA* mutation positive individuals over the two years of follow-up. At baseline, *GBA* mutation positive individuals showed significant differences in mean UPSIT and MoCA scores when compared to controls (McNeill et al., 2012b). At 2 years follow-up, *GBA* mutation positive individuals showed significant differences in mean UPSIT, UMSARS, MMSE, MoCA, UPDRS II and UPDRS III scores when compared to controls.

Table 2.3. Baseline and follow-up clinical markers in a pooled analysis comparing all *GBA* mutation positive individuals versus controls.

		Type 1 GD patients and Heterozygous *GBA* mutation carriers combined scores (n=58)	Controls (n=26)	*P* (between)[b]
				P^1
UPSIT	Baseline	31·85 (0·67)	35·32 (0·40)	
	Follow-up	30·71 (0·73)	33·95 (0·62)	<·001[c]
	P (within) [a]	·02	·13	
UMSARS	Baseline	0·38 (0·11)	0·08 (0·06)	
	Follow-up	0·58 (0·11)	0·13 (0·07)	·001[c]
	P (within) [a]	·15	·32	
RBDQ	Baseline	0·51 (0·20)	0·25 (0·14)	
	Follow-up	2·63 (0·33)	1·08 (0·30)	·06
	P (within) [a]	<·001[c]	·07	
MMSE	Baseline	29·23 (0·12)	29·28 (0·16)	
	Follow-up	28·51 (0·27)	29·50 (0·21)	·002[c]
	P (within) [a]	·02	·30	

MoCA	Baseline	25·7 (0·38)	27·32 (0·23)	
	Follow-up	26·3 (0·45)	27·73 (0·26)	<·001[c]
	P (within) [a]	·06	·20	
UPDRS II	Baseline	0·88 (0·39)	0·00 (0·00)	
	Follow-up	2·01 (0·36)	0·58 (0·19)	·02[c]
	P (within) [a]	<·001[c]	·006[c]	
UPDRS III	Baseline	3·09 (0·75)	0·21 (0·17)	
	Follow-up	6·10 (0·95)	0·92 (0·37)	<·001[c]
	P (within) [a]	<·001[c]	·06	
BDI	Baseline	1·72 (0·94)	0·33 (0·33)	
	Follow-up	4·44 (0·71)	0·58 (0·43)	·09
	P (within) [a]	·002[c]	·11	

Abbreviations: *UPSIT* Smell Identification Test, *UMSARS* Unified Multiple System Atrophy Rating Scale, *MoCA* Montreal Cognitive assessment, *MMSE* Mini-Mental State Examination, *RBDQ* Rapid Eye Movement Sleep Behaviour Disorder Questionnaire, *UPDRS* Unified Parkinson's Disease Rating Scale, *BDI* Becks Depression Inventory.

Results are presented as mean and SEM. Significance was taken at the 5% level for all variables. Only values which survived multiple comparisons with the FDR procedure were denoted significant.

Reported *P* values compare the mean values for clinical markers within groups (baseline and follow-up) and between groups (Controls and *GBA* mutation positive individuals) at follow-up.

[1] Controls versus *GBA* mutation positive individuals.
[a] Paired t- test.
[b] Two-way ANCOVA with Bonferroni correction.
[c] Statistically significant difference.

Specific GD patients and *GBA* heterozygotes show parkinsonian motor signs and significant deterioration across more than one clinical marker

At baseline, three GD patients had parkinsonian motor signs, but insufficient for a diagnosis of PD. As described previously (McNeill et al., 2012b), GD05 (male, 78 years old, Ashkenazi Jewish) had bilateral rigidity with activation manoeuvre, asymmetric bradykinesia of all limbs, and gait impairment. GD18 (male, 83 years old, Ashkenazi Jewish) had left arm rest tremor and bilateral arm rigidity with activation manoeuvre. GD27 (male, 69 years old, White British) had flexed posture, bilateral rigidity, and postural and kinetic tremor of the upper limbs. At follow-up, the parkinsonian signs present at baseline in these study subjects had worsened but did not meet the diagnostic criteria for PD (Lees et al., 2009). GD05 had developed a tremor in both hands (intermittent, present at rest and worse on intention). GD18 now had bilateral rigidity without activation manoeuvre and gait impairment. GD27 had developed a head tremor and the postural and kinetic tremor of the upper limbs had worsened (now present at rest). In addition, one subject that did not have parkinsonian signs at baseline had developed them at follow-up. GD11 (male, 73 years old, Ashkenazi Jewish) had developed a very slight tremor of his right thumb (non pill rolling) present at rest but with no other features of parkinsonism.

Similarly, two *GBA* carriers had parkinsonian motor signs at baseline. As described previously (McNeill et al., 2012b), C17 (female, 78 years old, White British) had bilateral rigidity, mask-like facies, and bradykinesia while C31 (male, 78 years old, White British) had masked facies, bilateral rigidity with activation manoeuvre, left arm kinetic tremor, and flexed posture. At follow-up, the parkinsonian signs present in these study subjects remained unchanged from baseline.

When specific GD patients and *GBA* heterozygotes with features of parkinsonism (6/58; 10·3%) were excluded, the follow-up data remained significant. The remaining GD patients and carriers (52/58; 89·7%) still showed a significant deterioration in RBDQ, UPDRS II and III, and BDI scores after two years (see **Table 2.4)**.

Premotor signs present at baseline that could predict parkinsonian motor signs

When clinical markers were compared between specific GD patients (GD05, GD11, GD18, and GD27) and *GBA* heterozygotes (C17 and C31) with parkinsonian motor signs and *GBA* mutation positive individuals without features of parkinsonism, there were significant differences in age (P = ·002) and cognition (P = ·009) at baseline (see **Table 2.5** and **Table 2.6**). Baseline UPSIT scores were also noted to be lower in those individuals with features of parkinsonism but this difference did not reach statistical significance.

Table 2.4. Two year follow-up data when *GBA* mutation positive individuals with parkinsonian signs are excluded.

		Type 1 GD patients (n=26)	Heterozygous *GBA* mutation carriers (n=26)	Controls (n=26)	*P* (between)[b]		
					P^1	P^2	P^3
UPSIT	Baseline	32·71 (1·09)	31·68 (0·89)	35·32 (0·40)			
	Follow-up	31·29 (1·13)	30·68 (1·15)	33·95 (0·62)	·001[c]	·001[c]	·96
	P (within) [a]	·03	·27	·13			
UMSARS	Baseline	0·31 (0·14)	0·39 (0·16)	0·08 (0·06)			
	Follow-up	0·42 (0·13)	0·46 (0·15)	0·13 (0·07)	·14	·05	1·00
	P (within) [a]	·32	·90	·32			
RBDQ	Baseline	0·88 (0·31)	0·11 (0·11)	0·25 (0·14)			
	Follow-up	2·96 (0·62)	2·36 (0·42)	1·08 (0·30)	·34	1·00	·32
	P (within) [a]	<·001[c]	<·001[c]	·07			
MMSE	Baseline	29·23 (0·16)	29·29 (0·19)	29·28 (0·16)			
	Follow-up	28·42 (0·54)	28·71 (0·33)	29·50 (0·21)	·001[c]	·006[c]	1·00
	P (within) [a]	·09	·11	·30			

MoCA	Baseline	26·23 (0·56)	25·93 (0·55)	27·32 (0·23)			
	Follow-up	26·42 (0·83)	26·44 (0·55)	27·73 (0·26)	<·001[c]	<·001[c]	1·00
	P (within) [a]	·70	·13	·20			
UPDRS II	Baseline	1·44 (0·96)	0.36 (0·23)	0·00 (0·00)			
	Follow-up	2.16 (0·65)	1·29 (0·32)	0·58 (0·19)	·04	1·00	·009
	P (within) [a]	·01[c]	·001[c]	·006[c]			
UPDRS III	Baseline	2·29 (0·62)	1·32 (0·43)	0·21 (0·17)			
	Follow-up	5·29 (1·25)	3·79 (0·59)	0·92 (0·37)	<·001[c]	·05	·07
	P (within) [a]	·001[c]	<·001[c]	·06			
BDI	Baseline	3·00 (1·99)	0·65 (0·41)	0·33 (0·33)			
	Follow-up	5·65 (1·27)	2·88 (0·68)	0·58 (0·43)	·15	1·00	·04
	P (within) [a]	·10	·01[c]	·11			

Abbreviations: *UPSIT* Smell Identification Test, *UMSARS* Unified Multiple System Atrophy Rating Scale, *MoCA* Montreal Cognitive assessment, *MMSE* Mini-Mental State Examination, *RBDQ* Rapid Eye Movement Sleep Behaviour Disorder Questionnaire, *UPDRS* Unified Parkinson's Disease Rating Scale, *BDI* Becks Depression Inventory.

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[1] Controls versus Type 1 GD patients.
[2] Controls versus heterozygote *GBA* mutation carriers.
[3] Type 1 GD patients versus heterozygote *GBA* mutation carriers.
[a] Paired t-test.
[b] Two-way ANCOVA with Bonferroni correction.
[c] Statistically significant difference.

Table 2.5. Demographic, clinical and genetic characteristics of *GBA* mutation positive individuals with parkinsonism versus those without parkinsonism.

	***GBA* mutation positive individuals**		
	Parkinsonism* (n=6)	**No Parkinsonism (n=52)**	***P* value**
Age, years	74·3 (3·2)	59·9 (1·5)	·002[ac]
Gender (F/M)	1/5	26/26	·14[b]
Ethnicity (Ashkenazi/White British)	3/3	3/7	·17[b]
Family history of PD, %	0	13·5	·32
Most frequent *GBA* allele	N370S	N370S	-

Results are presented as mean and SEM. Significance was taken at the 5% level.

Abbreviations: *ERT* Enzyme Replacement Therapy. *SRT* Substrate Reduction Therapy.

*Patients without Parkinson's disease but with subtle evolving features of parkinsonism.

[a] One-way ANOVA.

[b] Chi-squared test.

[c] Statistically significant difference.

Table 2.6. Baseline and follow-up clinical markers in a comparison of *GBA* mutation positive individuals with parkinsonism and *GBA* mutation positive individuals without parkinsonism.

		***GBA* mutation positive individuals**		***P* (between)[b]**
		Parkinsonism* (n=6)	**No Parkinsonism (n=52)**	***P*[1]**
UPSIT	Baseline	29·2 (2·1)	32·2 (0·7)	·16
	Follow-up	28·7 (1·6)	31·0 (0·8)	
	P (within) [a]	·67	·02	
UMSARS	Baseline	0·7 (0·5)	0·4 (0·1)	·37
	Follow-up	1·8 (0·5)	0·4 (0·1)	
	P (within) [a]	·10	·48	
RBDQ	Baseline	0·8 (0·8)	0·5 (0·2)	·60
	Follow-up	2·5 (0·9)	2·6 (0·4)	
	P (within) [a]	·04	<·001[c]	
MMSE	Baseline	29·0 (0·5)	29·3 (0·1)	·52
	Follow-up	28·0 (0·7)	28·6 (0·3)	
	P (within) [a]	·10	·02	

MoCA	Baseline	22·8 (1·3)	26·1 (0·4)	·009[c]
	Follow-up	24·8 (1·7)	26·4 (0·5)	
	P (within) [a]	·07	·17	
UPDRS II	Baseline	1·0 (0·8)	0·9 (0·4)	·92
	Follow-up	4·8 (1·4)	1·7 (0·3)	
	P (within) [a]	·03	<·001[c]	
UPDRS III	Baseline	14·5 (4·1)	1·8 (0·4)	<·001[c]
	Follow-up	20·2 (4·3)	4·5 (0·6)	
	P (within) [a]	·04	<·001[c]	
BDI	Baseline	0·0 (0·0)	1·8 (1·0)	·66
	Follow-up	7·5 (0·5)	4·3 (0·7)	
		·18	·005[c]	

Abbreviations: *UPSIT* Smell Identification Test, *UMSARS* Unified Multiple System Atrophy Rating Scale, *MoCA* Montreal Cognitive assessment, *MMSE* Mini-Mental State Examination, *RBDQ* Rapid Eye Movement Sleep Behaviour Disorder Questionnaire, *UPDRS* Unified Parkinson's Disease Rating Scale, *BDI* Becks Depression Inventory.

*Patients without Parkinson's disease but with subtle evolving features of parkinsonism.

Results are presented as mean and SEM. Significance was taken at the 5% level for all variables. Only values which survived multiple comparisons with the FDR procedure were denoted significant.

Reported *P* values compare the mean values for clinical markers within groups (baseline and follow-up) and between groups (Parkinsonism and no Parkinsonism) at baseline.

[1] Parkinsonism versus no Parkinsonism.
[a] Paired t-test.
[b] One-way ANOVA with Bonferroni correction.
[c] Statistically significant difference.

2.4.4 Discussion

This study was designed to investigate the progression of clinical biomarkers in a cohort of individuals at high risk for PD. My results demonstrate that clinical features associated with pre-motor PD, and motor features of PD have both evolved since the initial testing, and support the hypothesis that some *GBA* mutation positive individuals within this cohort are exhibiting clinical features of early neurodegeneration.

In the cohort studied, both Type 1 GD patients and heterozygous mutation carriers were, as a group, hyposmic at baseline (McNeill et al., 2012b). At 2 years, follow-up olfactory scores in GD patients and heterozygous carriers remained significantly lower than those reported for controls, but unchanged from baseline. This could reflect the short length of the follow-up period, considering olfactory impairment may progress slowly.

An impaired sense of smell does appear to correlate with other modalities in the prodromal phase of PD e.g. RBD (Berg et al., 2013). I did identify a significantly increased frequency of symptoms of RBD at the follow-up assessment in *GBA* mutation positive individuals compared to controls. It is arguable whether a score of five or six should be the cut off point for a scale that is structured to determine if there is RBD or not. It should be noted, however, that the proportion of RBDQ scores greater than five was higher in *GBA* mutation positive individuals compared to controls at follow-up, albeit this difference did not reach significance (P = 0·39, Chi-squared test).

There was an increase in the report of depressive symptoms in *GBA* mutation positive individuals at follow-up. Patients with GD can exhibit moderate to severe psychological complications, similar to patients with other long-term chronic illnesses (Packman et al., 2006). In addition, BDI scores of 1-10 are consistent with minimal depression and the specificity of depression alone as a clinical marker of prodromal PD is low, but may be usefully combined with other features (Liepelt-Scarfone et al., 2011).

There are several lines of evidence now for greater cognitive impairment in those with established GBA-PD versus sporadic PD (Brockmann et al., 2011; Alcalay et al., 2012),(Zokaei et al., 2014), and this may reflect a higher burden of LB disease in GBA-related parkinsonism (Clark et al., 2009; Neumann et al., 2009). Interestingly, in a subgroup of six *GBA* mutation positive individuals with parkinsonian motor signs, mild cognitive impairment (MoCA score </=24 in 5/6, 83·3%) was the main premotor sign present at baseline that could have predicted their motor deterioration. Compared to controls, the remaining *GBA* mutation positive individuals demonstrated significantly lower MMSE and MoCA scores at follow-up, albeit these were unchanged from baseline and still within the normal range for cognitive function.

Control subjects showed a small but significant change in the UPDRS part II score from baseline. I believe what drove the changes in the controls were a small group of individuals (n=6) who were older. The mean age of the 6 controls that had deteriorated on the UPDRS II was 70.5 years (range 62.6-77.8). The UPDRS II may not distinguish well between those symptoms occurring as part of normal aging and those related to PD, and I accept the UPDRS II was never designed or validated as

a tool for activities of daily living (ADL) in aging controls. Particular aspects of the UPDRS II that had worsened in controls included: 2.11 (getting out of a bed, a car or a deep chair), 2.12 (walking e.g. use of a walking aid), 2.5 (dressing e.g. help with buttons). I have had to take this into consideration when interpreting the analysis for the controls and also for our assessment of *GBA* mutation positive individuals who are older. When compared, a significantly higher follow-up UPDRS part II score in GD patients distinguished these individuals from age-matched controls.

There were some *GBA* mutation positive individuals (10%) with significant motor findings identified using the UPDRS part III, which did not overlap with normal physiology (e.g. bilateral postural tremor) or existing bone/joint abnormalities. These individuals did not meet the diagnostic criteria for PD but could represent a subgroup of *GBA* mutation positive individuals that are progressing towards clinical PD.

I considered the impact of GD or GD therapy on the clinical markers tested. There were GD individuals with significant motor findings identified using the UPDRS part III, but these did not overlap with existing bone or joint abnormalities. Cognitive dysfunction has been reported in Type I GD, and substrate reduction therapy (SRT) may induce memory problems (Elstein et al., 2005). However, only 2 GD patients were receiving SRT when evaluated at baseline and at follow-up, and neither had cognitive impairment. Olfactory abnormalities are found in those with PD with mutations in *GBA*, but are not a reported feature of GD or its treatment. The majority of Type 1 GD patients (83%) were receiving enzyme replacement therapy (ERT). This does not cross the blood brain barrier and has no reported neurological side effects. Furthermore, ERT has no known impact on dysautonomia.

My study is the first to undertake the longitudinal follow-up of a large cohort of *GBA* mutation positive individuals, prior to the development of PD. Much of the work published thus far in the literature has focussed on patients with established PD. This has been essential to make important comparisons between sporadic PD and GBA-related parkinsonism and to observe subtle differences. The opportunity to follow patients prospectively within a unique at-risk cohort such as this is essential for defining the optimal time to intervene with neuroprotective therapy.

One limitation of the study was that not all investigators were blind to the genetic status of individuals. The senior researcher was blinded to genotype but the remaining clinical researchers knew the medical and genetic status of the participants. Of the ninety individuals examined at baseline, I tried to follow-up all individuals. To minimise any observer bias, standardised scores were used and all follow-up data were re-examined. I did not exclude any individual who did not show a large change between time points 1 and 2. For the present study, this potential bias has been noted as a limitation.

Other potential criticisms are the use of prodromal markers and their sensitivity, specificity, and positive and negative predictive values. The presence of clinical markers alone may be insufficient accurately to predict a neurodegenerative disorder in the majority of cases. However, clinical markers may be used in combination with other biochemical or imaging markers for prodromal PD to develop a more reliable method for PD prediction.

In this study, I looked at the subtle changes in each clinical marker between the two time points rather than a clinically meaningful threshold. In *GBA* positive individuals, there were significant differences at follow-up when compared to controls but in most

cases, a clinically meaningful change has not been defined. When assigned to categories of severity based upon age- and sex-adjusted centiles (www.sensonics.com), Type 1 GD patients and heterozygous mutation carriers had, as a group, mild hyposmia (UPSIT score 30-34/40) at baseline and at follow-up. As a group, mean MMSE and MoCA scores remained within the normal range for cognitive function but were significantly lower when compared to controls at follow-up. BDI scores of 1-10 are consistent with minimal depression and the specificity of depression alone as a clinical marker of prodromal PD is low, but the frequency of depressive symptoms was higher in *GBA* positive individuals at follow-up. The Unified Parkinson's Disease Rating Scale (UPDRS) does not have a set cut point. It is a rating scale used to follow the longitudinal course of PD. Over 2 years, UPDRS III scores were significantly worse in GD patients and *GBA* heterozygotes. Following UPDRS scores in *GBA* mutation positive individuals over time should provide insight into the progression of the clinical prodrome of PD.

For individuals, rather than looking at scatter plots for each of the groups as a whole, I looked specifically at the trajectories for a subgroup of *GBA* positive individuals with parkinsonian motor signs. I felt that this would provide a better insight into the development of PD in this group of *GBA* positive individuals, but also in general how PD could develop in an individual over time.

The purpose of this study was the longitudinal clinical evaluation of a *GBA* mutation positive cohort and the evolution of the prodromal features of PD. This study indicates that as a group, *GBA* mutation positive individuals show deterioration in clinical markers consistent with the prodrome of PD. Within this group, 10% appear to be evolving at a more rapid rate.

Chapter 3: Interesting Observations from the Clinical Cohort Studied

3.1 A high prevalence of tremor in Type 1 Gaucher disease

While examining the study cohort, I also found a high prevalence of an essential tremor (ET)-like disorder in the Type 1 GD population, with increasing prevalence with advancing age associated with UPDRS changes, but not the full clinical picture of PD. Tremor is more common than would be expected in this cohort of *GBA* carriers and extends the clinical phenotype associated with this mutation.

3.1.1 Introduction

Prevalence data on different types of tremor among patients with Type 1 GD remain scarce. Here, for the first time, I report the prevalence of tremor in a Type 1 GD cohort.

3.1.2 Method

Participants

Patients were participants in a study of the prodrome of PD and were recruited between 2010 and 2011 from the Lysosomal Storage Disorder Unit at the Royal Free Hospital, London. Sixty Type 1 GD patients, with no prior diagnosis of PD or dementia, were evaluated. All participants provided written informed consent and the study was approved by Hampstead Research Ethics Committee. The mutation status in all participants was confirmed by molecular genetic analysis. Each participant underwent an assessment comprising: clinical history, Mini-Mental State Examination (MMSE), Montreal Cognitive Assessment (MoCA), Parts II-III of Unified Parkinson's Disease Rating Scale (UPDRS), Rapid Eye Movement Sleep Behaviour Disorder Questionnaire (RBDQ) and full neurological examination. A senior neurologist trained in movement disorders (A.H.V.S) confirmed the clinical diagnosis of tremor, and subsequent tremor analysis was performed in 2/7 patients.

Tremor recordings

Two of the seven patients consented to undergo tremor recordings. Patients were comfortably seated in a chair. A triaxial accelerometer (Biometrics Ltd; sensitivity ±50 mV/G) was attached to the dorsal surface index finger bilaterally. Patients were recorded for at least 1 minute at rest with arms relaxed and hands hanging freely from the arm rest; on posture, with the arms outstretched in front of the patient, parallel to the ground; this same position with 500g mass attached to the dorsum of the hand (weight loading); on action while performing a goal-directed task. Fast Fourier analysis of the signals derived from accelerometry was performed to define the peak tremor frequency and total power of the spectra between 1 and 30 Hz as a

measure of tremor amplitude. All parameters were calculated for each accelerometer axis then averaged.

Statistical analysis

The data was analysed using IBM SPSS Statistics (version 21). To assess the differences between the group means, I performed a two-way ANOVA with factors Group (Tremor vs. Non-tremor) and Time (Time 1 vs. Time 2), with the clinical marker as the dependent variable. Post-hoc tests were used to compare the groups. Differences in age, sex, and ethnicity between groups were checked using the One-way ANOVA and the Chi-squared test. I also accounted for performing multiple statistical tests across our dependent variables (RBDQ, MMSE, MoCA, UPDRS II, UPDRS III, BDI) by defining a significance threshold for statistical tests of $P < \cdot 05$, and correcting this for multiple comparisons using the Benjamini-Hochberg FDR (False Discovery Rate). In brief, this procedure involves ordering all P values in ascending order and applying a sequential threshold.

3.1.3 Results

Overall, tremor was observed in 7 of 60 patients, yielding a prevalence rate of 11.7%. Of those identified with tremor, 4 individuals were men and 3 were women (6.7% and 5.0%, respectively), 4/7 were Ashkenazi Jewish (57.1%), and 7 were available for follow-up. The mean age of GD patients without tremor was 51.5 years, while those with tremor were older (mean age 73.0 years), but this difference did not reach statistical significance ($P = 0.06$). Age, gender and ethnicity were not statistically different between the groups (26/60 (43.3%) female subjects, mean age 51.5 years, 12/60 (20.0%) Ashkenazi Jewish). Mean age at tremor onset was 66.9

years. The mean duration of tremor was 11.5 years. Prevalence for tremor appeared age-related, being present in 43.8% (7/16) in those aged ≥60 years. The prevalence rates for tremor were not higher for men than for women (6.7% and 5.0%, respectively). The most frequent genotype was N370S/N370S, but the presence of the N370S allele had no effect on tremor frequency (*P* = 0.58*,* Fisher's Exact test). Current treatment for patients with tremor included enzyme replacement therapy (ERT) in 6/7 (85.7%). No patient with tremor was currently receiving substrate reduction therapy (SRT) with Miglustat, but 3/7 had received Miglustat previously; the mean duration of Miglustat treatment was 1.7±2.1 years.

In 7 GD patients with tremor, 5 (71.4%) presented with a predominant postural and action tremor. Essential tremor (ET) was diagnosed by clinical examination in 3/5 subjects for a prevalence rate of 5.0% (3/60). ET involved the upper limbs in 2 patients, and the head and neck in 1 patient (yes-yes head titubation). In 2/5 subjects, ET was diagnosed but was accompanied by dystonic features. This involved mild dystonia of the neck in 1 patient and irregularity of tremor amplitude and frequency in another patient. Other types of tremor included a unilateral rest tremor of the right thumb (non pill rolling) (1.7%; n = 1) and a bilateral rest tremor of the upper limbs (1.7%; n = 1) with no other features of parkinsonism. There was a positive family history of tremor in 2/2 patients with ET and dystonia, and a family history of PD in 1/3 patients with ET.

Neuroimaging

Of the 7 Type 1 GD patients with tremor, DaT scans were performed in three patients: two patients with ET with dystonia and in one patient with a unilateral resting tremor of the right thumb. All scans were reported as normal.

Tremor analysis

Two patients underwent tremor analysis performed by Dr. Mark Edwards at the Sobell Department of Motor Neuroscience and Movement Disorders, UCL Institute of Neurology, London, UK. In one there was no recordable rest tremor and in the second patient very minimal rest tremor which was not clinically apparent. Both patients had a postural tremor with a frequency of approximately 7Hz. This tremor did not alter significantly in frequency or total power with weight loading. One patient was unable to complete recordings of tremor during action due to fatigue. In the patient who could complete this part of the recordings, the total power (amplitude) of the tremor was higher during action compared to posture and was noticeably asymmetrical (see **Figure 7.5**).

Comparison between tremulous and non-tremulous patients

The characteristics of patients with tremor at baseline and follow-up are reported in **Table 3.1**. The results of motor tests and the degree of motor decline at follow-up significantly separated tremulous patients from non-tremulous patients. As described previously (McNeill et al., 2012b), anosmia was interpreted using age- and sex-adjusted normative scores. Mean UPSIT scores were worse at follow-up, but did not differ significantly between those with tremor and those without tremor (P = 0.54). Follow-up RBDQ scores were significantly worse than those recorded at baseline, but scores were not statistically different between tremulous and non-tremulous patients (P = 0.35). Cognitive assessment scores were worse in the tremor group but this difference did not reach significance (P = .08), and results did not differ from baseline in either group. The mean UPDRS III score at follow-up was significantly worse than scores recorded at baseline for GD patients with tremor and those

without tremor (in both, $P < .05$). However, the degree of motor progression was significantly worse in the tremor group ($P < .001$). With tremor scores excluded, GD patients' UPDRS III scores remained higher in those with tremor than those without tremor ($P < .001$).

Table 3.1. Comparison between tremulous and non-tremulous patients within the GD group.

	Gaucher disease (n = 60)		
	Tremor (7)	**Non-tremor (53)**	***P* (between)[b]**
Age, years	73.0 (4.6)	51.5 (2.2)	.11
Gender (F/M)	3/4	23/30	.61
Ethnicity (White/Ashkenazi)	3/4	36/8	.13
Most frequent genotype	N370S/N370S	N370S/N370S	.58
History of receiving Miglustat, %	42.9	14.8	.07
Age of tremor onset, years	66.9 (5.6)	-	-
Family history of PD, %	14.3	15.1	.90
UPSIT			
Baseline	32.2 (1.5)	32.9 (0.9)	
Follow-up	30.3 (1.7)	31.5 (0.9)	.54
P (within)[a]	.21	.02[c]	
RBDQ			
Baseline	0.8 (0.8)	0.5 (0.2)	
Follow-up	3.2 (0.8)	2.2 (0.5)	.35
P (within)[a]	.04	< .001[c]	

MoCA			
Baseline	24.8 (0.9)	26.7 (0.5)	
Follow-up	25.2 (1.4)	26.8 (0.6)	.08
P (within)[a]	.85	.85	
UPDRS II			
Baseline	2.5 (0.9)	0.6 (0.6)	
Follow-up	6.2 (1.1)	1.1 (0.4)	< .001[c]
P (within)[a]	.04	.01[c]	
UPDRS III			
Baseline	13.3 (4.2)	0.7 (0.3)	
Follow-up	19.2 (4.7)	2.6 (0.7)	< .001[c]
P (within)[a]	.04[c]	.001[c]	

Abbreviations: *UPSIT* Smell Identification Test, *UMSARS* Unified Multiple System Atrophy Rating Scale, *MoCA* Montreal Cognitive assessment, *MMSE* Mini-Mental State Examination, *RBDQ* Rapid Eye Movement Sleep Behaviour Disorder Questionnaire, *UPDRS* Unified Parkinson's Disease Rating Scale, *BDI* Becks Depression Inventory.

Results are presented as mean and SEM. Significance was taken at the 5% level for all variables. Only values which survived multiple comparisons with the FDR procedure were denoted significant.

Reported *P* values compare the mean values for clinical markers within groups (baseline and follow-up) and between groups (tremor and non-tremor) at follow-up.

[a] Paired t-test.
[b] Student *t* test and Chi squared test for differences in age, sex, ethnicity, treatment, family history; Fisher's exact test for differences in genotype; Two-way ANOVA with Bonferroni correction for differences between groups and time of evaluation for UPSIT, MoCA, RBDQ and UPDRS scores.
[c] Statistically significant difference.

3.1.4 Discussion

This study is the first estimate of the prevalence of tremor in Type 1 GD. I found an unexpectedly high prevalence of postural and action tremor (8.3% all ages; 20.0% age ≥65 years) in this population.

ET accounted for the majority of tremor, for a prevalence rate of 5.0% (3/60). There has been much speculation regarding the possibility of a relationship between ET and PD. In a recent prospective study, patients with ET were four times more likely to develop PD than controls during follow-up (Benito-León et al., 2009). However, others argue this may well be coincidence and the actual cause of PD in these cases could relate to other factors (Adler et al., 2011). The finding that ET is an aging-associated disease, together with an observed concurrence of both PD and Alzheimer's disease does lend support to the suggestion that ET is a neurodegenerative disease (LaRoia and Louis, 2011). Neurodegenerative diseases have been linked to defects in autophagy and the ubiquitin-proteasome system (UPS) (Korolchuk et al., 2009), and it has been proposed that lysosomal storage disorders could also be diseases of autophagy. Of interest is a recent study, which observed changes in macroautophagy within the cerebellum of ET cases compared to that of age-matched controls, and suggests perturbed macroautophagy could contribute to Purkinje cell pathology in ET (Kuo et al., 2012). This highlights a potential role for the lysosome in ET pathogenesis, an organelle increasingly connected to neurodegenerative disease.

Normal DaT scans were reported in three of the tremor patients. These patients could represent an important subgroup of patients suspected clinically to have early PD, but who have scans without evidence of dopaminergic deficit (SWEDD). It can be difficult to distinguish these patients from those with PD on clinical grounds, but

SWEDD cases can present with atypical or dystonic tremor (Bajaj et al., 2010). However, there is debate as to whether these patients have early PD or whether adult-onset dystonia is the underlying diagnosis (Schwingenschuh et al., 2010).

I considered a diagnosis of drug-induced tremor, particularly if there was a history of receiving substrate reduction therapy (SRT) with Miglustat, a known cause of tremor (Kuter et al., 2013). In 3/7 tremor cases, SRT had been administered previously but it should be noted that in all cases, tremor was present before the treatment commenced.

The high prevalence of tremor in this type 1 GD population, with prevalence rates increasing with age, combined with a significant decline on motor tests during follow-up, could lend support to the association of GD with PD that has been highlighted in the literature (Bultron et al., 2010), although larger numbers and longer follow-up will be needed to confirm this, and to determine if the tremor in these cases has any link to PD, or simply represents an incidental finding. The tremor in this cohort was atypical for PD tremor, and in some patients the tremor has been longstanding, making PD less likely as a cause. My work could, therefore, suggest that *GBA* represents a shared risk factor for two pathophysiologically separate movement disorders. This remains to be supported by genetic studies. For instance, in the first comprehensive analysis of the frequency of the *GBA* gene mutation in ET, authors found a similar frequency of *GBA* mutations in ET cases versus controls (Clark et al., 2010). Larger studies are needed to determine whether the *GBA* gene mutation is specifically associated with PD, or whether it affects the risk for other neurodegenerative diseases and related movement disorders.

3.2 A case of type 1 Gaucher disease with parkinsonism

3.2.1 Introduction

The majority of patients with type I GD never develop neurological signs or symptoms. However, several case reports of PD disease associated with type I GD have been published, confirming a genetic link between the two diseases. In the clinical setting, it can be difficult to distinguish GBA-related PD from sporadic PD. Here I present a right handed Sri Lankan man in whom parkinsonism was associated with type I GD. Findings of the brain DaT scan are presented.

3.2.2 Case report

A 53-year-old man with known type I GD was a participant in a study of the aetiology and prodrome of PD. He was recruited in 2010 from the Lysosomal Storage Disorder Unit at the Royal Free Hospital, London. He had no prior diagnosis of PD or dementia. He was working as a Tesco checkout assistant.

At the age of 12, he had removal of an enlarged spleen. Thereafter, he also developed an enlarged liver, by which time he was living in the United Kingdom. A diagnosis of GD was made based on the following typical findings; hepatosplenomegaly, Gaucher cells on bone marrow biopsy, reduction in blood β-glucosidase activity, and N370S/L444P mutation of the glucocerebrosidase gene on genetic analysis. He has been on enzyme replacement therapy with 1200 IU of Velaglucerase alpha every 2 weeks (recently reduced to 800 IU as he felt his current presentation was worse following his infusions).

At baseline in 2010, he had no existing neurological disease. He was found to have moderate hyposmia with a score of 20/40 on the University of Pennsylvania Smell Identification Test. At two years follow-up, he had severe hyposmia with a score of 14/40. At three years into the research study, he had noticed a deterioration in his walking for 1 year. This had become slow and he had taken to using a walking stick. He had noticed a tremor in his right leg and occasional tremor in his right arm. The tremor was present almost continuously. His speech had become slow and he felt he had become more forgetful. His sleep was particularly fragmented but there were no features of RBD. For two years, he had noticed a reduction in his sense of smell. His writing had become micrographic but bladder function remained normal. There was no family history of PD and I understand his mother has motor neurone disease.

Examination confirmed hypophonia. Cranial nerve examination revealed some reduction in saccadic eye movement but the external ocular movements were full. There was a resting tremor in the right leg and evidence of mild to moderate bradykinesia and rigidity, particularly on the right. The remainder of the neurological examination was normal. Examination of his gait revealed poor arm swing and small steps. His postural reflexes were still intact. The clinical features were indicative of PD.

DaT scan (see **Figure 7.6**) revealed clear-cut reduction of uptake within the putamen bilaterally. In addition, there was reduced uptake within the right caudate nucleus. Findings were consistent with idiopathic PD. He was commenced on L-dopa therapy with Sinemet. This has led to some improvement but it is felt that he will need increasing concentrations of L-dopa therapy.

At four years into the research study, he has now developed moderate cognitive impairment with a score of 21/30 on the Montreal Cognitive Assessment (MoCA) and 20/30 on the Mini Mental State Examination (MMSE). This compares to a MoCA score of 29/30 and MMSE score of 30/30 at his initial baseline evaluation in 2010.

3.2.3 Discussion

In this case report of PD associated with type 1 GD, I describe the type of trajectory a patient with GBA-PD is likely to have. PD is typically characterized by the motor symptoms of resting tremor, bradykinesia, rigidity, and postural instability. Patients with GD who develop parkinsonism typically exhibit the symptoms of sporadic PD. The majority of patients with type 1 GD who develop PD therefore present with asymmetric onset, tremor, and bradykinesia (Goker-Alpan et al., 2008), the cardinal features of 'idiopathic' PD. However, there are some notable differences. In most published cases, the age at onset of PD is younger than that seen in typical PD by 5 years and there is a higher frequency of cognitive impairment. In an early case series of six type 1 GD patients (Neudorfer et al., 1996), parkinsonism developed at a mean age of 49 years and manifested atypical findings such as psychiatric symptoms, myoclonus, and an unresponsiveness to L-dopa therapy. Other atypical features have also been observed such as perceptive deafness (Bembi et al., 2003), dementia (Tayebi et al., 2003), and horizontal saccadic eye movements (Bembi et al., 2003). In the present case, parkinsonism responded well to L-dopa therapy, and dopamine imaging by single photon emission tomography (SPECT) of the dopamine transporter was abnormal in this patient with GBA-PD in a pattern identical to idiopathic PD. However, onset as early as the 5th decade of life and significant cognitive impairment are not typical features of idiopathic PD.

The evidence from longitudinal studies demonstrate that GBA-PD patients show a more rapid disease progression and a greater preponderance to dementia or cognitive decline (Brockmann et al., 2014) (Winder-Rhodes et al., 2013). The association between the distribution of LB pathology and the cognitive impairment seen in GBA-PD is well established. Brains from PD patients with *GBA* mutations reveal more diffuse neocortical LB–type pathology compared with those from sporadic PD patients (Neumann et al., 2009).

It would be interesting to know when the process of rapid progression in GBA-associated PD starts. At the time of diagnosis, it is difficult to distinguish PD patients with and without *GBA* mutations. In a recent prospective longitudinal study to evaluate progression of motor and non-motor symptoms in sporadic PD patients depending on the mutational *GBA* status, the *GBA* positive subgroup were more severely affected by motor impairment, disease staging, and cognitive decline (Brockmann et al., 2014). In GBA-PD patients, the acceleration of disease progression appeared to be relatively late (8-9 years disease duration). Authors suggest this could be due to compensatory mechanisms in the first years of the disease but that at some point this system becomes overburdened, resulting in rapid progression.

Experimental evidence puts forward a self-perpetuating model and suggests a bidirectional pathogenic loop: GCase interacts directly with SNCA and this binding between the wild type enzyme and SNCA, under lysosomal conditions, could have a beneficial effect by either promoting SNCA degradation by the lysosome and/or preventing aggregation (Yap et al., 2011). Mutations in *GBA* or reduced levels of wild

type GCase reaching the lysosome could weaken the beneficial interaction between SNCA and GCase, reduce lysosomal protein degradation, and contribute to the SNCA aggregation characteristic of PD pathology. The resultant accumulation of substrate and SNCA would then mediate the interaction of the SNCA-GCase complex on intralysosomal vesicles, leading to a further loss of GCase activity (Yap et al., 2013).

Overall, this case supports the idea that the *GBA* mutation could represent a poor prognostic marker for an individual with PD.

Chapter 4: The Biochemical Signature of the *GBA* Mutation

4.1 Development and characterization of cell culture models from individuals within the clinical cohort studied

OBJECTIVE

To establish the relevant cell models from controls and individuals carrying the *GBA* mutations, with or without PD, in order to characterise the biochemical defects associated to mutant *GBA* and to PD.

4.1.1 Establishing human cell models for Parkinson's disease

I want to define the downstream biochemical signature of *GBA* mutations, and fibroblasts provide a good model to do that – studying a disease in patient-specific cells. However, fibroblasts are limited for interrogating the complete pathway in PD in comparison to neuronal models, owing to the lack of SNCA expression in fibroblasts and other neuronal features. A neuronal model would be ideal where levels of SNCA can also be measured in the dopaminergic (DA) neurons, and which will more accurately reflect what is happening in the brain. To this end, I plan to investigate

whether small molecular chaperones, ambroxol (ABX) and isofagomine (IFG), increase GCase activity and reduce SNCA levels, in a human DA neuronal model of PD.

Many different neuronal cell models have been developed in an attempt to recapitulate PD. One of the major limitations to research into PD has been the inaccessibility of dopaminergic (DA) neurons and the lack of genuine *in vitro* models. One technology that has emerged is the use of induced pluripotent stem cells (iPSC) generated from patients themselves. To create patient-specific DA neuronal cultures, iPSCs can be generated by reprogramming patient fibroblasts using pluripotency factors such as Oct4, SOX2, Nanog and Klf4. One of the major limitations to this technology is the variability between iPSC lines. From each culture of reprogrammed fibroblasts, colonies are selected and are subjected to quality-control measures such as transgene silencing and karyotype analysis. However, these clones, colonies derived from the same human donor, still exhibit large variability (Devine et al., 2011). This can lead to considerable phenotypic variability that is unrelated to their genotype and a differing efficiency to which iPSC lines differentiate into neurons (Sánchez-Danés et al., 2012). Genome editing has become a necessary step to introduce point mutations into the genome, resulting in clonal cell lines that are isogenic (Soldner et al., 2011). Variability between clones can also be reduced by using non-disruptive methods of reprogramming, such as episomal plasmids (Okita et al., 2011) or non-integrating viruses, e.g. Sendai virus (FUSAKI et al., 2009). This variability between lines can reduce the ability to achieve meaningful results, especially when only a few lines are used for study. One other issue is the potential consequence of long-term culture of iPSCs over time. This can result in the generation of chromosomal abnormalities, and although each line is subjected to

analyses of karyotype, detailed examination reveals small copy number variants in iPSC lines that may go undetected (Soldner et al., 2011). In summary, iPSC are a good way of studying the effects of a human mutation in human cells, but there remain some important limitations including a low transfection efficiency (~10% or incomplete reprogramming), genomic instability, tumour formation in vivo, and some argue that the iPSC-derived DA neurons are immature and do not behave like neurons.

A novel technique has been developed with which neural crest stem cells (NCSC) can be isolated from patient's fat tissue. Human adipose tissue has distinct developmental origins including mesoderm and neural crest lineage, and contains many different types of cells. Human adipose NCSC have the capability of differentiation to mesodermal, endodermal and ectodermal lineages (Hauser et al., 2012; Motohashi and Kunisada, 2015). This ability of adipose-derived stem cells to differentiate into DA neurons opens a new window into developing a physiologically relevant, *in vitro* model of PD. This approach will allow the exploitation of a readily available and abundant source of adult stem cells, and bypasses the time consuming reprogramming and cloning phases associated with iPSC technology. I used these NCSC to develop human NCSC-derived DA neurons as a model for the study of PD. This cell model was developed and prepared for my study through collaboration with Dr. Shi-Yu Yang.

4.1.2 Sample collection

All cells were generated *in vitro* after written informed consent using protocols approved by the Royal Free research ethics committee, Royal Free Hospital,

London, UK. PD was diagnosed according to UK Brain Bank criteria (Litvan et al., 2003). The *GBA* mutation status in all participants was confirmed by Sanger sequencing of the *GBA* gene, as previously described (McNeill et al., 2012b). Any clinical sign of a neurodegenerative disorder led to exclusion from the study. Differences in age and sex between groups were checked using the One-way ANOVA. See Appendix **Table 9.6** for the full list of skin biopsies obtained from the patient cohort.

4.1.3 Patient-derived fibroblasts

Human skin fibroblasts (**Table 4.1**) were obtained from the patient cohort and included five healthy controls, five heterozygous *GBA* mutation carriers with PD (N370S/wt; L444P/wt), five heterozygous *GBA* mutation carriers without PD (N370S/wt; L444P/wt), and five PD patients negative for the *GBA* mutation. Fibroblasts were grown in the standard conditions: Dulbecco's modified Eagle medium (DMEM) supplemented with 10% fetal bovine serum (FBS), 5% sodium pyruvate, 1% fungizone and 1% penicillin–streptomycin at 37 °C in the presence of 5% CO_2, with medium changed every 2 days. Cultures were not left to become confluent. Passage numbers were limited from 8 to 12, and cell cultures were matched for passage number.

4.1.4 Identification and isolation of neural crest stem cells from human adipose tissues

Human adipose NCSC (**Table 4.2**) were isolated from the subcutaneous fat collected from wild type, heterozygous *GBA* mutation positive (N370S/wt) carrier and PD

patients who underwent a skin punch biopsy, and differentiated into functional human dopaminergic neurons.

Table 4.1. Summary of patient-derived fibroblast lines

Subject Code	Genotype	Gender	Diagnosis	Age at biopsy
C1	wt/wt	Female	CTRL	67
C2	wt/wt	Female	CTRL	70
C3	wt/wt	Male	CTRL	81
C4	wt/wt	Female	CTRL	82
C5	wt/wt	Male	CTRL	52
Het1	N370S/wt	Female	*GBA* Carrier	67
Het2	N370S/wt	Female	*GBA* Carrier	60
Het5	N370S/wt	Male	GBA-PD	80
Het6	N370S/wt	Female	GBA-PD	75
Het7	N370S/wt	Female	GBA-PD	53
Het8	L444P/wt	Male	*GBA* Carrier	62
Het9	L444P/wt	Female	*GBA* Carrier	45
Het10	L444P/wt	Male	*GBA* Carrier	67
Het11	L444P/wt	Male	GBA-PD	72
Het12	L444P/wt	Male	GBA-PD	85
GD1	N370S/N370S	Male	GD	70
PD1	wt/wt	Female	IPD	85
PD2	wt/wt	Male	IPD	79
PD3	wt/wt	Female	IPD	81
PD4	wt/wt	Female	IPD	72
PD5	wt/wt	Male	IPD	80

CTRL, healthy control; IPD, Idiopathic Parkinson's disease; *GBA* Carrier, heterozygous *GBA* mutation carrier; GBA-PD, heterozygous *GBA* mutation carrier with PD; GD, Gaucher disease.

Table 4.2. Summary of patient-derived adipose neural crest stem cells

Subject Code	Genotype	Gender	Diagnosis	Age at biopsy
C1	wt/wt	Female	CTRL	67
C2	wt/wt	Female	CTRL	70
C3	wt/wt	Male	CTRL	81
Het1	N370S/wt	Female	*GBA* Carrier	67
Het3	N370S/wt	Female	*GBA* Carrier	82
Het4	N370S/wt	Male	*GBA* Carrier	52
PD5	wt/wt	Male	IPD	80
PD6	wt/wt	Female	IPD	75
PD7	wt/wt	Female	IPD	53

CTRL, healthy control; IPD, Idiopathic Parkinson's disease; *GBA* Carrier, heterozygous *GBA* mutation carrier.

4.1.5 Human adipose neural crest stem cell culture

Human adipose NCSC were cultured in DMEM supplemented with 10% FBS, 5% sodium pyruvate, 0.5% uridine, 1% fungizone and 1% penicillin–streptomycin at 37 °C in the presence of 5% CO_2, with medium changed every 2-3 days. Cultures were not left to become confluent. Passage numbers were limited from 3 to15, and cell cultures were matched for passage number.

4.1.6 Characterisation of adipose neural crest stem cells

The adipose NCSC used for this study have been extensively characterised by Dr. Shi-Yu Yang who has shown that adipose NCSC are capable of forming neurospheres and are positive on immunostaining and RT-PCR for neural crest stem cell markers (data not shown here). Immunostaining was performed for pluripotency markers Nanog, Oct-4, P75, SOX10, SSEA4 and TRA-1-18 to characterise

pluripotent properties of healthy and *GBA* mutant positive NCSC (see **Figure 7.7** and **Figure 7.8**).

4.1.7 Karyotyping

Karyotyping was performed by full G-band analysis of 20 cells in 3 human neural crest stem cell cultures (1x wild type, 1x heterozygous *GBA* mutation carrier, and 1x PD) to examine the integrity of the chromosomes by The Doctor's Laboratory at Whitfield Street, London. All cells tested showed a normal banding pattern.

4.1.8 Dopaminergic neuronal differentiation of human adipose neural crest stem cells

Adipose NCSC were differentiated to functional DA neuronal cells using a fully defined step-wise differentiation protocol (see section **4.3.2**). Following differentiation, human adipose NCSC adopt a neuronal morphology. These cells express specific neuronal markers such as Nestin, NeuN, class III β-tubulin, and DA neuronal markers tyrosine hydroxylase and the DA transporter. The neurons respond to glutamate- and KCL-induced plasma membrane depolarisations with robust Ca2+ and Na+ fluxes, confirming that, in terms of Ca2+ and Na+ homeostasis, the neurons are functional.

4.2 Characterisation of the fibroblast model related to Parkinson's disease and the biochemical effect of the *GBA* mutation

OBJECTIVE

To use the relevant cell models from controls and individuals carrying the *GBA* mutations, with or without PD, in order to characterise the biochemical defects associated to mutant *GBA* and to PD.

4.2.1 Introduction

The discovery of an association of the *GBA* mutation with PD (Sidransky et al., 2009a) has proved an important development for research on the pathogenesis of PD, and has provided support for increasing evidence that lysosomal pathways are implicated in PD (Dehay et al., 2013).

The relationship between GD and PD is not yet fully understood. It could arise from the gain of a new toxic function by mutated *GBA*, a loss of enzyme function, or a combination of both. Most mutations in *GBA* cause GCase to fold abnormally. The misfolded protein is retained in the endoplasmic reticulum (ER) and after unsuccessful attempts at refolding, finally undergoes degradation by the ER-associated degradation (ERAD) system. This reduces the GCase reaching the lysosome, could compromise lysosome function and have consequences for the lysosomal-autophagy pathway, considered to be the main pathway through which SNCA is degraded (Vogiatzi et al., 2008; Machiya et al., 2010). In GD patients homozygous for the *GBA* mutation, there is a significant reduction in GCase activity. In the asymptomatic heterozygous carrier, there is varying degrees of activity

ranging from 50% to normal. However, even this moderate reduction of GCase activity may be sufficient to lead to suboptimal lysosomal function, SNCA accumulation and an increased frequency of PD (Mazzulli et al., 2011).

Previously our lab has demonstrated some of the molecular changes associated with the *GBA* mutation that might predispose to PD. Here, I have extended the investigation into the biochemical consequences of the *GBA* mutation relevant to PD pathogenesis. I have shown for the first time that there is a defective localisation of GBA in *GBA* mutant fibroblasts by immunocytochemistry. I have also shown for the first time that the UPR protein BiP/GRP78BiP, a marker of the UPR, is reduced in heterozygous *GBA* mutation carriers and could suggest an inadequate UPR in these cells. Furthermore, I have identified defects in lysosomal autophagy in *GBA* mutant fibroblasts which were not identified previously (McNeill et al., 2014).

4.2.2 Method

Lysosomal enzymatic activity assays

Cell pellets were re-suspended in water and sonicated in a water-bath sonicator for 1 minute. GCase activity was determined in cell lysate of about 20µg protein by hydrolysis of 5mM 4-methylumbelliferyl-β-D-glucopyranoside in McIlvaine buffer (pH 5.4) in the presence of 22mM sodium taurocholate at 37°C for 1 hour (Wenger et al., 1978). The reaction was stopped by addition of 0.25M glycine (pH 10.4) and 4-methylumbelliferone fluorescence measured, against the reference at excitation 365nm, emission 450nm. Contribution from non-lysosomal GCase (*GBA2*) was distinguished by using the corresponding inhibitor conduritol-β-epoxide (CβE) (see Appendix **Figure 9.6** and **Figure 9.7**).

β-Hexosaminidase was assayed in the above cell lysate of about 2µg protein using the fluorogenic substrate 4-methylumbelliferyl-N-acetyl-glucosamide substrate (2mM) in sodium citrate buffer (pH 4.2) at 37°C for 30 minutes. The reaction was stopped by addition of 0.25M glycine (pH 10.4), and fluorescence was measured as above (Wendeler and Sandhoff, 2009).

β-Galactosidase was assayed in the above cell lysate of about 2µg protein using the fluorogenic substrate 4-methylumbelliferyl-β-D-galactopyranoside (2mM) in sodium citrate buffer (pH 4.1) at 37°C for 30 minutes. The reaction was stopped by addition of 0.25M glycine (pH 10.4), and 4-methylumbelliferone fluorescence measured at excitation 365nm, emission 450nm.

The total protein concentration of the cell lysate was determined by the Bicinchoninic Acid (BCA) method. 5µl of the above cell lysate was loaded in 20µl of water. A set of protein standards were also measured. The fluorescence was measured at 562 nm. The protein concentration of the standards was used to create a standard curve. The standard curve was used to calculate the protein concentration of the cell lysate in mg/ml.

Enzyme activities were calculated by substracting the background fluorescence from the mean fluorescence measured for a given cell lysate, then dividing by the standard to calculate the nmol/hr/ml activity. This result was then divided by the total protein concentration, as determined using the BCA method, to calculate the enzymatic activity in nmol/hr/mg. All enzyme assays were validated (see Appendix **Chapter 9**:).

Western blot analysis

Cells were harvested, washed with PBS, and processed as previously described (McNeill et al., 2014). In total, 20µg of the protein lysate was loaded on a 4–12% Bis-Tris Gel (NuPAGE, Invitrogen) and transferred to a polyvinylidene difluoride membrane (Millipore, Watford, England). The following antibodies were used: mouse anti-GBA (Calbiochem), rabbit anti-β-actin (Abcam), rabbit anti-BiP (Abcam), rabbit anti-LAMP1 (Abcam), rabbit anti-LAMP2a (Abcam), rabbit anti-Hsc70 (Abcam), rabbit anti-GAPDH (Abcam), rabbit anti-LC3 (Cell signaling), and mouse anti-p62 (BD Biosciences). Horseradish peroxidase goat anti-mouse (Dako), anti-rabbit (Dako) or anti-goat (Dako) secondary antibodies and Clarity Western ECL kits (BioRad) were used. Analyses were prepared using Image Lab software (BioRad). See Appendix **Table 9.4** for the full list of antibodies used. Full-length images of immunoblots are shown in Appendix **Figure 9.10**.

Autophagy studies

Where indicated, cells were treated with 0.2µM bafilomycin (EMD, Millipore) or with DMEM without supplements (starvation) for 4 hours at 37 °C in the presence of 5% CO_2, and then fixed or lysed for western blot analysis. LC3/LC3-II levels were quantified by densitometry and normalized for β-actin. LC3 flux was quantified by dividing levels of LC3-II after bafilomycin treatment for 4 h by the level of LC3-II without treatment.

Quantitative Real-Time PCR

Total RNA was extracted with the RNeasy® kit (Qiagen) and converted to complementary DNA with the QuantiTect reverse transcription kit (Qiagen). Quantitative RT–PCR reaction was performed using a TaqMan Assay (Life Technologies). Fold-change in gene expression was calculated using the $2^{-\Delta\Delta CT}$method, based on biological reference samples and β-actin messenger RNA levels for normalization. All the results were obtained from the evaluation of two technical duplicates of three independent experiments. See Appendix **Table 9.5** for the list of primers used.

Immunocytochemistry

Cells were fixed in 4% paraformaldehyde for 15 min, permeabilized in ice cold methanol, rinsed with PBS and blocked by 10% normal goat serum before incubation with primary and secondary antibodies. Anti-glucocerebrosidase (Sigma) was used at a concentration of 1:50 and anti-cathepsin D (Abcam) at 1:200. Images were captured with a Zeiss LSM 510 (Carl Zeiss) confocal microscope using a 40 × 1.3NA plan-apochromat oil objective, at an excitation of 488nm (for Alexa Fluor Green) or 568nm (for Alexa Fluor Red). Individual cells were marked and mean fluorescence of individual cells measured by the ImageJ software (NIH, USA); where the image was captured with z-stack, *z*-projection was performed using maximum intensity and net fluorescence was obtained by subtracting the background fluorescence. Co-localization of GBA/cathepsin D in fibroblasts was quantified with the 'JACoP' plug-in of the ImageJ software. See Appendix **Table 9.4** for the list of antibodies used.

Statistical analysis

Data are expressed as mean ± SEM and statistical significance between groups determined by one-way ANOVA followed by the two-tailed t-Test. A *p* value of < 0.05 was considered as significantly different. All data were analysed by GraphPad Prism 6 statistical software (GraphPad Software).

4.2.3 Results

Reduced GCase activity, protein and gene expression levels in *GBA* mutant fibroblasts

GCase activity was significantly reduced in heterozygous *GBA* mutation carrier fibroblasts compared to healthy control cells. Quantitative analysis of intracellular GBA protein levels in cells of different patients by western-blotting revealed that protein levels of the enzyme were also reduced in heterozygous *GBA* mutation carriers compared with healthy control cells. I compared the levels of *GBA* mRNA using quantitative PCR. I found that the amount of *GBA* mRNA expressed in heterozygous *GBA* mutation carriers was significantly reduced compared to controls (**Figure 7.9**).

Defective localization of GBA protein in *GBA* mutant fibroblasts

To evaluate the localization of GBA, I performed immunocytochemistry for each cell line using antibodies to cathepsin D, a lysosomal marker, and GBA. The GBA signals were clearly stronger in control and idiopathic PD (IPD) cells compared to heterozygous *GBA* mutation carrier cells. Control and IPD cells showed colocalization of GBA and cathepsin D. Overall, the strength of signal and the

distribution of GBA did not differ between control and IPD cell lines. In contrast, I observed a decreased degree of co-localization between GBA and cathepsin D in heterozygous *GBA* mutation carrier cells when compared to control cells, which may suggest impaired trafficking of GBA in *GBA* mutant fibroblasts (**Figure 7.10**).

Inadequate unfolded protein response (UPR) in *GBA* mutant and PD fibroblasts

To evaluate the effect of the *GBA* mutation on the UPR, I performed western blot analysis of the major UPR protein BiP/GRP78. My results demonstrated a marked reduction in UPR signal in heterozygous *GBA* mutation carriers (**Figure 7.11**). This reduction was also seen in PD, independent of *GBA* mutation status. Heterozygous *GBA* mutation carriers and PD cells show reduced GRP78 expression, suggesting an inadequate UPR.

Lysosomal content and function is unaltered in *GBA* mutant fibroblasts

To assess the effect of the *GBA* mutation on lysosomal content and function, I analysed the autophagic/lysosomal machinery in *GBA* mutant fibroblasts. Enzyme assays were performed for hexosaminidase and β-galactosidase activity. There were no changes in the activity of the lysosomal enzymes between the cell lines; hexosaminidase and β-galactosidase activity were not significantly different between control, heterozygous *GBA* mutation carrier or IPD cells (**Figure 7.12**). To extend my investigation, I measured the lysosomal content of the cells by LAMP1 levels. Immunoblotting for LAMP1, a lysosomal marker, did not reveal any significant increase in LAMP1 protein in heterozygous *GBA* mutation carriers, IPD and control

fibroblasts, suggesting no accumulation of lysosomes **(Figure 7.12**). Taken together, these data indicate that there is no alteration of lysosomal mass or number in heterozygous *GBA* mutation carriers.

Lysosomal autophagic defects in *GBA* mutant fibroblasts

Autophagy has a role in quality control and activation of autophagy forms part of the cellular response to ER stress (Schneider and Cuervo, 2014a). To examine lysosomal function and macroautophagy, I first investigated the autophagosome content by measuring levels of endogenous light chain type 3 protein (LC3), a marker of autophagosomes by western blot. This revealed a significant decrease in basal levels of LC3-II in *GBA* mutation carrier fibroblasts, suggesting an impairment of the rate of autophagosome formation. Treatment with bafilomycin revealed significant increases in LC3-II flux in all cell lines, and therefore no impairment of the fusion of autophagosomes with lysosomes in *GBA* mutant fibroblasts (**Figure 7.13**). In comparison, under basal conditions there was a further significant decrease in p62 levels in heterozygous *GBA* mutation carriers compared to controls (data not shown).

Given the potential cross-talk among proteolytic systems and the evidence of potential macroautophagic compromise, I investigated the chaperone mediated autophagy (CMA) pathway. Using the heat shock cognate protein of 70kDa (Hsc70) which targets substrates to the lysosome surface, and the lysosome-associated membrane protein type 2A (LAMP2a) receptor as markers for CMA, a significant reduction of LAMP2a protein associated with the presence of *GBA* mutation was

seen in heterozygous *GBA* mutation carriers, while levels of Hsc70 did not differ between controls, heterozygous *GBA* mutation carriers, or PD cells (**Figure 7.13**).

4.2.4 Discussion

The contribution of mutations of *GBA* and reductions in GCase activity in fibroblasts was recently studied in samples from PD patients with and without the *GBA* mutation, and in GD (McNeill et al., 2014). I have extended these investigations to help define the exact mechanisms by which *GBA* mutations predispose to neurodegeneration in order to facilitate the development of a novel treatment strategy. I have shown for the first time that there is a defective localisation of GBA in *GBA* mutant fibroblasts, the UPR protein BiP is reduced and could reflect an inadequate UPR, and finally, *GBA* mutant fibroblasts demonstrate defects in lysosomal autophagy.

My data supports those of others (Sun et al., 2012; Bendikov-Bar et al., 2013; McNeill et al., 2014) by confirming that the *GBA* mutation is associated with a reduction in GCase activity. However, the level of GCase activity varied between mutations and is to some degree, patient dependent. I measured GCase activity predominantly from those heterozygous for the *GBA* mutation, where levels ranged between 50-70% of the wild type level. In a patient homozygous for the *GBA* mutation, the GCase activity was ~2.5% of the wild type level (**Figure 7.27**), which matches previous data (McNeill et al., 2014). Loss of GCase activity was associated with decreased protein levels of GBA and mRNA levels indicating this loss of activity was induced by decreased transcription. In a recent study, other lysosomal enzymes

were altered in fibroblasts from PD patients positive for the *GBA* mutation with increases seen in cathepsin D and β-hexosaminidase, laying support for a global dysregulation of lysosome functioning (McNeill et al., 2014). In the present study, the activity of β-hexosaminidase and β-galactosidase did not show significant change in any of the fibroblasts studied. There was minimal change identified in lysosomal marker LAMP1. These data suggest that the defect in GCase may be selective in PD, with no overall change in lysosomal number or mass.

Studies of PD brain have identified reductions in GCase activity involving substantia nigra (reduced 58%), putamen (48%), amygdala (40%) and cerebellum (47%) in those positive for the *GBA* mutation. What was perhaps surprising was the finding that GCase activity was also significantly decreased in the substantia nigra (33% decrease) and cerebellum (24% decrease) of sporadic PD brain. This implied that reductions in GCase activity could hold a wider significance, not just for those carrying the *GBA* mutation, but for PD in general. I also noted reductions of GCase activity and protein in skin cells obtained from PD patients negative for the *GBA* mutation, albeit this reduction was not statistically significant. There is now evidence not only for decreasing lysosomal numbers and function with age, but an age-dependent reduction in GCase activity which may lower the threshold for developing PD (Rocha et al., 2015). It is worth noting that the PD patient group in this study were older, and if the GCase activity measurements were repeated, I would ensure that patient-derived fibroblasts were more closely age matched.

The majority of *GBA* mutations result in a misfolded protein which is retained in the endoplasmic reticulum (ER) demonstrated by an increase in the endoglycosidase-H

sensitive fraction of GCase (McNeill et al., 2014). The presence of misfolded proteins, such as GCase, in the ER is proposed to induce ER stress and contribute to the development of PD (McNeill et al., 2014). ER stress activates a signalling network known as the UPR to reduce ER stress and restore ER homeostasis (Oslowski and Urano, 2011). Accumulation of protein can activate the unfolded protein response (UPR) and endoplasmic reticulum associated degradation (ERAD). The 2 most common *GBA* mutations associated with PD (N370S and L444P) have been reported to become trapped in the ER and trigger the UPR and ERAD (Ron and Horowitz, 2005; Mu et al., 2008; Lu et al., 2010). Markers of UPR/ERAD were found to be increased in the GBA-PD brain, supporting a role for this pathway in the pathogenesis of PD in these patients. Aberrant trafficking of GBA might contribute to this increase in UPR/ERAD. However, ER stress can also be a consequence of perturbed calcium homeostasis, oxidative stress, or mitochondrial dysfunction, all of which are implicated in the pathogenesis of PD. In 2 GD mouse studies, there was no evidence of UPR/ERAD activation in the brain (Farfel-Becker et al., 2009; Cullen et al., 2011). Furthermore, a recent study of post-mortem frontal cortex tissue from 7 *GBA* mutation carriers with DLB, and 5 *GBA* mutation carriers with no signs of neurological disease, reports the UPR regulator BiP/GRP78 is reduced by the *GBA* mutation and this is a general phenomenon in DLB. Indeed, my data also demonstrates reductions in UPR signal in heterozygous *GBA* mutation carriers but also PD in general, independent of *GBA* mutation status. Individuals with *GBA* mutations and those with PD show reduced GRP78 expression, suggesting an inadequate UPR.

Recent data has provided increasing evidence of a role for altered lysosomal function in the pathogenesis of PD (Tofaris, 2012; McNeill et al., 2014; Murphy et al., 2014a). Reduced GCase activity has been reported in PD brain (Gegg et al., 2012; Murphy et al., 2014a), with and without mutations in the *GBA* gene. It has also been demonstrated that lysosomal dysfunction occurs early in *GBA* mutation-negative PD. In early stage PD anterior cingulate cortex, there is a selective upregulation of cathepsin A and D protein and reductions in LAMP2, a marker of chaperone-mediated autophagy (CMA) (Murphy et al., 2014a). Authors imply that this might be relevant to reduced degradation of SNCA. My study of fibroblasts from *GBA* mutation-positive patients, and those of others (McNeill et al., 2014), also demonstrate evidence of altered lysosomal function. My results reveal a significant decrease in basal levels of LC3-II in *GBA* mutation carrier fibroblasts indicating an impairment of macroautophagy. Cells can compensate for loss of macroautophagy by upregulating CMA or the ubiquitin-proteasome system (Schneider and Cuervo, 2014a). I further identified reductions of CMA. The selective loss of lysosomal GCase appears to be directly related to defects of lysosomal autophagy.

SNCA is predominantly turned over through chaperone mediated autophagy (CMA) and to a lesser degree through macroautophagy. Abnormalities of CMA have been identified in PD brain (Cuervo et al., 2004; Alvarez-Erviti et al., 2010). Our lab has also shown that impaired lysosomal function can enhance the exosomal release of SNCA (Alvarez-Erviti et al., 2011). Furthermore, decreased GCase activity promotes propagation of SNCA aggregates (Bae et al., 2014). In my studies, LAMP2a was reduced in *GBA* mutation carrier fibroblasts but this did not correlate with a coexistent reduction/increase in Hsc70. However, it appears that the rate limiting

step for CMA is the binding of the substrate proteins to LAMP2a and, consequently, levels of LAMP2a at the lysosomal membrane correlate directly with CMA activity (Schneider and Cuervo, 2014b). Therefore, to modulate the activity of this autophagic pathway, the cell stringently regulates the levels of the CMA receptor at the lysosomal membrane by controlling the degradation rates of LAMP2a monomers in lysosomes and by de novo synthesis of LAMP2a molecules. In addition, transport of substrates also depends on the efficiency of the assembly of LAMP2a into the translocation complex. This could explain why reductions of LAMP2a were noted with no coexistent change in Hsc70.

In conclusion, I observed a 50-70% decrease in the enzymatic activity of glucocerebrosidase, protein level and gene expression in *GBA* mutation carriers. This was associated with lysosomal abnormalities including defective localization of GBA protein and impaired lysosomal autophagy. These data provide support for a link between *GBA* mutations and changes in the autophagic/lysosomal system, which could predispose to neurodegeneration. My results indicate that mutations in *GBA* could lead to lysosomal abnormalities, enhanced by an impaired UPR, and therefore, have the potential to cause SNCA accumulation. The discovery of a connection between *GBA* mutations and PD has provided invaluable insights into the pathogenesis of this disease and provides opportunities to target the GCase–lysosomal pathway for the development of neuroprotective drugs in PD.

4.3 Characterisation of the adipose NCSC-derived dopaminergic model related to Parkinson's disease and the biochemical effect of the *GBA* mutation

OBJECTIVE

To use human dopaminergic neurons derived from their adipose-derived neural crest stem cells from controls and individuals carrying the *GBA* mutation, in order to characterise the biochemical defects associated to mutant *GBA* and to PD, and to investigate the relationship between GCase and SNCA in PD.

4.3.1 Introduction

The pathological hallmarks of PD include the presence of insoluble oligomeric and fibrillar SNCA positive inclusions, the formation of LBs and the loss of dopaminergic neurons in the SNpc. Numerically, the most important genetic risk factor for the development of PD is the presence of a mutation of *GBA*.

Both gain-of-function and loss-of-function of GCase effects have been proposed as mechanisms contributing to PD pathogenesis, leading to substrate accumulation, disturbances in lysosomal function/trafficking, and SNCA aggregation. It has been suggested that reductions in GCase could contribute to SNCA aggregation through direct interaction with SNCA or interference with SNCA homeostasis pathways such as the unfolded protein response or autophagy. However, the exact molecular mechanisms involved in the interaction between GCase and SNCA remain unresolved.

Important progress in understanding PD pathogenesis has been based on human post-mortem samples, chemically-lesioned animals, transgenic animals and *in vitro* models; however these models all have limitations. I used a novel human neuronal cell model originating from heterozygous *GBA* mutation positive subjects, in which neural crest stem cells were isolated from *GBA* heterozygous mutated subjects' adipose tissues. The isolated neural crest stem cells were differentiated to functional dopaminergic neurons with high efficiency. The activities of GCase enzyme in differentiated neurons were measured; and the mRNA and protein level of GCase and SNCA were determined. The effect of GCase activity on lysosomal function, and the expression and aggregation of SNCA was then investigated.

This model has successfully mimicked specific biochemical features of GBA-associated PD and offers a unique opportunity to examine cell-type specific pathology in the vulnerable dopaminergic neurons derived from PD patients. These cells provide a suitable platform for drug screening for drugs that may target selected pathways in PD pathogenesis.

Finally, these data provide insight into the relationship between GCase, SNCA and lysosomal impairment, and helps to delineate the cellular function of GCase in the neuropathogenesis of synucleinopathies.

4.3.2 Method

Differentiation of adipose NCSC to dopaminergic neurons

Adipose NCSC were differentiated to functional DA neuronal cells using a fully defined step-wise differentiation protocol. Cells were collected by centrifugation and seeded at a density of 8000 cells per 35mm^2 dish coated with collagen (Sigma-Aldrich). On the next day, DMEM was changed to Neurobasal/B27 containing medium supplemented with 1% fungizone and 1% penicillin–streptomycin. For dopaminergic differentiation, I used a cocktail of sonic hedgehog (SHH), fibroblast growth factor 8 (FGF-8), and basic fibroblast growth factor (FGF-β) under a low serum condition for 10 days. On day 10, brain-derived neurotrophic factor (BDNF) was added to the differentiation medium. After 12 days, differentiated human adipose NCSC adopted a neuronal morphology. By day 40, the differentiated NCSC lines were characterised by expression of β-Tubulin III (a marker of neurons), tyrosine hydroxylase (TH) (a marker of dopaminergic neurons), and nuclear receptor related 1 protein (NURR1) (a marker of midbrain DA phenotype) (**Figure 7.14**). No significant differences in differentiation potential were observed among control, *GBA* mutation positive carrier, and PD lines. Immunostaining revealed the expression of β-Tubulin III proteins in the differentiated adipose NCSC (see **Figure 7.15**). Functional analysis demonstrated that 56% of cells responded to dopamine (**Figure 7.16**).

Lysosomal enzymatic activity assays

See section **4.2.2.**

Western blot analysis

Cells were harvested, washed with PBS, and processed as previously described (McNeill et al., 2014). Proteins were extracted using urea/SDS buffer (8M urea, 2% SDS, 10mM Tris) containing protease inhibitors on ice. In total, 40µg of the protein lysate was loaded on a 4–12% Bis-Tris Gel (NuPAGE, Invitrogen) and transferred to a polyvinylidene difluoride membrane (Millipore, Watford, England). The following antibodies were used: mouse anti-GBA (Calbiochem), rabbit anti-β-actin (Abcam), rabbit anti-LAMP1 (Abcam), rabbit anti-LAMP2a (Abcam), rabbit anti-Hsc70 (Abcam), rabbit anti-GAPDH (Abcam), rabbit anti-LC3 (Cell signaling), mouse anti-Synuclein (Abcam), rabbit anti-tublulin (Abcam), and mouse anti-p62 (BD Biosciences). Horseradish peroxidase goat anti-mouse (Dako), anti-rabbit (Dako) or anti-goat (Dako) secondary antibodies and Clarity Western ECL kits (BioRad) were used. Analyses were prepared using Image Lab software (BioRad). See Appendix **Table 9.4** for the full list of antibodies used. Full-length images of immunoblots are shown in Appendix **Figure 9.11**.

Autophagy studies

Where indicated, cells were treated with 0.2µM bafilomycin (EMD, Millipore) for 6 hours at 37 °C in the presence of 5% CO_2, and then fixed or lysed for western blot analysis. LC3/LC3-II levels were quantified by densitometry and normalized for β-actin. LC3 flux was quantified by dividing levels of LC3-II after bafilomycin treatment for 6 h by the level of LC3-II without treatment.

Immunocytochemistry

Cells were fixed in 4% paraformaldehyde for 10 min, rinsed with PBS, permeabilized in 0.25% triton x100 for 10 mins, rinsed with PBS again and blocked by 10% normal goat serum before incubation with primary and secondary antibodies. Anti-synuclein (Abcam) was used at a concentration of 1:500, anti-tubulin (Abcam) at 1:500, anti-Nurr1 (Abcam) at 1:200 and anti-tyrosine hydroxylase (Abcam) at 1:1000. Images were captured with a fluorescent microscope using a 40 × objective, at an excitation of 488nm (for Alexa Fluor Green) or 568nm (for Alexa Fluor Red). Individual cells were marked. See Appendix **Table 9.4** for the list of antibodies used.

Statistical analysis

Data are expressed as mean ± SEM and statistical significance between groups determined by one-way ANOVA followed by the two-tailed t-Test. A p value of < 0.05 was considered as significantly different. All data were analysed by GraphPad Prism 6 statistical software (GraphPad Software).

4.3.3 Results

To characterise the chosen model, preliminary experiments were performed at different stages of neuronal differentiation (0, 12, 20 and 40 days) in a control and a *GBA* mutant NCSC line. These measurements confirmed that adipose NCSC-derived neurons model many of the biochemical defects seen in the fibroblast cell model and agree with iPSC data obtained by other groups (Schöndorf et al., 2014; Woodard et al., 2014). Following these preliminary experiments, I went on to perform

the main measurements for all cell lines at differentiation day 40 in mature dopaminergic neurons.

GCase activity at different stages of neuronal differentiation

Control and heterozygous *GBA* mutation positive carrier NCSCs were differentiated to DA neurons for 12, 20, and 40 days. The GCase activity was measured at day 0, 12, 20 and 40. The GCase activity was reduced in heterozygous *GBA* mutation positive neurons compared with healthy control cells for all time points of neuronal differentiation (**Figure 7.17**).

GCase protein levels at different stages of neuronal differentiation

Quantitative analysis of intracellular GCase protein levels in cells of different patients by western-blotting revealed that protein levels of the enzyme were also reduced in heterozygous *GBA* mutation positive carriers compared with control individuals for all time points of neuronal differentiation (**Figure 7.18**).

SNCA levels at different stages of neuronal differentitation

The SNCA protein level was increased in heterozygous *GBA* mutation positive neurons compared with healthy control cells for all time points of neuronal differentiation (**Figure 7.19**). This increase was maximal at an early stage of neuronal differentiation (day 20) which could suggest a predisposition to PD.

Macroautophagy at different stages of neuronal differentiation

Quantitative analysis of LC3 protein levels in cells of different patients by western-blotting revealed that protein levels of LC3-II were markedly reduced in heterozygous *GBA* mutation positive carriers compared with control individuals for all time points of neuronal differentiation suggesting an impairment of macroautophagy (**Figure 7.20**).

Chaperone mediated autophagy at different stages of neuronal differentiation

Given the potential cross-talk among proteolytic systems and the evidence of potential macroautophagic compromise, I investigated chaperone mediated autophagy (CMA). Using lysosome-associated membrane protein type 2A (LAMP2a) receptor as a marker for the CMA pathway, a significant increase of LAMP2a protein associated with the presence of *GBA* mutation was seen in heterozygous *GBA* mutation carriers at 20 days neuronal differentiation (**Figure 7.21**). This suggests that CMA may act as an alternative way to compensate for the deficiency of macroautophagy in *GBA* mutant NCSC-derived neuronal cells. The levels of LAMP2a decreased significantly in *GBA* mutant stem cell-derived neuronal cells at 40 days indicating the chaperone-mediated autophagy pathway may be upregulated at an early stage of neuronal differentiation to compensate for impaired macroautophagy as a way to clear SNCA.

Reduced GCase in *GBA* mutant neurons

Control, heterozygous *GBA* mutation positive carrier, and IPD NCSCs were differentiated for 40 days and neuronal cultures were predominantly dopaminergic.

The GCase activity was significantly reduced in heterozygous *GBA* mutation positive neurons compared with healthy control cells (**Figure 7.22**). Importantly, control lines showed a significantly higher average of enzymatic activity compared with *GBA* positive lines. Quantitative analysis by western-blotting revealed that GBA protein levels were also reduced in heterozygous *GBA* mutation positive carriers compared with control individuals (**Figure 7.22**).

Increased α-synuclein levels in *GBA* mutant neurons

A direct link between GCase and SNCA accumulation has previously been shown (Manning-Boğ et al., 2009; Mazzulli et al., 2011; Sardi et al., 2011). Furthermore, it has been shown that heterozygous *GBA* mutations are linked to increased levels of SNCA. I sought to confirm the effects of *GBA* mutations on SNCA accumulation in differentiated NCSCs. NCSCs from controls, heterozygous *GBA* mutation positive carriers and PD subjects were differentiated for 40 days to DA neurons, and SNCA levels were measured by western blot. It is known that differences in expression levels of SNCA are influenced by individual genetic background, which could explain the high variability of SNCA expression in the general population (Fuchs et al., 2008). I measured SNCA levels in neuronal cultures from *GBA* mutation positive NCSC-derived neurons and corresponding *GBA* negative controls. A significant increase in SNCA protein levels was found in *GBA* mutation positive neurons carrying the N370S allele compared with controls (**Figure 7.23**). Immunofluorescent staining for SNCA revealed a significant increase in SNCA in *GBA* mutation positive NCSC-derived neurons compared with healthy individuals, suggesting an accumulation of SNCA (**Figure 7.23**). Reduced glucocerebrosidase protein was associated with increased SNCA protein levels in *GBA* mutant neurons,

while higher levels of glucocerebrosidase protein were associated with reduced SNCA protein levels in control cells. This data suggests the relationship between GCase and SNCA may be leveraged to reduce SNCA levels in PD by enhancing GCase levels and activity (**Figure 7.23**).

Lysosomal and autophagic defects in *GBA* mutant neurons

To extend these findings, I analysed the autophagic/lysosomal machinery in *GBA* mutant neurons differentiated for 40 days. To examine macroautophagy, I first investigated the autophagosome content by immunoblot for basal levels of light chain type 3 protein (LC3), a marker of autophagosomes. My analysis revealed a decrease, albeit not statistically significant, of LC3-II in *GBA* mutation positive carrier neurons compared with control neurons (**Figure 7.24**). These results were confirmed on further immunoblotting, which showed a coexistent decrease in basal levels of p62 in heterozygous *GBA* mutation positive carrier neurons (**Figure 7.24**). Bafilomycin treatment revealed that LC3-II was significantly increased in *GBA* mutation positive carrier neurons compared to control cells (**Figure 7.25**). This suggested no impairment of autophagosome-lysosome fusion.

Lysosomal content is unaltered in *GBA* mutant neurons

Immunoblot staining for LAMP1, a lysosomal marker, revealed no significant change in LAMP1 protein in patient NCSC-derived neurons compared with healthy individuals, suggesting no significant change of lysosomal content (**Figure 7.26**). To examine chaperone mediated autophagy (CMA), I performed immunostaining for LAMP2a, a marker of CMA. My analysis revealed no significant change in basal

levels of LAMP2a in *GBA* mutation positive carrier neurons when compared to control cells (**Figure 7.26**).

4.3.4 Discussion

In this study, human adipose NCSC were isolated from the subcutaneous fat collected from wild type (n =3), heterozygous *GBA* mutation positive (N370S/wt) carrier (n = 3) and PD patients (n = 3), and differentiated into functional human dopaminergic (DA) neurons. N370S point mutations are the most frequent GD-causing alleles that have been linked to PD (Sidransky et al., 2009b). NCSCs were efficiently differentiated to DA neurons without significant differences.

The neuronal model used in this study recapitulates many of the defects I identified in the fibroblast model (section 4.2.3), as well as those observed in the human brain (Gegg et al., 2012; Murphy et al., 2014b), including reductions in GCase enzyme activity and protein level, and lysosomal abnormalities including impairments of lysosomal autophagy. In addition, elevated levels of SNCA have been seen in adipose NCSC-derived neuronal cultures from *GBA* N370S patients. This supports my hypothesis that abnormalities of relevance to PD pathogenesis expressed in human fibroblasts are also seen in dopaminergic neurons derived from their adipose-derived NCSC and secondly, reduced GCase is associated with increased SNCA.

PD patients carrying *GBA* mutations show classic PD pathology with widespread SNCA inclusions (Velayati et al., 2010). The exact mechanism is unknown, and both gain of function and loss of function hypotheses have been put forward. It has been suggested that a relationship could exist between the two proteins and that *GBA* mutations could contribute to SNCA aggregation through a

direct interaction. In support of such a gain of function hypothesis is the presence of mutant GBA in SNCA-positive LBs, as demonstrated by immunohistochemistry studies (Goker-Alpan et al., 2010). In support for a loss of function hypothesis, a deficiency of GCase could lead to an accumulation of substrate, which could in turn influence the amyloid formation of SNCA, and the resulting toxic build-up of insoluble SNCA within lysosomes could in turn compromise lysosomal protein degradation, and further impair the trafficking of GCase, exacerbating substrate accumulation and SNCA aggregation (Mazzulli et al., 2011).

The link between GCase and SNCA was first demonstrated in vitro by the overexpression of mutant *GBA*, whereupon mutant forms increased SNCA levels (Cullen et al., 2011). GCase deficiency, overexpression of mutant *GBA*, or inhibition of GCase by condruitol β-epoxide (CβE) can all interfere with the clearance of or promote the aggregation of SNCA (Manning-Boğ et al., 2009; Cullen et al., 2011; Mazzulli et al., 2011; Xu et al., 2011; Gegg et al., 2012; Cleeter et al., 2013; Osellame et al., 2013). Although, short-term exposure to CβE does not increase SNCA levels in rat cortical neurons (Dermentzaki et al., 2013). In contrast, the overexpression of SNCA leads to a reduction in GCase activity in SH-SY5Y cells (Gegg et al., 2012).

There appears to be an inverse relationship between LB density load and GCase activity in PD brain (Murphy et al., 2014b). In *GBA* deficient mice (D409V/D409V genotype), increasing GCase activity, through viral mediated expression of *GBA*, corrected the accumulation of SNCA in the brain (Sardi et al., 2011). In addition, SNCA knockout mice demonstrate increases in GCase activity, and heterozygosity

for a GD-associated mutation in *GBA* interferes with SNCA degradation and contributes to its accumulation, exacerbating the phenotype in a mouse model of PD.

I utilized NCSC-derived neurons carrying heterozygous *GBA* mutations and showed that *GBA* mutations are directly linked to an increase of SNCA levels. This model confirms reports from other studies in pluripotent stem cells. The functional loss of GCase in human iPS neurons compromises lysosomal protein degradation, and causes the accumulation of SNCA (Mazzulli et al., 2011). Cell types derived from GD human iPSC can also effectively recapitulate pathologic hallmarks of the disease in terms of reduced GCase activity, impaired lysosomal function and increased sphingolipids (Panicker et al., 2014). In monozygotic twins harbouring the heterozygous *GBA* mutation N370S but clinically discordant for PD, iPSC-derived DA neurons from both twins had ~50% GCase enzymatic activity, ~3-fold elevated SNCA protein levels, and a reduced capacity to synthesize and release dopamine. Interestingly, the affected twin's neurons showed an even lower dopamine level, increased monoamine oxidase B (MAO-B) expression, and impaired intrinsic network activity. Overexpression of wild type *GBA* and treatment with MAO-B inhibitors normalized SNCA and dopamine levels, suggesting a combination therapy for the affected twin. In another iPS model, abnormal calcium homeostasis and lysosomal dysfunction were identified in cells derived from *GBA* mutant individuals and strengthens the evidence for a link between *GBA* mutations and complex changes in the autophagic/lysosomal system, which could underlie vulnerability to neurodegeneration (Schöndorf et al., 2014). In my study, I did not investigate levels of glucosylceramide, the substrate of GCase, and it is interesting to note an increase of glucosylceramide in iPSC-derived neurons from PD patients carrying

heterozygous *GBA* mutations, which in turn may influence SNCA levels and aggregation.

The interplay between GCase and SNCA has yet to be fully understood in terms of the molecular and biochemical mechanisms involved. Some suggest that GCase interacts directly with SNCA at membranes, within lysosomes (Yap et al., 2013). Others propose a disturbance of GCase trafficking (Cooper et al., 2006; Thayanidhi et al., 2010).

My data shows that *GBA* mutations are linked to defects in the autophagic/lysosomal system. I found reductions in the basal levels of LC3-II but no impairment of the autophagic flux in response to bafilomycin. These data are consistent with the results in fibroblast cells and suggest a deficit in autophagosome number but no impairment of the fusion between autophagosomes and lysosomes. This impairment of macroautophagy may explain the increased levels of SNCA seen in *GBA* mutant neurons. The decrement in lysosomal activity in NCSC-derived neurons was accompanied by a reduction in the enzymatic activity of GCase in *GBA* mutation positive patients compared with control individuals. Parkinsonism has been described in many lysosomal storage diseases, including GD and Anderson-Fabry disease. This suggests that impairment of the lysosome could correlate with early neurodegenerative processes. In this context, *GBA* mutations may alter the lysosomal function through one of several pathways. The loss of GCase could have a secondary effect on lysosomal function and lead to SNCA accumulation (Manning-Boğ et al., 2009; Mazzulli et al., 2011). In addition, an accumulation of lipid substrate could influence lysosome formation as well as SNCA aggregation (Jo et al., 2000; Koga et al., 2010). In contrast, increased levels of SNCA may cause lysosomal

dysfunction in *GBA* mutant neurons via a positive feedback loop (Mazzulli et al., 2011). It has been demonstrated that SNCA overexpression impairs macroautophagy via Rab1a inhibition in mammalian cells and in transgenic mice, and Rab1a overexpression rescues the autophagy defect caused by SNCA (Winslow et al., 2010).

In view of the crosstalk between macroautophagy and CMA (Schneider and Cuervo, 2014b), I looked for a measurable change in CMA in the neuronal model. CMA serves as a backup mechanism for the removal of malfunctioning proteins (i.e. aberrant SNCA) when macroautophagy is compromised, and vice versa (Wu et al., 2014). In fibroblasts, the selective loss of lysosomal GCase was directly related to reduced lysosomal CMA. In mature NCSC-derived DA neurons at differentiation day 40, levels of LAMP2a were unchanged. Perhaps this does suggest some adaptations in the neuronal model, in the presence of SNCA, where levels of LAMP2a may initially increase in order to compensate for a reduction in macroautophagy. This was certainly seen during the early stages of neuronal differentiation, where levels of LAMP2a were significantly higher in *GBA* mutant neurons at differentiation day 20. This compensatory increase, however, being insufficient to overcome the SNCA accumulation observed in *GBA* heterozygotes, LAMP2a levels may return to the basal level by differentiation day 40.

In summary my studies show that heterozygous *GBA* mutation positive NCSC-derived neurons have increased levels of SNCA as well as complex changes in the autophagic/lysosomal system, which may underlie the selective vulnerability of DA neurons to degeneration in PD. Taken together, my results suggest possible targets for the development of disease-modifying drugs for patients with familial and

idiopathic PD. My results lay support to intended strategies to influence lysosomal function and SNCA deposition in PD, as a mechanism of neuroprotection (Schapira et al., 2014).

Chapter 5: Pharmacological Chaperones to Obtain the Maximal Enhancement of GCase Function

5.1 Pharmacological chaperones for GCase

The relationship between GD and PD is not yet fully understood. It could arise from the gain of a new toxic function by mutated *GBA* (Gregersen N et al., 2006), a loss of enzyme function (Thompson AJ et al., 2002), or a combination of both. Approximately 203/300 (~70%) *GBA* mutations are missense, which result in the synthesis of GCase, but with decreased catalytic function and stability. Missense mutations in *GBA* cause GCase to fold abnormally. This results in a misfolded protein which becomes retained in the endoplasmic reticulum (ER) and after unsuccessful attempts at refolding, finally undergoes degradation by the ER-associated degradation (ERAD) system. This reduces the GCase reaching the lysosome, and could have consequences for lysosomal function, by overwhelming the ubiquitin-proteasome pathway (UPS) or by impairment of autophagy. Alternatively, misfolded GCase could lead to the development of PD directly by promoting SNCA accumulation or preventing its degradation. Parkinsonism could also arise from a deficiency/reduction of wild-type (WT) GCase resulting from a loss-of-function mutation, with subsequent substrate accumulation and altered lipid homeostasis.

Recent data has placed the spotlight on pharmacological chaperones (PC) as an emerging therapeutic strategy for GD (Bendikov-Bar et al., 2013), but also their potential use in PD (McNeill et al., 2014). PC are small molecules that are able to bind mutant misfolded GCase stalled in the ER and enhance its folding, stability and trafficking to the lysosome, where the residual activity of the enzyme can hydrolyse its substrate. This sequence of events has been termed chaperoning. The mechanism of action for any chaperone compound is competitive binding to the active site of the mutant enzyme.

GBA mutations cause a reduction in enzyme activity, but this may not be the mechanism that mediates the pathogenesis of GD. Protein misfolding, mis-trafficking of GCase and endoplasmic reticulum stress are considered some of the mechanisms contributing to the pathophysiology of GD. Evidence to support protein misfolding as a putative cause of GD is the observation that common *GBA* mutations are processed by endoplasmic-reticulum-associated degradation. In PD, the abnormal accumulation of SNCA and other ubiquitinated proteins in LBs has also implicated protein mishandling as a cause.

The N370S missense mutation of the *GBA* gene is the most prevalent mutation causing GD, after the L444P mutation. For mutant GCase, including N370S, PC are being pursued as potential small-molecule '*enzyme enhancement*' therapeutics. These active-site-specific inhibitors have been proposed to 'rescue' a proportion of the newly synthesized mutant enzyme when present at sub-inhibitory concentrations, promoting their maturation and lysosomal trafficking (Sawkar et al., 2002, 2005; Fan, 2008).

PC therapy has been proposed for lysosomal storage disorders, including GD, Anderson-Fabry, and Tay–Sachs diseases. Current treatment for patients with GD include enzyme replacement therapy (ERT). Although ERT is highly effective in reversing the visceral and hematologic manifestations, skeletal disease is slow to respond, pulmonary involvement is relatively resistant, and ERT does not cross the blood brain barrier and therefore, cannot treat the neurological manifestations of GD. PC therapy is an attractive approach because of its potential for more uniform tissue distribution and its ability to penetrate the blood–brain barrier.

The effect of PC is to some degree mutation specific, and can also be affected by the cellular environment, for instance upon the individual genetic background. Thus, PC may not be suitable for every patient with GD or PD, even among those with the same genotypes.

The first molecule identified as a potential chaperone for mutant GCase was N- (n-Nonyl) 1-deoxynojirimycin (NN-DNJ), a member of the iminosugar family, naturally occurring glycosidase inhibitors (Sawkar et al., 2002). The majority of PC are enzyme inhibitors and can be classed as iminosugars or similar analogs of the natural substrate, glucosylceramide. Iminosugars, including isofagomine (IFG), are potent competitive inhibitors that bind to the active site of GCase with high affinity at the neutral pH of the endoplasmic reticulum, where GCase has decreased stability, and with low affinity in the acidic conditions of the lysosome where the chaperone-enzyme complex dissociates.

In a simple yet reliable enzyme assay using capillary electrophoresis for inhibitor screening of five putative PC (IFG, ABX, bromhexine, diltiazem, and fluphenazine) that target the lysosomal enzyme GCase, IFG was confirmed as a potent competitive

inhibitor of recombinant GCase. In contrast, the four other non-carbohydrate amines were demonstrated to function as mixed-type inhibitors with high micromolar activity at neutral pH relative to acidic pH conditions reflective of the lysosome (Shanmuganathan and Britz-McKibbin, 2011).

There is extensive research on the use of PC to enhance lysosomal GCase activity, and the screening of chemical libraries in order to identify novel chaperone molecules with improved efficiency and efficacy. Ambroxol (ABX), a drug used to treat airway mucus hypersecretion and hyaline membrane disease in humans, was isolated in a screen of a library of 1,040 Food and Drug Administration (FDA)-approved drugs from the chemical library of the National Institute for Neurological Diseases and Stroke (NINDS) (Maegawa et al., 2009). Several researchers have demonstrated the effectiveness of chemical chaperone treatment in GD e.g. Nojirimycin analogus, iminosugars etc. However, these chaperones have not got FDA approval for treating GD.

ABX, traditionally used in humans as an oral over-the-counter cough medicine (pills, syrup, or caplets), has been used for more than three decades in many countries as a mucolytic agent. ABX, a mixed-type inhibitor of GCase (Maegawa et al., 2009), has been shown to interact with both the active and non-active sites of GCase in a pH-dependent manner. It has a theoretical advantage over other iminosugars because it does not require wash-out periods, which enables continued dosing. As a mixed inhibitor, ABX has a similar mode of action to an enzyme enhancement therapy agent. It has been shown to enhance the activity of several mutant GCase variants (Bendikov-Bar et al., 2013). Moreover, enhancement of GCase activity is selective and activities of other lysosomal enzymes are not affected.

In an experiment to examine the “chaperoning” half-life of ABX, GD fibroblasts were grown in medium containing 60μm ABX or DMSO (mock-treated) for 5 days (Maegawa et al., 2009). The cells were then “chased” for 8 days by replacing the ABX/DMSO-containing media with fresh media lacking the compounds and thereafter every other day. The persistence of GCase enhancement produced by ABX after drug removal in N370S/N370S fibroblasts was then measured and chased over a period of 8 days. This “pulse-chase” experiment was helpful in showing that the enhanced N370S GCase activity gradually decreases back to baseline levels within approximately 6 days (Maegawa et al., 2009). This suggests a reasonably long half-life of ABX in cells. It should also be noted that wild type GCase in skin fibroblasts has been reported to have a half-life of approximately 60 hours.

There are major advantages of using ABX and IFG as PC in this study. Both have been shown to significantly increase GCase activity in human fibroblasts (Sun et al., 2012; Bendikov-Bar et al., 2013). Studies in fibroblasts derived from homozygous and heterozygous *GBA* mutation carriers, revealed the expected range of reduction in GCase activity: severe in GD patients, intermediate in heterozygous carriers and unaffected in PD patients without mutations (McNeill et al., 2014). After 5 days treatment with the GCase chaperone ABX, there was a significant increase in GCase activity in GD patients, although in absolute terms this was small. There was a further significant increase of GCase activity and protein levels in carriers. Of particular interest was the increase in GCase activity seen in PD patient cells without mutations. This effect was also seen in controls. IFG has also been shown to increase residual GCase activity in fibroblasts originating from type 1 GD patients (Chang et al., 2006; Steet et al., 2006) and also in mice homozygous for *GBA* mutations (Sun et al., 2012).

ABX has a long history of use in humans, which documents its very low level of toxicity. This is in contrast to most other small molecules, identified as chaperones for GCase, but which have never been tested in humans. In the first pilot study of ABX in GD, Zimran et al. gave ambroxol to 12 subjects with GD, naïve to therapy, with symptoms of moderate severity, for a duration of 6 months (Zimran et al., 2013). This pilot investigation was conducted to assess the safety, tolerability and efficacy of ABX in patients with type 1 GD, in order to provide proof of concept and/or ascertain suitability of ABX for a larger clinical trial. There were no reports of adverse events in this 6 month study (Zimran et al., 2013). Some of the shortcomings of this pilot study were the small sample size, lack of data on different dosing regimens, pharmacologic distribution and bioavailability of ABX. However, there were clinically significant improvements. In 25% of subjects (3/12), there was a 20% decrease in spleen volume and a 50% decrease in the activity of chitotriosidase that persisted for more than 6 months. Albeit, these improvements were seen in the 2 thinnest patients suggesting the need for higher concentrations. Isofagomine has also been used orally in a phase 2 clinical trial of 6 months duration (Amicus Therapeutics). Only 1 patient showed clinically meaningful improvement. Again, the optimal concentration, regimen and treatment duration still needs to be determined.

Some of the advantages of ABX are its simple oral administration, the fact that it is readily available, and its low cost. However, these same properties may hinder ABX from being tested by a sponsoring drug company as an orphan drug through costly trials if there is not a sufficient benefit/cost margin.

PC are hydrophobic in nature and of low molecular mass and therefore, it was proposed that these small molecules would be able to cross the blood brain barrier. The oral administration of ABX and IFG in GD mice has confirmed their wide distribution and chaperone activity in tissues, including the brain, and lack of acute toxicity (Sun et al., 2011; Luan et al., 2013). In one such mouse model, IFG delayed the onset of neurological disease and extended the life span of mice (Sun et al., 2011). Furthermore, ABX was recently shown to decrease ER stress and recover morphology in *Drosophila eye,* in which expression of *GBA* generated neurodevelopmental defects and ER stress (Suzuki et al., 2013). However, the response to ABX does appear to be patient dependent, and this could be due to individual differences in ER quality control machinery.

There are other ways to modulate GCase activity e.g. with the use of histone deactylase inhibitors, to reduce recognition of misfolded GCase by chaperone Hsp90β, decrease degradation, and increase residual enzyme levels. This strategy has been validated in GD patient fibroblasts. Another approach is to increase GCase expression e.g. using gene therapy. However, there is a vast amount of publications on the use of small molecular chaperones to enhance lysosomal GCase activity and the main advantages for choosing ABX and IFG as PC is that they are safe and well tolerated, binding to GCase is highly pH dependent, both are readily available, both have been shown to significantly increase GCase activity in human fibroblasts, and in a mouse model, IFG delayed onset of neurological disease and extended their life span demonstrating its potential to exhibit a similar effect in humans.

The GCase–lysosomal–SNCA pathway has become a new target for drug intervention. Improving the trafficking of mutant GCase by PC will reduce ER-associated degradation (ERAD), and increase the lysosomal localisation but also the expression of GCase. This latter effect could be relevant to those PD patients without *GBA* mutations. By improving the lysosomal localisation or expression of GCase, this could have a beneficial effect by improving lysosomal function which will increase the turnover of SNCA by autophagy, thereby reducing its propensity to aggregate. It is thought now that enhancing lysosomal function and reducing SNCA levels will reduce the release and spread of SNCA and its related pathology, and that importantly, these effects will not be restricted to dopaminergic neurons, an essential feature of a successful neuroprotective agent (Schapira, 2015).

5.2 The optimal concentration and duration of chaperone treatment for maximal enhancement of GCase activity

OBJECTIVE

To investigate the optimal concentration and duration of drug treatment (in days) for maximal enhancement of GCase activity in a type 1 GD (N370S/N370S) and a control fibroblast line.

5.2.1 Introduction

Both human skin fibroblasts and human adipose NCSC were developed from skin biopsies obtained from patients within the clinical cohort at high risk for PD. The fibroblast cell model was used in the first instance to investigate the potential for PC treatment to restore GCase function.

Experiments were performed to identify the optimal concentration and duration of drug treatment (in days) for maximal enhancement of GCase activity in a type 1 GD (N370S/N370S) and a control fibroblast line. The concentrations used for ABX and IFG were guided by the literature.

It was important to study the influence of PC treatment in a control line because of the potential benefit for PD patients negative for the *GBA* mutation. Reductions in GCase have been noted in sporadic PD brain (Gegg et al., 2012). Moreover, the results from animal and cellular studies demonstrate that knockdown or inhibition of GCase results in the accumulation of SNCA and furthermore, augmenting GCase activity can reduce levels of SNCA (Mazzulli et al., 2011). Therefore, the use of PC

to restore GCase function, could lead to a reduction in SNCA accumulation. This could be significant for those with GBA-PD, asymptomatic *GBA* positive carriers at risk of conversion to clinical PD, or even those with sporadic PD.

5.2.2 Method

Cell lines

Human primary skin fibroblast cultures that contained the wild type (WT) and the homozygous N370S/N370S *GBA* mutation were obtained from the clinical study cohort. Preliminary analysis of fibroblast cultures following treatment with ABX has previously demonstrated a 4-fold increase in GCase activity measured in the N370S/N370S GD line, and a steady-state GBA protein level ~50% of the WT. The cells were grown in the standard conditions: DMEM supplemented with 10% FBS, at 37 °C in the presence of 5% CO_2, with medium changed every 2-3 days. Cultures were not left to become confluent. Passage numbers were limited from 8 to 12.

Drug treatment

Sub-confluent human skin fibroblast cultures were treated with selected concentrations of ABX (A9797, Sigma-Aldrich) and IFG (Santa Cruz) for an increasing number of days. ABX was dissolved in dimethylsulphoxide (DMSO) and diluted in cell culture media to give final concentrations of 3.3μM, 10μM and 60μM. IFG (water soluble) was diluted in cell culture media to give final concentrations of 10μM and 50μM. Media was changed alternate daily for PC treated and control lines. Controls were treated with DMSO or media only. At day 10, the cells were washed

twice with phosphate–buffered saline (PBS) prior to harvest, to collect as cell pellets that were frozen in -80˚C for storage.

Lysosomal enzymatic activity

See section **4.2.2.**

5.2.3 Results

I evaluated the effect of ABX and IFG treatment on residual GCase activity. Treatment of skin fibroblast cultures that originated from a control and a type 1 GD patient, with selected concentrations of ABX over 10 days, resulted in a 2-fold increase in activity of the control line and a 4-fold increase in activity of the mutant line with 60μM ABX at 6 days. Concentrations of IFG of 10μM in the media enhanced GCase activities significantly in the wild type at 6, 8 and 10 days as shown in **Figure 7.27**, but did not enhance GCase activity significantly in the mutant line for any time point. In comparison, treatment with 50μM IFG resulted in a 2-fold increase in activity of the control line and a 3-fold increase in activity of the mutant line at 6 days; these enhanced activities were ~5% of untreated WT levels. The concentrations to achieve peak GCase activity in the mutant line for ABX and IFG were 60μM and 50μM respectively. The duration of treatment to achieve peak GCase activity was 6 days for ABX and IFG. It is worth noting that treatment of skin fibroblast cultures that originated from a control and a type 1 GD patient, with increasing concentrations of ABX and IFG resulted in no significant change of

lysosomal hexosaminidase or β–galactosidase activity, for any given concentration, and at all time points studied as shown in **Figure 7.28** and **Figure 7.29**.

5.2.4 Discussion

The results presented here confirm previous reports (Sun et al., 2012; Bendikov-Bar et al., 2013) demonstrating the ability of small molecular chaperones to enhance GCase enzymatic activity. The data is new in providing additional information on the optimum concentration and duration of treatment that would be recommended for the maximal enhancement of GCase function. The data illustrates that the effect of the two drugs for enhancing GCase activity in both the WT and the GD cell line is comparable. Furthermore, the PC molecules tested appear selective for enhancing GCase function. It is interesting to note that although GCase activity is enhanced up to 4-fold in the mutant cell line, it remains at ~5% of the WT level. Despite this, this small enhancement may be sufficient to produce significant physiological effects.

Current treatment for patients with GD include enzyme replacement therapy (ERT), but this does not cross into the brain and is very expensive, and alternatives such as substrate reduction therapy (SRT) and the use of small molecule chaperone therapy. The latter has the added benefit of crossing the blood-brain barrier, providing an opportunity to treat additional features of the disease e.g. the neurological manifestations of GD. Enhancing GCase activity using PC and improving lysosomal function therefore holds particular relevance for those with GD, but could also be significant for those with PD where the impairment of lysosomal pathways is increasingly viewed as a major pathogenic event (Dehay et al., 2013) and the ability

to modulate GCase activity has important therapeutic implications (Schapira and Gegg, 2013).

My results on the skin fibroblast cultures reproduce the GCase enhancement seen from previous studies following treatment with chaperone molecules. Good progress has been made to determine the optimal concentration and duration of drug treatment to achieve the maximal increase in GCase activity in both a representative control and a type 1 GD (N370S/N370S) cell line. However, full restoration of GCase has not been obtained from the mutant *GBA* line, when compared to the level found in the control cell line.

5.3 The efficacy of pharmacological chaperone treatment in restoring GCase function, and reversing the underlying molecular events

OBJECTIVE

To investigate whether the small molecular chaperones, ABX and IFG, restore GCase activity and reduce SNCA levels, in human cell models.

5.3.1 Introduction

I aim to demonstrate that the PC ABX and IFG are able to modulate glucocerebrosidase function in fibroblasts. It is unclear whether treatment alone may be able to overcome all biochemical defects due to mutations in *GBA*, due to the limitation of fibroblasts as a model for interrogating the complete pathway in PD. The ability of ABX to increase GCase activity in fibroblast cells and to reduce SNCA levels in ectopically expressing SNCA SH-SY5Y cells first highlighted the potential benefit of manipulating the lysosomal-GCase pathway to lower SNCA levels (McNeill et al., 2014). Due to the artificial nature of the overexpressing SH-SY5Y cell model, I felt it was important to repeat measurements in human dopaminergic neurons, which in the presence of SNCA would better recapitulate what is happening in the brain.

5.3.2 Method

See sections 4.2.2 and 4.3.2.

Drug lots

To evaluate the effect of ABX and IFG from different suppliers, I cultured control and mutant (N370S/N370S) fibroblasts in the presence of 60μM ABX from Sigma Aldrich or LKT Laboratories, and 50μM IFG from Santa Cruz Biotechnology or Amicus Therapeutics. The results demonstrated that GCase activity was maximal in both a control and mutant line for ABX from Sigma Aldrich, while GCase activity was maximal in a mutant line for IFG from Santa Cruz Biotechnology. See Appendix **Figure 9.13** and **Figure 9.14**. I therefore used ABX supplied by Sigma Aldrich (A9797) and IFG supplied by Santa Cruz Biotechnology for all of my experiments.

Ambroxol and isofagomine treatment of human fibroblasts

Sub-confluent human skin fibroblast cultures were treated with pre-determined concentrations of ABX (A9797, Sigma-Aldrich) and IFG (Santa Cruz) alternate daily for 6 days. ABX was dissolved in dimethylsulphoxide (DMSO) and diluted in cell culture media to give a final concentration of 60μM. IFG (water soluble) was diluted in cell culture media to give a final concentration of 50μM. Media was changed alternate daily for PC treated and control lines. Controls were treated with DMSO or media only. At day 6, the cells were washed twice with phosphate–buffered saline (PBS) prior to harvest, to collect as cell pellets that were frozen in -80˚C for storage.

Ambroxol treatment of human adipose NCSC-derived neurons

Adipose NCSC-derived neuronal cultures were treated at day 40 of neuronal differentiation with the pre-determined concentration of ABX (A9797, Sigma-Aldrich) alternate daily for 6 days. ABX was dissolved in dimethylsulphoxide (DMSO) and

diluted in cell culture media to give a final concentration of 60μM. Media was changed alternate daily for PC treated and control lines. Controls were treated with media only. At day 6, the cells were washed twice with phosphate–buffered saline (PBS) prior to harvest, to collect as cell pellets that were frozen in -80˚C for storage.

5.3.3 Results

Pharmacological chaperones enhance enzymatic activity and lysosomal localization of GCase in *GBA* mutant fibroblasts

Treatment of skin fibroblasts that originated from heterozygous *GBA* mutation carriers, IPD patients, and healthy controls with ABX or IFG resulted in 50–100% increases in the activity of the mutant protein in all tested lines (**Figure 7.30**). The data illustrate that the effect of PC for enhancing GCase activity in all cell lines is comparable. Furthermore, the PC molecules tested appear selective for enhancing *GBA* function (contribution from non-lysosomal GCase (*GBA2*) was distinguished by using the corresponding inhibitor conduritol-β-epoxide (CβE) (see Appendix **Figure 9.6** and **Figure 9.7**). PC treatment also resulted in a significant elevation of GCase protein and messenger RNA levels (**Figure 7.30**) in heterozygous *GBA* mutation carriers, IPD patients, and healthy controls. The greatest effect on GCase activity, protein and mRNA levels was seen with the drug ABX. To evaluate the effects of PC on GBA intracellular trafficking, fibroblasts were treated with ABX and IFG. ABX treatment increased GBA protein trafficking into the lysosome and resulted in partial GBA/cathepsin D co-localization. I observed an increased degree of co-localization between GBA and cathepsin D in all cell lines following ABX treatment. For heterozygous *GBA* carriers, the effect of ABX was similar to the untreated WT. IFG

treatment did not significantly increase the degree of co-localization in all cell lines (**Figure 7.31**).

Pharmacological chaperones expand the lysosomal compartment in *GBA* mutant fibroblasts

Treatment of skin fibroblasts that originated from heterozygous *GBA* mutation carriers, IPD patients, and healthy controls with pre-determined concentrations of ABX or IFG resulted in no significant change of lysosomal hexosaminidase or β–galactosidase activity, for any given drug, as shown in **Figure 7.32**. These data suggest PC are selective for enhancing the activity of the lysosomal enzyme, GCase. In addition, immunoblotting for lysosomal marker, LAMP1, revealed a significant increase in LAMP1 protein following ABX treatment in heterozygous *GBA* mutation carrier, IPD patients and controls (**Figure 7.32**). This suggests a general increase in lysosomal mass and is consistent with previous reports (McNeill et al., 2014). Taken together, these data illustrate that ABX may cause an overall expansion of the lysosomal compartment.

Ambroxol is selected for the treatment of *GBA* mutant neurons.

The testing of 2 different PC molecules in human fibroblast cells demonstrated that ABX produced the greatest enhancement of GCase activity and showed the most potential for reversing the underlying molecular events relevant to the *GBA* mutation. ABX was the most effective PC for enhancing the enzymatic activity and lysosomal localization of GBA in all cell types. Due to the superior efficacy of ABX over IFG, and the limited neuronal material available as a result of the cost and time to differentiate NCSC to DA neurons, neuronal cultures were treated with ABX only.

Ambroxol enhances GCase enzyme activity in *GBA* mutant neurons

Control, heterozygous GBA mutation positive carrier, and IPD NCSCs were differentiated for 40 days and neuronal cultures were predominantly dopaminergic. The GCase activity was significantly reduced in heterozygous GBA mutation positive neurons compared with healthy control cells (**Figure 7.22**). Importantly, control lines showed a significantly higher average of enzymatic activity compared with GBA positive lines. Treatment of NCSC-derived neuronal cultures that originated from heterozygous GBA mutation carriers, IPD patients, and healthy controls with 60µM ABX resulted in significant increases of GCase activity in all tested lines (**Figure 7.33**). The data illustrate that the effect of ABX for enhancing GCase activity was greatest in GBA mutation positive carrier and PD cell lines.

Ambroxol increases GCase protein levels in *GBA* mutant neurons

Analysis of intracellular GCase protein levels in cells of different patients by western-blotting revealed that protein levels of the enzyme were reduced in heterozygous *GBA* mutation positive carriers compared with control individuals (**Figure 7.22**). ABX treatment resulted in a significant increase of GBA protein (**Figure 7.34**) in heterozygous *GBA* mutation carriers and IPD patients.

Ambroxol reduces SNCA levels in *GBA* mutant neurons

A significant increase in SNCA protein levels was found in *GBA* mutation positive neurons carrying the N370S allele compared with controls (**Figure 7.23**). Immunofluorescent staining for SNCA revealed a significant increase in SNCA in *GBA* mutation positive NCSC-derived neurons compared with healthy individuals,

suggesting an accumulation of SNCA (**Figure 7.23**). This data suggested the relationship between GCase and SNCA could be manipulated to reduce SNCA levels in PD by enhancing GCase levels and activity. Following 6 days of ABX treatment, I measured SNCA levels in neuronal cultures from *GBA* mutation positive NCSC-derived neurons, IPD and *GBA* negative controls. Treatment with ABX led to a significant reduction in SNCA protein levels in *GBA* mutation positive and PD neurons (**Figure 7.35**).

Ambroxol upregulates macroautophagy in *GBA* mutant neurons

Analysis of the autophagic/lysosomal machinery in *GBA* mutant neurons revealed that basal levels of light chain type 3 protein (LC3), a marker of autophagosomes, were reduced in heterozygous *GBA* mutation positive carriers compared with control individuals (**Figure 7.24**). Bafilomycin treatment revealed that the autophagic flux was not significantly reduced in *GBA* mutation positive carrier neurons compared to control cells (**Figure 7.25**) suggesting no impairment of autophagosome-lysosome fusion. Following 6 days of ABX treatment, I measured LC3 and p62 levels in neuronal cultures from *GBA* mutation positive NCSC-derived neurons, IPD and *GBA* negative controls. Treatment with ABX led to a significant increase in both LC3-II and p62 protein levels in *GBA* mutation positive and PD neurons (**Figure 7.36**).

Reducing the concentration of ambroxol produces the same key biochemical effects in *GBA* mutant neurons

Control, heterozygous *GBA* mutation positive carrier, and IPD NCSCs were differentiated for 40 days. I have previously shown that 10μM ABX can produce a 3-

fold increase in GCase activity in a mutant fibroblast cell line (**Figure 7.27**). Treatment of NCSC-derived neuronal cultures that originated from heterozygous *GBA* mutation carriers, IPD patients, and healthy controls with selected concentrations of ABX over 6 days, resulted in a ~1.5 fold increase in GCase activity with 10μM ABX and ~2-fold increase in activity of all cell lines with 30μM ABX (**Figure 7.37**). This corresponded with a significant increase in levels of GBA protein in *GBA* mutation positive NCSC-derived neurons (**Figure 7.38**). To determine whether the increase of GCase activity and protein was sufficient to reduce SNCA accumulation in *GBA* mutant cells, I measured levels of SNCA in neuronal cultures from *GBA* mutation positive NCSC-derived neurons. Both 10μM and 30μM ABX led to significant reductions in SNCA protein levels (**Figure 7.39**).

5.3.4 Discussion

Fibroblast cultures derived from human skin biopsies provide an opportunity to study the contribution of mutations in *GBA* in PD as a cell model. I hypothesized that the biochemical defects present in the GBA-PD brain would also be expressed in the fibroblasts (Gegg et al., 2012). My data suggest they do express the same biochemical defects, for example reduced GCase activity. They are also a useful tool to test drugs in a high-throughput setting. The results presented here confirm previous reports (Sun et al., 2012; Bendikov-Bar et al., 2013; McNeill et al., 2014) demonstrating the ability of small molecular chaperones to enhance the enzymatic activity, protein and expression of GCase. The data is new in providing a more extensive report on the effects of ABX and an additional chaperone molecule, IFG, on the upregulation of GCase as a novel therapeutic strategy for modulating lysosomal function in human disease. The data is also new in testing PC in patient-

derived dopaminergic neurons, as a model for their potential therapeutic effects in the brain.

I have demonstrated that ABX and IFG improve GCase activity and protein levels in *GBA* mutation fibroblasts, as well as in PD and control fibroblasts. The greatest increase in GCase activity was upon ABX treatment. This could be because ABX is a mixed-type inhibitor, while IFG is a potent inhibitor of recombinant GCase. Although a PC is supposed to dissociate in the acidic pH of the lysosome, some researchers recommend that media containing IFG is removed and replaced with media alone to help remove any residual inhibition in the cells (a 'washout') (Khanna et al., 2010). This is not ideal for a drug given to patients, where a washout would not be practical. My data shows that ABX increases the trafficking of GBA to the lysosome. While IFG increases the overall GCase activity, there is no significant improvement in the fraction co-localized with the lysosome. It could be that IFG is an activator of GCase and improves the stability of the enzyme, but does not increase the trafficking of the protein to the lysosome. However, others have shown that IFG does increase the lysosomal pool of GCase (Khanna et al., 2010). It is important to note that authors performed measurements after a 7-day incubation in the presence of 30μM IFG, followed by a 3-day washout period to minimize potential GCase inhibition by IFG *in situ*. I incubated fibroblasts for 6 days in the presence of 50μM IFG but did not follow with a washout period. The degree of potential GCase inhibition may have been greater at the higher concentration of IFG. However, in an experiment to compare the GCase activity of fibroblast cells following a 24-hour washout versus no washout, I found no significant difference in the GCase activity measured (**Figure 9.15**). In fact, the GCase activity was slightly lower after the washout period.

It has been suggested that the function of ABX may not be restricted to a 'chaperoning effect'. ABX may be inducing a more global effect on lysosomal function. For instance, it has been postulated that ABX activates the CLEAR network through the action of Transcription Factor EB (TFEB) (McNeill et al., 2014). TFEB is a transcription factor that controls at least 471 genes and is the master regulator of lysosomal biogenesis (Palmieri et al., 2011). In a recent study, it was shown that ABX treatment led to a significant upregulation of TFEB messenger RNA levels in all treated cells (McNeill et al., 2014). Furthermore, authors demonstrated that gene expression profiling in ABX treated control cells demonstrated strong upregulation of lysosomal and autophagy genes. This provided strong evidence for an activation of the CLEAR network by ABX and a resultant upregulation of TFEB transcript levels. Therefore, ABX could have an additional mechanism of action aside from its role as a chaperone. ABX could be increasing GCase activity in *GBA* mutant fibroblasts by increasing the expression of TFEB, which is associated with activating components of the CLEAR network (Sardiello et al., 2009). My data, and those of others (McNeill et al., 2014), does suggest a general expansion of the lysosomal compartment. I saw significant increases in LAMP1 upon ABX treatment in *GBA* mutant fibroblasts. Lysosomes are a major site of SNCA degradation. It is plausible that an increased lysosomal mass could result in an increased SNCA degradation rate (Alvarez-Erviti et al., 2010). TFEB directly regulates the biogenesis of autophagosomes, the fusion of autophagosomes with lysosomes and the autophagic flux (Palmieri et al., 2011). If ABX is mediating its affects through an activation of TFEB, this could explain the upregulation of LC3, a marker of autophagosomes, seen upon ABX treatment in *GBA* mutant neurons. This upregulation of macroautophagy could increase the turnover of SNCA, which could be a critical step in *GBA* mutant neurons where

levels of SNCA are higher, and if aberrant SNCA is not removed, this could accumulate and predispose to PD.

For any potential therapy, it is important to pick a concentration which produces the desired effect but with the least side effects. Treatment of fibroblasts with 60μM ABX produced the greatest increase in GCase activity (**Figure 7.27**). However, cytotoxicity appeared upon every day treatment at this concentration (**Figure 9.12**). In a study of the chaperone activity and toxicity of ABX in GD cells, the viability of fibroblast cells was unchanged after incubation with ABX at concentrations up to 30μM (Luan et al., 2013). Cytotoxicity to GD and normal cells appeared only at the concentrations of 60μM and 100μM. For my experiments, I overcame potential cytotoxicity of the 60μM concentration by treating cells alternate daily. This 48-hour treatment did not compromise the cell viability (**Figure 9.12**). However, for a treatment that is intended to be given daily, I decided to evaluate whether a concentration of 10μM or 30μM ABX was sufficient to reproduce the same biochemical effects observed. My data have shown that at concentrations low enough not to cause cytotoxicity (Luan et al., 2013), significant increases of GCase activity and protein levels, and significant reductions of SNCA were still observed.

In summary, the small molecular chaperone, ABX, restores GCase activity in human fibroblasts and dopaminergic neurons derived from their fat-derived NCSC. Optimised treatment with ABX resulted in significant restoration of GCase enzyme activity, protein and gene expression in all cell lines, and an upregulation of macroautophagy and a reduction of SNCA in DA neurons. ABX could be mediating its effects by activating components of the CLEAR network through TFEB combined with its known chaperone activity (McNeill et al., 2014). My data highlight the potential benefits of manipulating the GCase pathway to lower SNCA levels in PD.

For patients, the optimal concentration, regimen and treatment duration of a PC still needs to be determined. The concentration of ABX as a mucolytic may not be adequate for PD. It would be important to make some correlation between the concentrations used in patient-derived cells with the equivalent oral concentration administered to patients.

Chapter 6: Discussion and Conclusions

6.1 A clinical road map of the pre-motor, pre-diagnostic prodrome of Parkinson's disease

Mutations in *GBA* are widely recognized to be an important and common genetic risk factor for PD (Sidransky et al., 2009b), and are found in British subjects with PD at a higher frequency than any other known PD-associated gene (Neumann et al., 2009). Of particular interest is the evidence accumulating that *GBA* mutant homozygote and heterozygote carriers without clinical evidence of PD, exhibit the prodromal features of the disease. In 2012, I continued the clinical evaluation of a unique cohort of *GBA* mutation positive individuals for the early prodromal features of PD (Beavan et al., 2015). This study cohort is an ideal population to study a neuroprotective strategy or disease modifying therapy. What was perhaps surprising about this non-parkinsonian *GBA* positive cohort at the initial assessment in 2010 was a significant difference from controls even at the baseline evaluation (McNeill et al., 2012b). At baseline, *GBA* mutation-positive individuals demonstrated hyposmia and cognitive impairment (McNeill et al., 2012b). Importantly, I found that olfaction and cognition remained significantly lower in *GBA* mutation-positive individuals compared to controls at two years follow-up (Beavan et al., 2015). I further identified a significant deterioration across clinical markers in *GBA* mutation–positive individuals consistent with the prodrome of PD. Within this group, 10% appeared to be progressing at a

more rapid rate. It is interesting to compare my results which identified 10% of *GBA* mutation-positive individuals developing parkinsonian motor features, with the reported estimate that 10% of PD patients carry a mutation in *GBA* (Sidransky et al., 2009b). In this subgroup of *GBA* mutation positive individuals with parkinsonian motor signs, mild cognitive impairment was the main premotor sign present at baseline that could have predicted their motor deterioration.

The aim of the present study was to investigate the development and evolution of clinical biomarkers for PD in a high-risk population, in order to ascertain a spectrum/pattern of changes that would enable me to develop an early biomarker set that could predict PD. In a subgroup of individuals with parkinsonian signs, I have gone on to perform analysis to identify premotor signs at baseline that could predict the motor signs at follow-up. However, I have also planned future follow-up assessments to confirm that the differences I am seeing are indeed predictive of future PD.

My data indicate that individuals with *GBA* mutations exhibit identical prodromal abnormalities to those with idiopathic PD. There appears to be a relatively rapid evolution of non-motor and motor features in this cohort. Further follow up of this group will enable early diagnosis in those progressing to clinical PD and perhaps allow identification of a specific clinical or biochemical pattern that distinguishes those with *GBA* mutations who will and those who will not develop PD. In terms of the specific alterations that lead to developing PD in a group of *GBA* mutation-positive individuals, I can only hypothesize that other genetic or possibly environmental factors may be of relevance here. Study of *GBA* mutation-positive

individuals that do not go on to develop PD could provide insight into factors that may afford protection from PD.

Improved insight into possible disease mechanisms, lays the basis for the development of new therapies to slow the rate of progression or stop the disease process (Schapira et al., 2014). It is thus imperative to develop an early biomarker set to identify individuals at greatest risk for the development of PD. In my PhD, I have attempted to identify subjects likely to develop PD through the use of early clinical markers (Beavan et al., 2015). The data from this cohort suggest that hyposmia is the earliest and most sensitive prodromal marker. Cognitive impairment is also an early feature and this may relate to the increased cognitive impairment observed with GBA-PD. Symptoms of RBD, the most specific clinical marker, are now present in *GBA* mutation positive individuals. Depressive symptoms have also surfaced but must be interpreted with some caution considering their low specificity as a marker for PD. There has also and perhaps most importantly, been a significant decline on the UPDRS, which together with impaired RBD and depression, suggest that clinical markers in some individuals of this *GBA* mutation positive cohort have evolved, in a pattern consistent with the clinical prodrome of PD.

6.2 *GBA* mutation escalates the onset of Parkinson's disease

Patients with a *GBA* mutation have clinical deterioration consistent with the early stages of PD. In a prospective cohort study, both patients with type 1 GD and those with heterozygous *GBA* mutations have shown a gradual clinical deterioration consistent with the clinical prodrome of PD (Beavan et al., 2015).

In the clinical setting, it can be difficult to distinguish individual PD patients with *GBA* mutations versus those without *GBA* mutations. GBA-PD patients show the classic triad of bradykinesia, rigidity and tremor, with an asymmetric onset (Goker-Alpan et al., 2008). However, there are some subtle differences. In *GBA* mutation positive carriers, the age of onset tends to be slightly younger and there is a greater risk for earlier and more prevalent cognitive impairment (Sidransky et al., 2009b; Winder-Rhodes et al., 2013). In addition, the pattern of cognitive impairment seen in *GBA* mutation positive carriers with PD is slightly different and present even in those without PD at the time of investigation (Zokaei et al., 2014).

Neuroimaging in GBA-PD with positron emission tomography or single photon emission computed tomography for the visualisation of radioactive dopamine transporter ligands demonstrates an asymmetry of radioligand uptake indistinguishable from idiopathic PD (Goker-Alpan et al., 2012; McNeill et al., 2013a). Furthermore, the pathology of GBA-PD is identical to that of idiopathic disease. GBA-PD brains exhibit the characteristic pathology of PD such as brainstem and cortical LBs (Wong et al., 2004; Neumann et al., 2009). GBA has also been found in LBs, and more frequently in those with the *GBA* mutation (Goker-Alpan et al., 2010).

The response to dopaminergic therapy in patients with PD with or without mutations in *GBA* appears to be the same, including the development of motor complications (Ziegler et al., 2007). Most studies report a good or excellent response to L-dopa treatment in heterozygote *GBA* mutation carriers (Ziegler et al., 2007; Neumann et al., 2009), but some report a less favourable response (Tayebi et al., 2003).

My data favours a link between the *GBA* mutation and a higher frequency of non-motor symptoms and rate of clinical progression. My work (Beavan et al., 2015) and

those of others (Winder-Rhodes et al., 2013; Brockmann et al., 2014) may suggest that the *GBA* mutation represents a poor prognostic marker for an individual who develops PD. The evidence from longitudinal studies demonstrate that *GBA*-associated PD patients show a more rapid disease progression dominated by accelerated motor impairment with greater risk for progression to Hoehn and Yahr stage 3 (Winder-Rhodes et al., 2013), and a greater preponderance to dementia or cognitive decline (Brockmann et al., 2014). Some studies have suggested that LB deposition is more extensive in *GBA* mutant positive brains (Clark et al., 2009) but this is not universally found (Parkkinen et al., 2011). Therefore, one might argue that it is the *GBA* mutation status, rather than advanced age at onset, that presents a predictor for disease progression in PD. In population-based longitudinal studies, motor impairment, dementia and advanced age all predict mortality in PD patients. In the same vein, those carrying the *GBA* mutation who exhibit more rapid progression of motor impairment and cognitive decline, also show increased rates of mortality (Brockmann et al., 2014).

This could have significant socioeconomic implications. PD is the second most common neurodegenerative disease after Alzheimer's disease and affects approximately seven million people globally. PD is more common in the elderly and prevalence rises from 1% in those over 60 years of age to 4% of the population over 80. The incidence of PD is between 8 and 18 per 100,000 person–years. The costs of PD to society are high, but precise calculations are difficult due to methodological issues in research and differences between countries. The annual cost in the UK is estimated to be between 449 million and 3.3 billion pounds. This variance reflects whether costs are limited to drugs and devices (deep brain stimulation) or also include institutional support. A study in Europe has shown that delaying the course of

PD by 3 months alone, and delaying institutionalisation by the same amount could pay for the life-time cost of a PD patient's drugs. Hence the focus of research in PD is on slowing progression of the disease. In those carrying the *GBA* mutation, a more rapid progression of motor impairment and cognitive decline, could increase the demand for nursing care and/or institutional support.

In a recent longitudinal study, authors raised the question of whether screening patients with PD for *GBA* mutations should become part of the routine diagnostic work-up in clinical practice (Winder-Rhodes et al., 2013). It is still relatively expensive to perform whole exome and/or whole genome screening on every patient with PD, but this could be a future consideration as we move towards an era of preventative genetic screening and the costs go down with increased demand. However, there are ethical considerations with mass screening, such as genetic counselling of family members, albeit the penetrance of *GBA* mutations is low, and carrying the *GBA* mutation is not a prerequisite for developing PD in later life.

In summary, *GBA* mutations are found in 10% of the PD population worldwide (Sidransky et al., 2009b) and therefore, represent a significant proportion of patients with PD who might reasonably be encountered by the non-specialist clinician. Both mutations and polymorphisms in *GBA* could adversely affect the trajectory of PD (Winder-Rhodes et al., 2013), and therefore, the presence or absence of the gene could be an important red flag for physicians.

6.3 Delineating the molecular mechanism of mutant *GBA* to the pathogenesis of Parkinson's disease

My data suggest that *GBA* mutations interfere with SNCA clearance. I have demonstrated that mutations in *GBA* cause reductions in GCase activity, impairment of the UPR, and dysfunction of lysosomal autophagy, potentially leading to insufficient SNCA turnover. The analysis of GBA-PD brain on post mortem has shown that LBs are immune-reactive for GBA, which suggests that *GBA* mutations are sufficient for SNCA aggregation (Goker-Alpan et al., 2010).

The pathological hallmark of PD is SNCA aggregation. This phenotype has been demonstrated in dopaminergic neurons derived from patients with genetic PD including those with SNCA triplications (Devine et al., 2011), *Parkin* (Imaizumi et al., 2012) and *LRRK2* G2019S (Nguyen et al., 2011), and *GBA* N370S/84GG (Mazzulli et al., 2011). In twins harbouring *GBA* N370S, an increase in monomeric SNCA levels was observed (Woodard et al., 2014). In my studies, when differentiated into DA neurons, cell lines harbouring *GBA* N370S had significantly higher SNCA levels in the absence of disease, suggesting that the *GBA* mutation perturbs SNCA processing. It would be prudent to check whether SNCA mRNA levels differ in these cell lines, as an indication of whether the *GBA* mutation interferes with SNCA transcription. Recent data suggests that SNCA mRNA levels do not differ in *GBA* N370S positive cell lines (Woodard et al., 2014). It would also be important to determine SNCA mRNA levels in the presence of ABX. This could help elucidate whether the drug is affecting the synthesis or, as implied by an upregulation of

autophagy, the turnover of SNCA protein. In addition, measurements for SNCA release will help demonstrate whether more of the protein is being secreted.

In neurons derived from twins carrying heterozygous *GBA* N370S, there was an approximately 50% decrease in GCase activity. However, despite an increase in monomeric SNCA levels, authors did not discover SNCA aggregation. In an animal model, homozygous *GBA* mutations enhanced SNCA aggregation in mouse brains (Sardi et al., 2011). This implies that more severe GCase deficiency may be required for SNCA aggregation and/or additional factors are important for the synucleinopathy in PD brains carrying heterozygous *GBA* mutations.

Human adipose derived NCSC have the potential to differentiate into functional dopaminergic neurons. This finding offers the opportunity to generate a patient-specific DA neuronal model. Using this patient-specific DA neuronal model to investigate the relationship between GCase, SNCA and lysosome impairment has confirmed *GBA* mutation carriers demonstrate decreased *GBA* protein levels, reduced GCase activity, and impaired neuronal cell macroautophagy; these pathological processes in turn may increase SNCA levels in neuronal cells with the potential for leading to SNCA aggregation. Therefore, DA neuronal cell models derived from *GBA* mutation positive patient adipose tissues could mimic the PD pathological process. NCSC-derived DA neurons represent individual human nerve cells; recapitulate some key PD pathological features, particularly SNCA accumulation, and provide a useful model for developing a novel therapeutic strategy.

6.4 Using the data collected could pharmacological chaperones represent a novel therapeutic approach for Parkinson's disease?

At present, all treatment for PD is symptomatic. It does not slow down the progression of the disease. A drug that can slow or stop the disease is desperately needed. There are many causes and factors that increase the risk of PD, but mutations of the *GBA* gene are the most important. Mutations of this gene increase the risk for PD by 20-30 times, and are found in at least 10% of PD cases.

Studies of brains from patients who have died with PD, cell models and animal models of *GBA* mutations and PD indicate that there is a close link between glucocerebrosidase enzyme GCase, the product of the *GBA* gene and SNCA, the main protein involved in PD pathology. This relationship is reciprocal in that reduced GCase activity, for instance as a consequence of a mutation in the *GBA* gene, causes an increase in SNCA such as that seen in PD. Alternatively, increased SNCA, for instance as a consequence of mutations in the SNCA gene, result in a reduction in GCase activity.

My data support the suggested interaction between GCase and SNCA. In addition, mutations in *GBA* have been shown to cause autophagic dysfunction leading to insufficient SNCA turnover. The relationship between GCase and SNCA may be leveraged to reduce SNCA levels in PD by enhancing GCase levels and activity. This hypothesis has previously been suggested by preliminary data in cells overexpressing SNCA (McNeill et al., 2014), and offers an important target pathway for future neuroprotection therapy in PD. Importantly, I have shown in human cells that this relationship can in fact be manipulated so that increasing GCase activity

induces a reduction in SNCA levels. This is of considerable relevance to PD, and offers the opportunity to develop novel drugs to target this relationship and decrease SNCA levels and spread to slow or stop the progression of PD.

In my PhD study, I have used a drug which is currently available for use in patients for an entirely different illness (as a cough medicine), and with a very good safety profile, in human cells from PD patients to show that it can increase GCase activity. ABX is a drug class known as a small molecule chaperone that interacts with GCase and increases activity of both the mutant and normal forms of this enzyme. The next stage will move towards developing this drug for PD patients. The drug has now been tested further in cell cultures from individuals with *GBA* mutations, with and without PD. The results have confirmed the effect of small molecule chaperones to increase GCase activity. In addition, my studies have confirmed that this drug can reduce SNCA levels in neuronal cells. The data highlights the potential effect of the drug on brain SNCA to reduce levels and potentially the spread of SNCA pathology. However, the pathogenesis of PD is complex, and any successful therapeutic strategy will require stratification of genetic and non-genetic factors.

I have analysed how *GBA* mutations increase the risk of PD and how an individual's symptoms and signs can signal their risk for the disease. This will be very important in identifying those at risk and who would be best suited to receive a drug prior to the development of PD. If the results of subsequent studies in this laboratory are positive, and my pilot data strongly indicate they will be, the next stage will be to move towards a clinical trial of ABX in PD patients, and in parallel, to collaborate with industry to develop novel highly active small molecule chaperones for further testing and use in patients.

6.5 If I could do this PhD again, what would I do differently now?

If I went back to re-examine the clinical cohort now, I would consider recruiting a larger number of subjects as it is difficult to reach meaningful conclusions from a small sample number. I would also look into whether some of the assessments could be performed online by subjects at home or on a computer. This might encourage wider participation and improve follow-up rates. I would also ensure that all investigators were blind to the genetic status of individuals, as this is a potential bias when it comes to the final data analysis. One other improvement would be to ensure that it is the same assessor evaluating subjects to reduce any inter-observer differences. However, this may be difficult in view of the fact that it is often a clinical fellow performing these assessments and who is employed for a limited time period to complete a PhD etc. Finally, due to the mixed specificity and sensitivity of clinical biomarkers, it would have been useful to combine these with biochemical and imaging markers in order to more accurately predict PD in this at-risk cohort.

For my laboratory studies, I focused on human material but it would have been nice to have tested the drug compounds in an animal model e.g. a mouse model, which would have been complimentary and perhaps reinforced my studies. In addition, it is always beneficial to collect and test as many samples as possible to achieve meaningful results and robust conclusions. I would have liked to have tested more patient samples for a higher n number, but I did the minimum (n=3) for my neuronal studies due to the limitations of cost and time. On a final note, if I had more time, I would have liked to have made a more distinct correlation between the concentration of ABX that produced the desired biochemical effect in cells with the quantity required for a patient to take as an oral tablet to reduce SNCA in PD.

Chapter 7: Figures

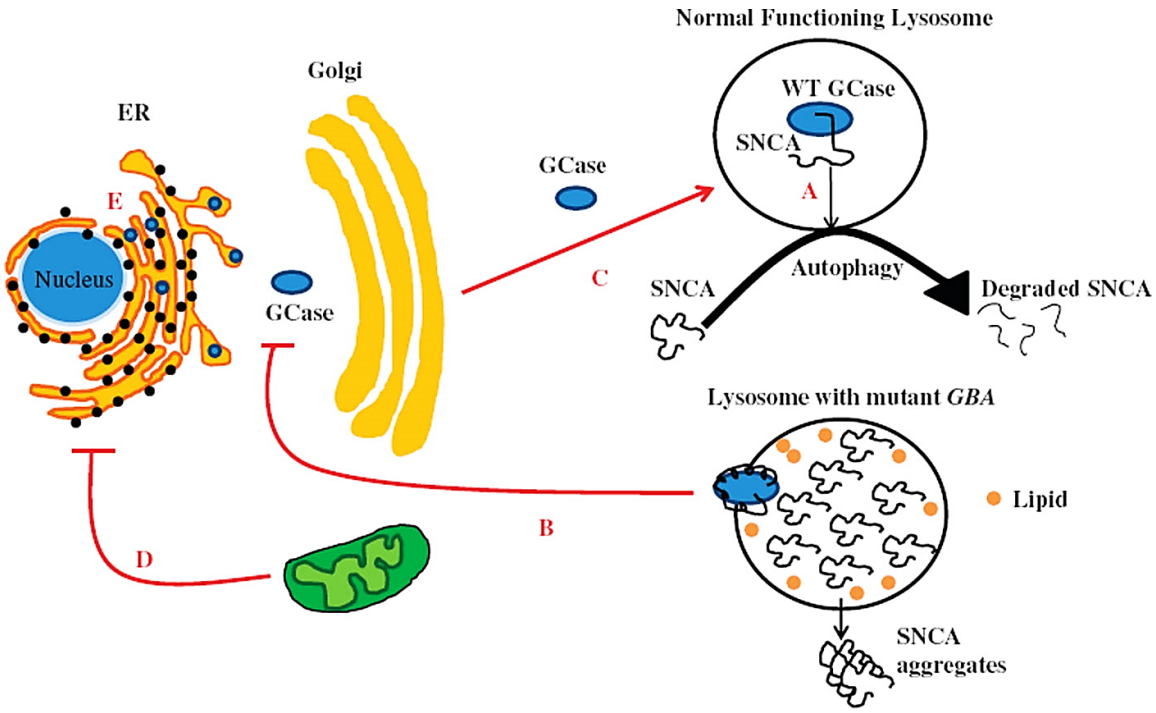


Figure 7.1. The pathogenesis of *GBA* mutations in Parkinson's disease.

In normal functioning lysosomes, wild-type (WT) GCase interacts with α-synuclein (SNCA), and this beneficial interaction could promote the degradation of SNCA through lysosomal autophagy (A). In some cases, mutant GCase or decreased levels of WT GCase reaching the lysosome weaken this interaction, increasing the likelihood of SNCA accumulation. This event is more likely with increasing age, when the numbers and function of lysosomes decrease and cellular SNCA concentrations increase. The inhibitory effect of SNCA can further impair the trafficking of WT GCase via the endoplasmic reticulum (ER)/Golgi (B), resulting in less WT GCase being delivered to the lysosome via the lysosomal integral membrane protein-2 (LIMP-2) transporter (C), compounding the problem. Mutations in *GBA* could also increase the risk of PD through abnormalities in mitochondrial function by an unknown mechanism (D). Misfolded GCase trapped in the ER could trigger the unfolded protein response (UPR) and/or activate endoplasmic reticulum-associated degradation (ERAD) (E). Reproduced with permission from Beavan MS, Schapira AH. Ann Med. 2013; 45:511-21.

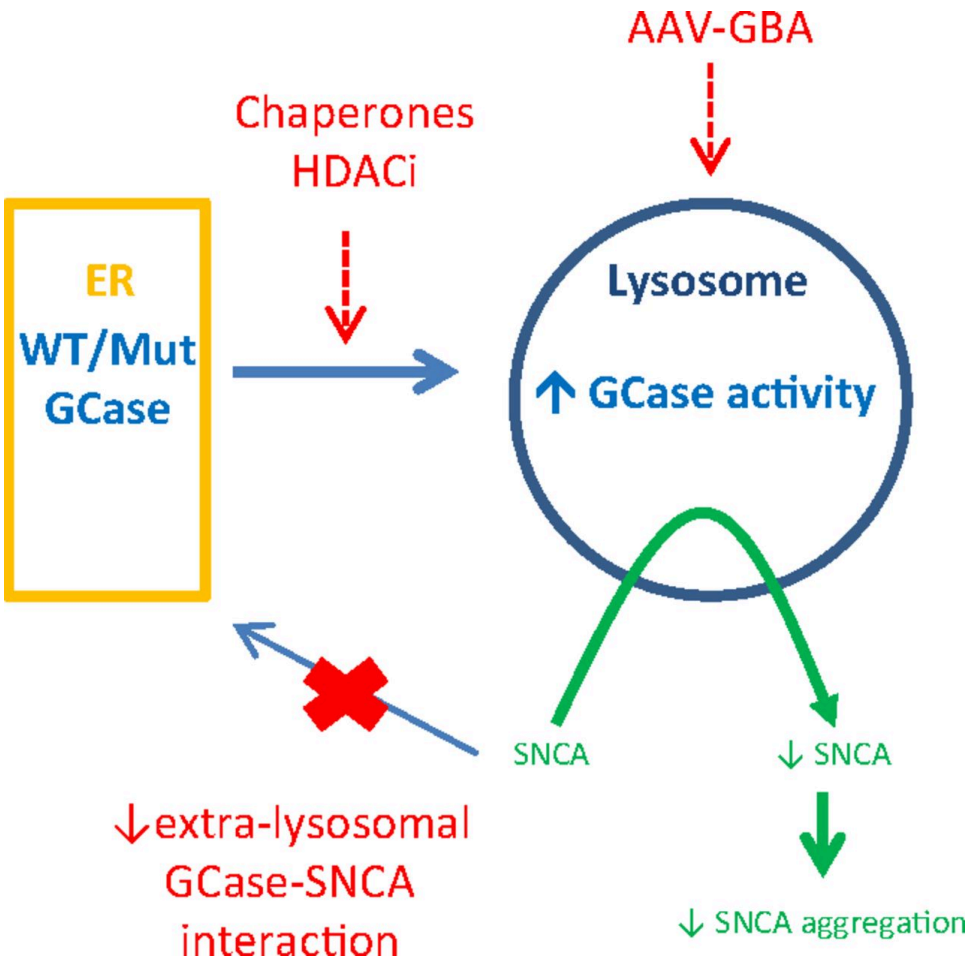


Figure 7.2. The glucocerebrosidase/SNCA axis, potential targets for therapeutic intervention for PD.

SNCA interacts with both wild-type (WT) and mutant (Mut) GCase, and modulation of this may serve to reduce SNCA concentrations and delay or reverse PD-related pathology. Strategies to increase lysosomal GCase or to decrease extralysosomal GCase–SNCA interactions would be anticipated to reduce SNCA concentrations. Chaperones, histone deacetylase inhibitors (HDACi), or increased expression of by, for example, gene therapy (AAV-*GBA*) may be examples of such strategies. Reproduced with permission from Schapira AH, Gegg ME. Proc Natl Acad Sci U S A. 2013;110:3214–15.

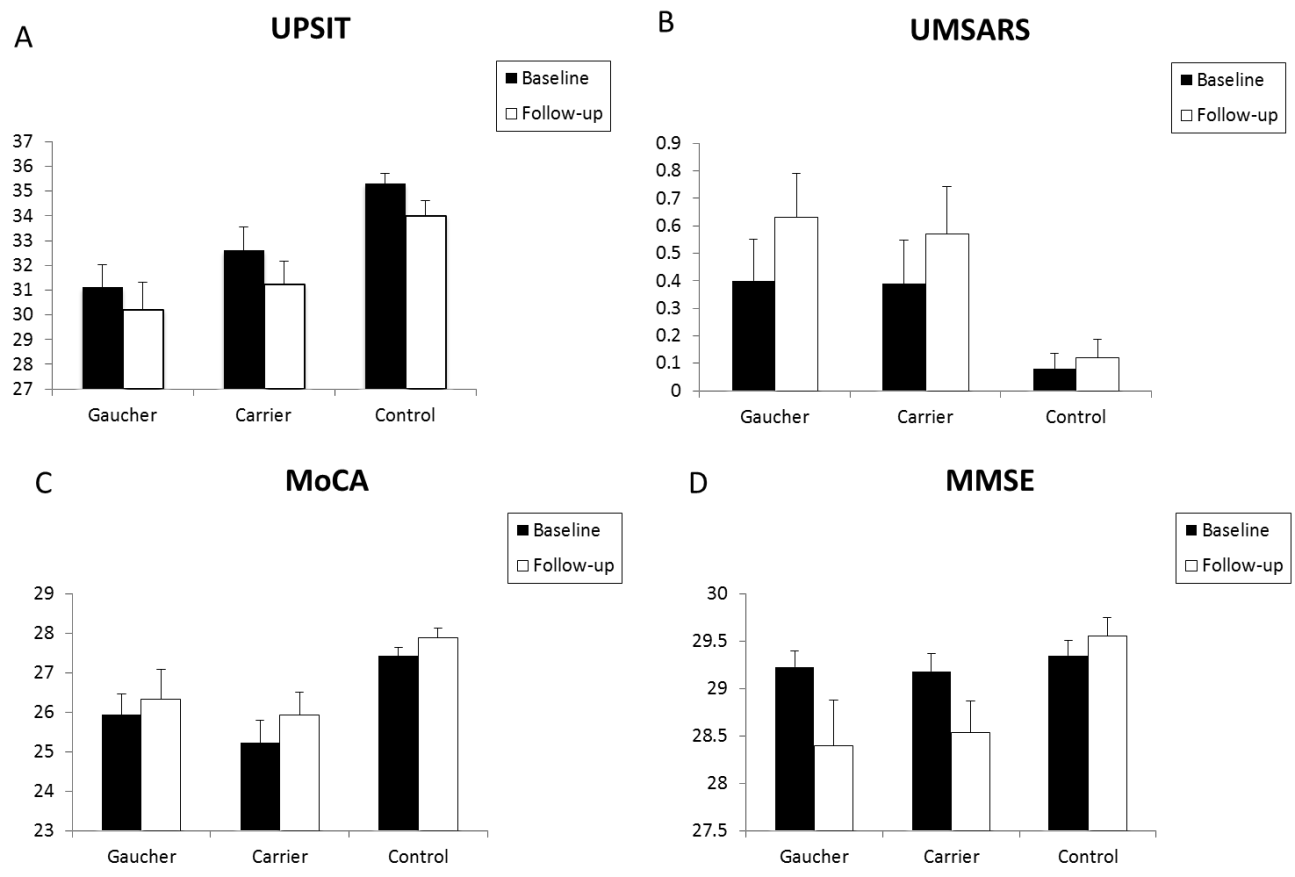


Figure 7.3. Clinical markers show progression in *GBA* mutation positive individuals.

Figures demonstrate mean baseline and follow-up scores for olfaction (A), mean baseline and follow-up scores for autonomic dysfunction (B), mean baseline and follow-up MoCA scores (C) and mean baseline and follow-up MMSE scores (D) for Type 1 GD patients and heterozygous *GBA* mutation positive carriers compared to controls. Means are plotted together with the SEM.

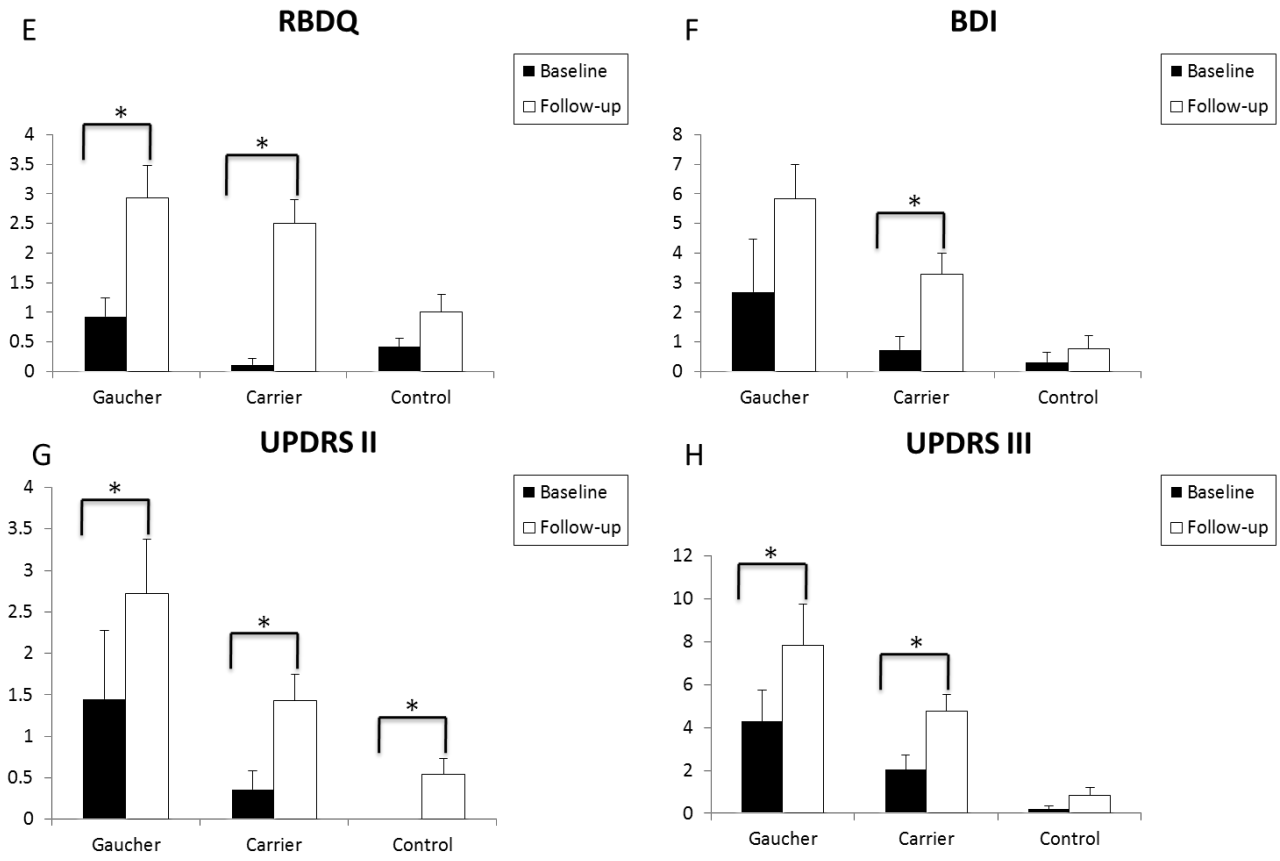


Figure 7.4. Clinical markers show progression in *GBA* mutation positive individuals.

Figures demonstrate a statistically significant increase in depressive symptoms for carriers at the follow-up evaluation (F), a statistically significant increase in mean follow-up RBDQ scores (E) and UPDRS III scores (H) in Type 1 GD patients and heterozygous *GBA* mutation positive carriers compared to controls, and a statistically significant increase in mean follow-up UPDRS II scores in Type 1 GD patients, heterozygous *GBA* mutation positive carriers, and controls (G). Means are plotted together with the SEM. *$p<0.05$.

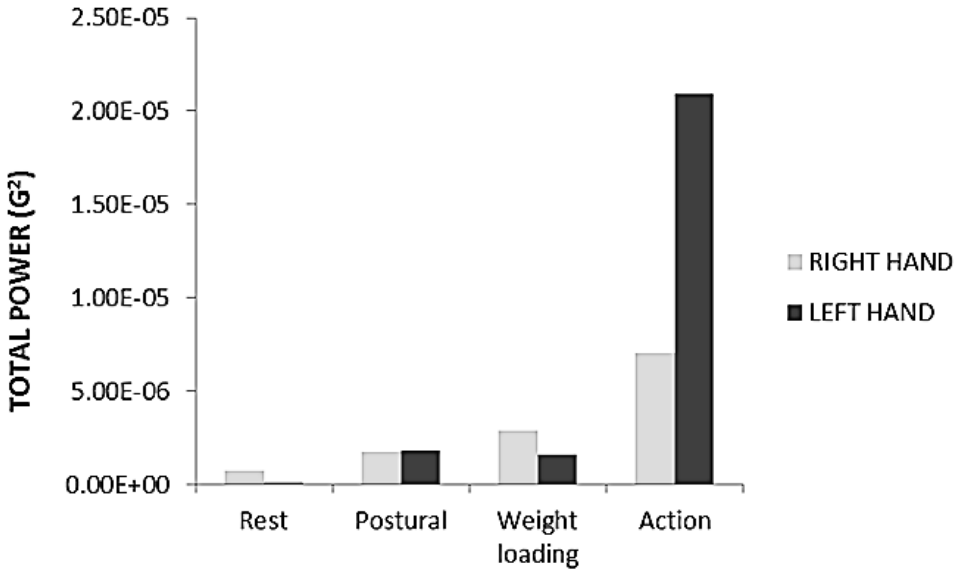


Figure 7.5. Tremor recording in a patient with essential tremor.

Total power (G^2) of the tremor in one patient at rest, on posture, during loading and during action. Power for right and left hands is shown separately.

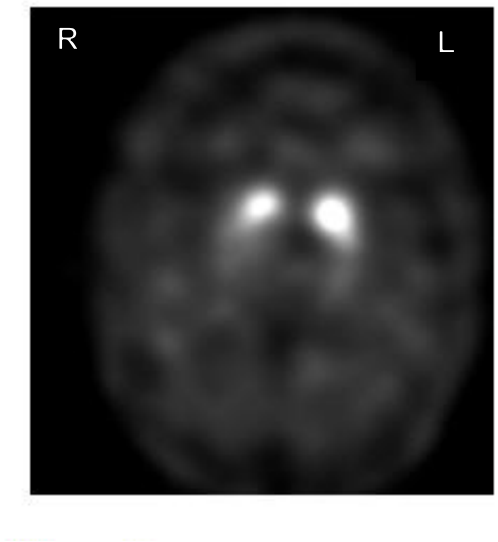


Figure 7.6. Dopamine Transporter (DaT) scan in patient JT.

Single-photon emission tomography (SPECT) demonstrated clear-cut reduction of uptake within the putamina bilaterally, and reduced uptake within the right caudate nucleus. Findings were consistent with Parkinson's disease.

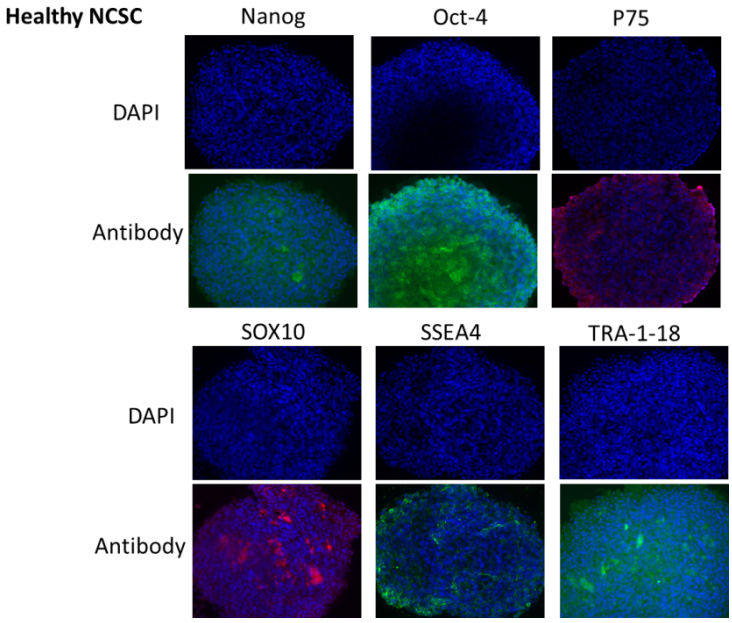


Figure 7.7. Characterisation of pluripotent properties in healthy NCSC

Immunostaining of wild type NCSC cultures. Cells were stained for P75, SOX10 (red), and Nanog, Oct-4, SSEA4 and TRA-1-18 (all green). Nuclei were counterstained with DAPI (blue).

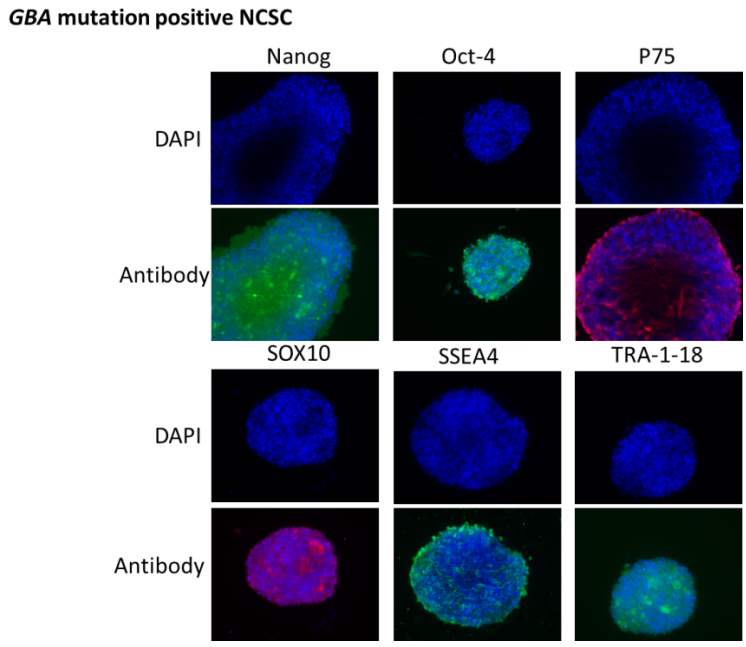


Figure 7.8. Characterisation of pluripotent properties in *GBA* mutant NCSC

Immunostaining of *GBA* mutation positive NCSC cultures. Cells were stained for P75, SOX10 (red), and Nanog, Oct-4, SSEA4 and TRA-1-18 (all green). Nuclei were counterstained with DAPI (blue).

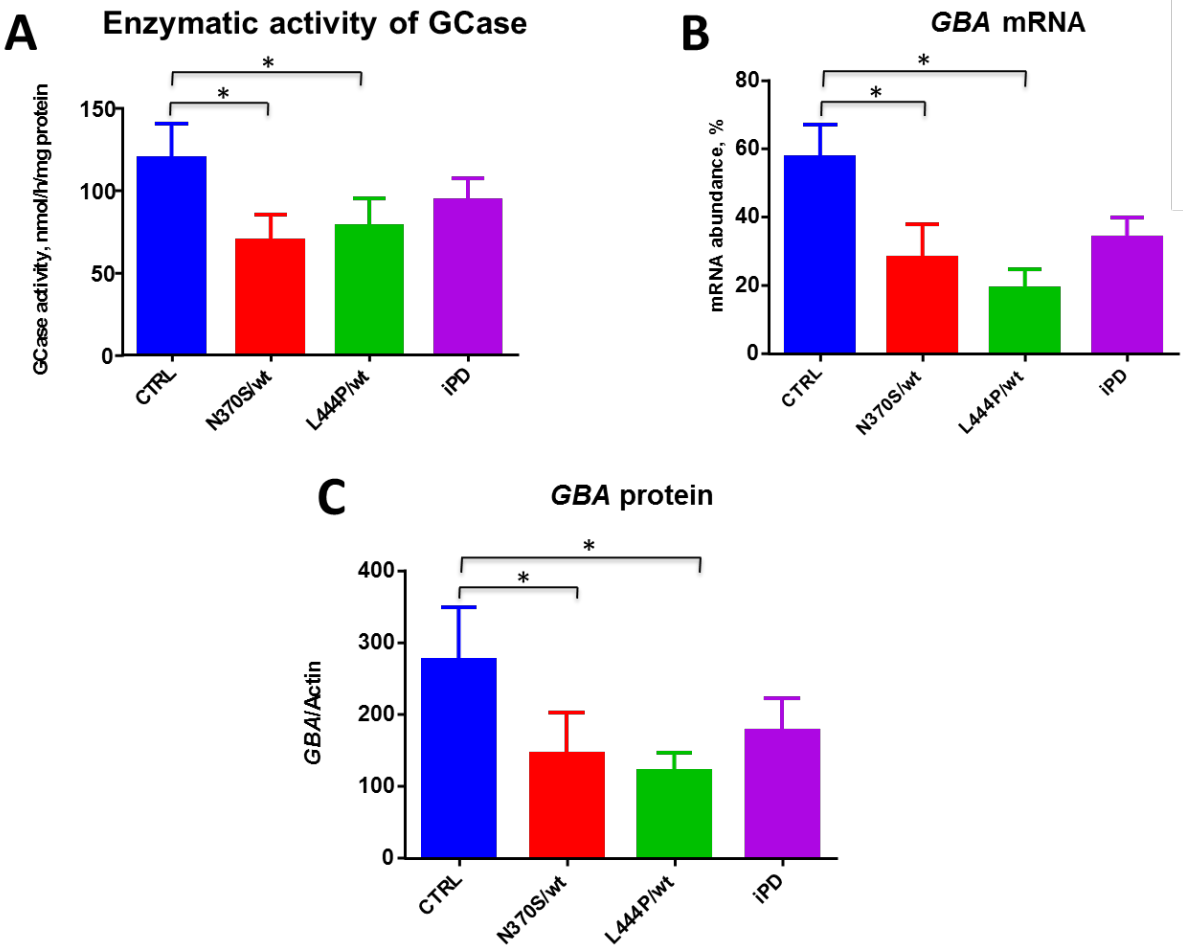


Figure 7.9. *GBA* mutation carriers show decreased GCase enzyme activity, protein levels and gene expression.

Glucocerebrosidase enzyme (GCase) activity was measured in fibroblasts from control, *GBA* mutation carriers with and without PD, and idiopathic PD patients (A). Enzyme activities were expressed as nmol/hr/mg protein. GCase activity was significantly reduced in heterozygous *GBA* mutation carrier fibroblasts compared with healthy control cells. *GBA* mRNA levels in disease lines compared to controls (B)*;* there were reduced mRNA levels in *GBA* mutation carriers compared to controls. Quantification of GBA protein in each variant was determined by western blot with human-specific antibodies (see **Figure 7.30** for representative western blots). There were significant reductions of GBA protein in *GBA* mutation carriers compared to controls (C). Results represent the mean ± SEM, of five independent experiments. *p<0.05; one-way ANOVA and Student's t-test.

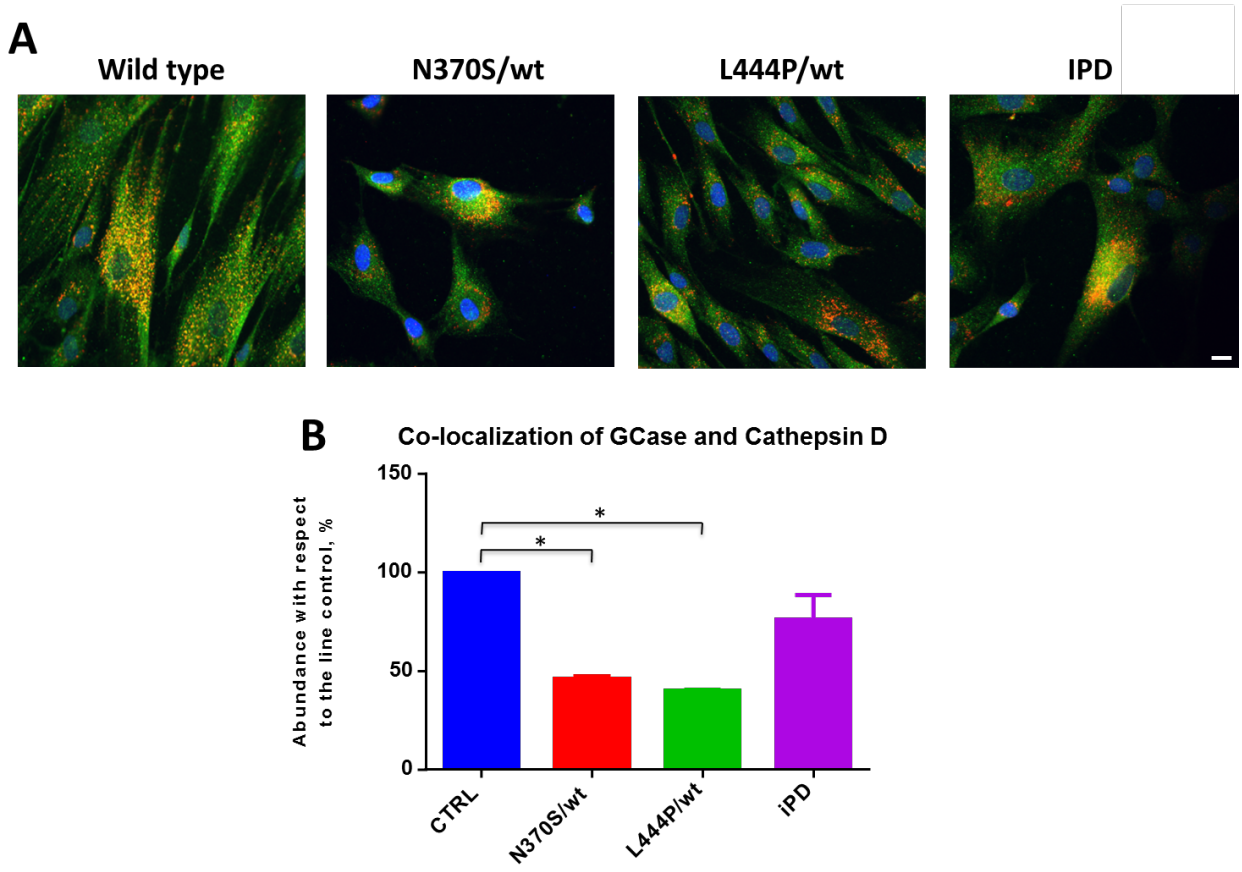


Figure 7.10 *GBA* mutation carriers show defective localization of GBA protein.

Representative slices (depth 3μM) showing colocalization *(yellow)* of GBA (*green*) with lysosomal marker cathepsin D (*red*), conducted by immunofluorescence staining (A). Nuclei were counterstained with DAPI (*blue*). GBA signals were significantly stronger in control and IPD cells compared to heterozygous *GBA* mutation carrier cells, where GBA was nearly undetectable. Untreated control GBA colocalized (*yellow*) with cathepsin D in the lysosome. In contrast, colocalization of GBA with cathepsin D (*yellow*) was significantly reduced by 50% in heterozygous *GBA* mutation carrier cells compared to control cells. Quantification of the level of colocalization relative to the control (B). Data are represented as mean + SEM; of three independent experiments. *p<0.05; one-way ANOVA and Student's *t*-test. *Scale bar*, 10 μm.

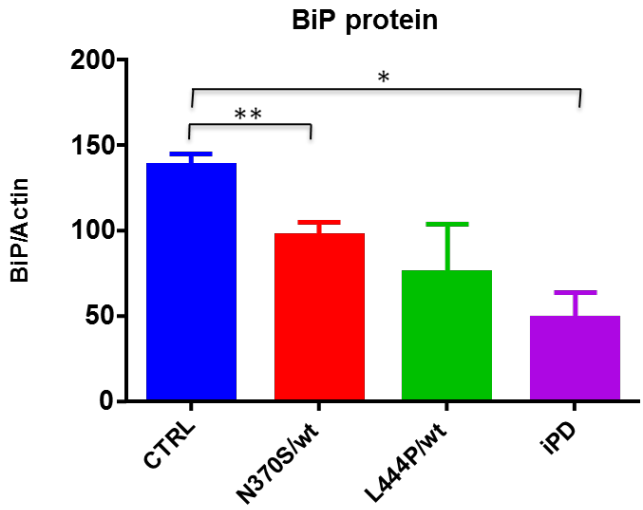


Figure 7.11. *GBA* mutation carriers and PD cells show reduced GRP78 expression, suggesting an inadequate UPR.

The levels of ER protein BiP/GRP78 were determined in fibroblasts from *GBA* mutation carriers with and without PD, idiopathic PD patients, and in normal control individuals without *GBA* mutations. Quantification of the levels of BiP relative to β-actin (see graph shown). Relative levels of BiP/GRP78 were reduced in PD cases, independent of mutation status, by approximately 35-50% and reduced in *GBA* mutation carriers by approximately 50–70% compared to controls. Data are represented as the mean ± SEM, of three independent experiments. *p<0.05; **p<0.01; one-way ANOVA and Student's t-test.

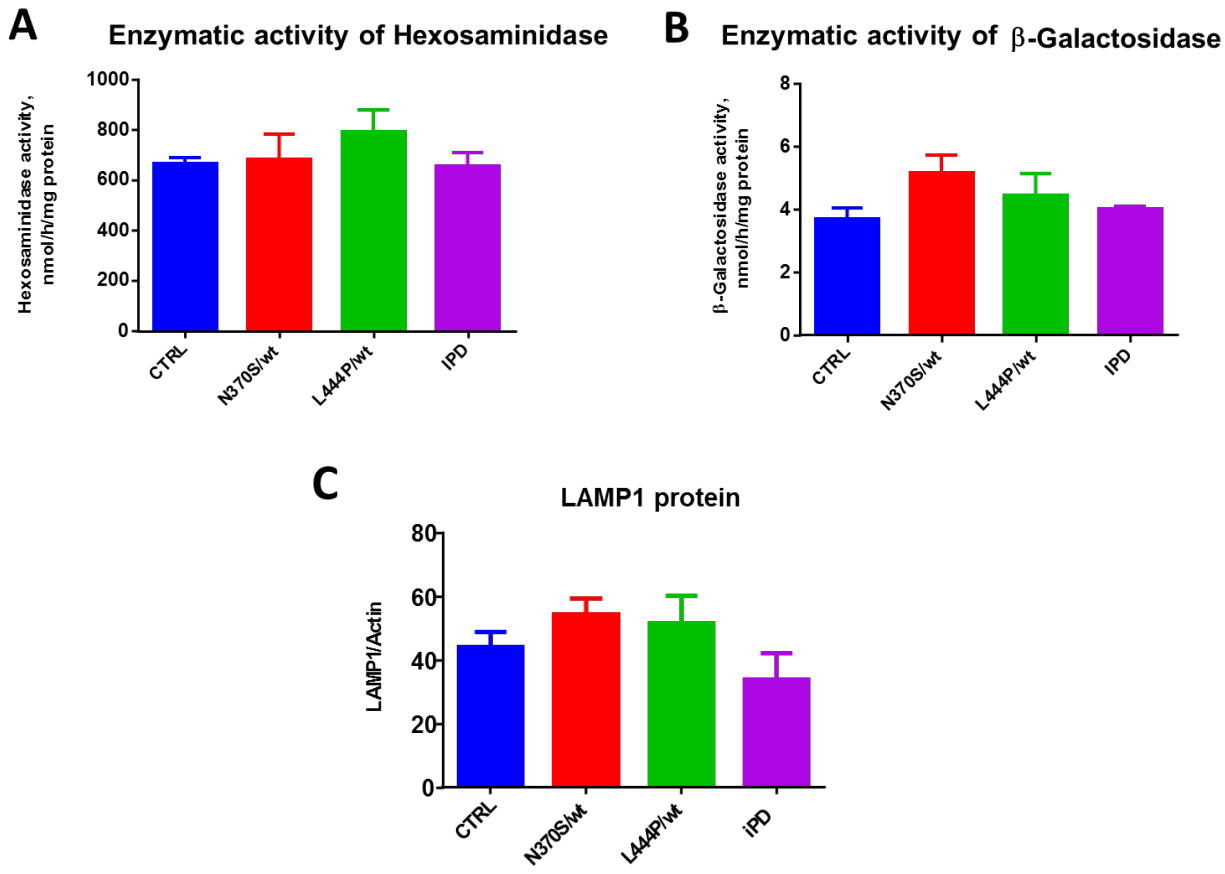


Figure 7.12. Lysosomal content and function is unchanged in *GBA* mutation carriers.

Hexosaminidase and β-galactosidase activity were unchanged in heterozygous *GBA* mutation carrier fibroblasts compared with healthy control cells (A) (B). Quantification of the basal levels of LAMP1 relative to β-actin (C). See **Figure 7.32** for representative western blots. Data are represented as the mean ± SEM, of three independent experiments.

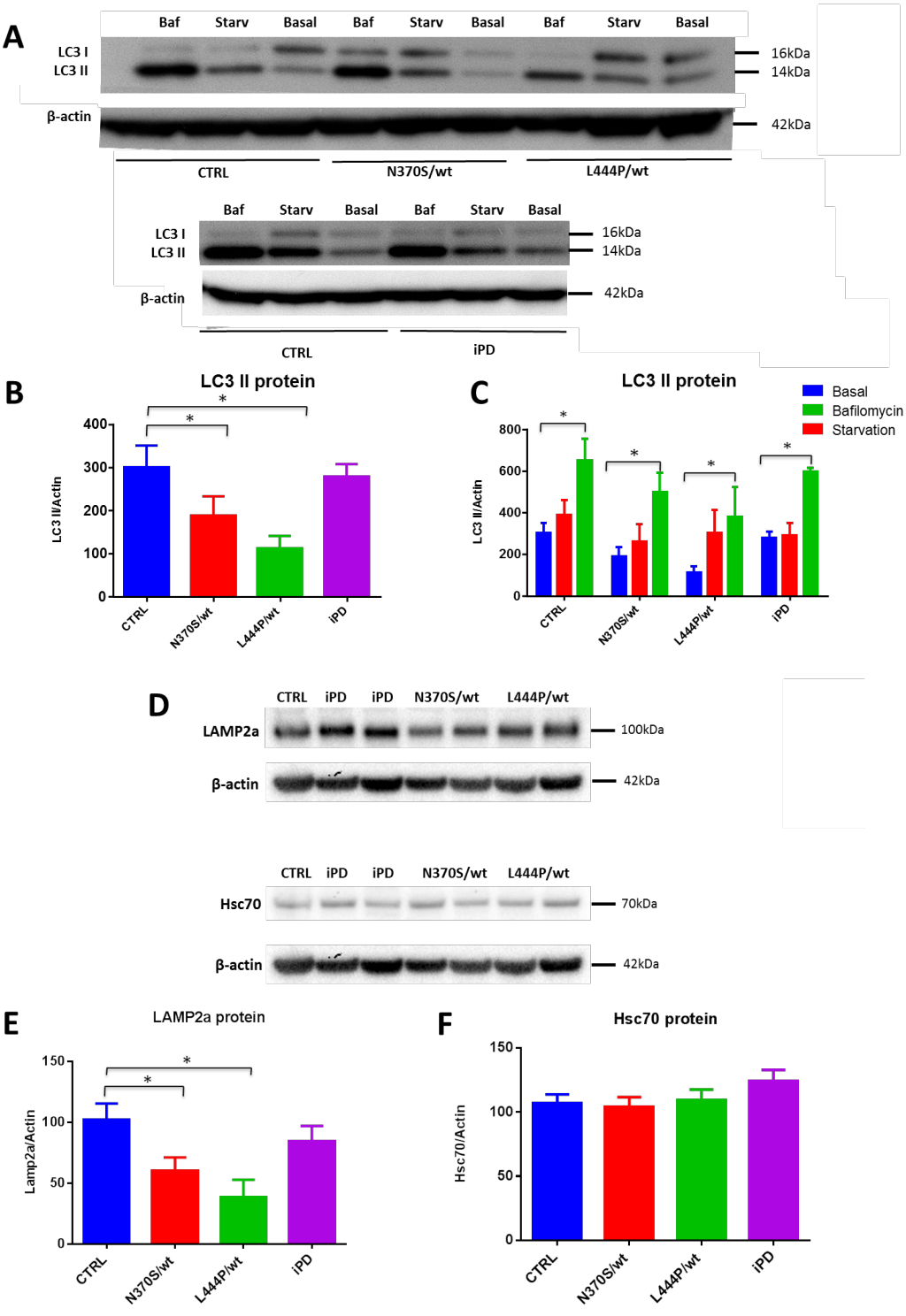


Figure 7.13. *GBA* mutation carriers show defects in lysosomal autophagy.

Representative western blots for LC3 in fibroblasts, untreated (Basal) or treated with 0.2μM bafilomycin (Baf) or DMEM without supplements (Starv) for 4 hours (A). Quantification of the basal levels of LC3-II relative to β-actin (B). Quantification of LC3 flux relative to β-actin (C). Representative western blots for LAMP2a and Hsc70 protein in human fibroblasts (D). Quantification of the basal

levels of LAMP2a relative to β-actin (E). Levels of LAMP2a protein were significantly reduced in heterozygous *GBA* mutation carriers compared with healthy control cells. Quantification of the basal levels of Hsc70 relative to β-actin (F). Data are represented as the mean ± SEM, of three independent experiments. *$p<0.05$; one-way ANOVA and Student's *t*-test.

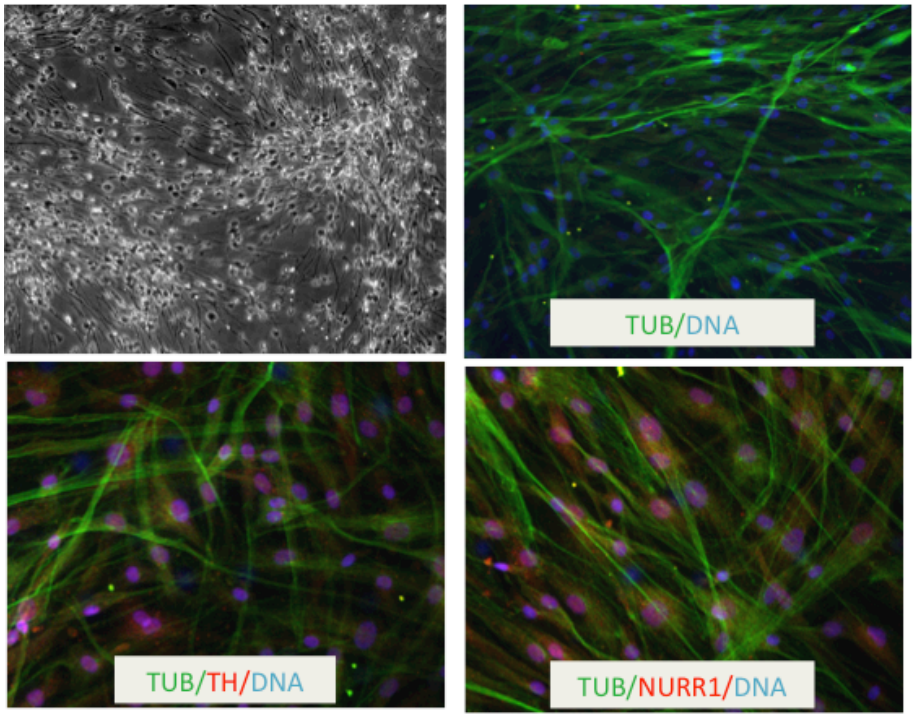


Figure 7.14. Immunocytochemistry of differentiated neuronal cells

Immunostaining of differentiated wild type NCSC cultures at day 40. Cells were stained for β-Tubulin III (TUB) (green), tyrosine hydroxylase (TH) or NURR1 (both red). Nuclei were counterstained with DAPI (blue).

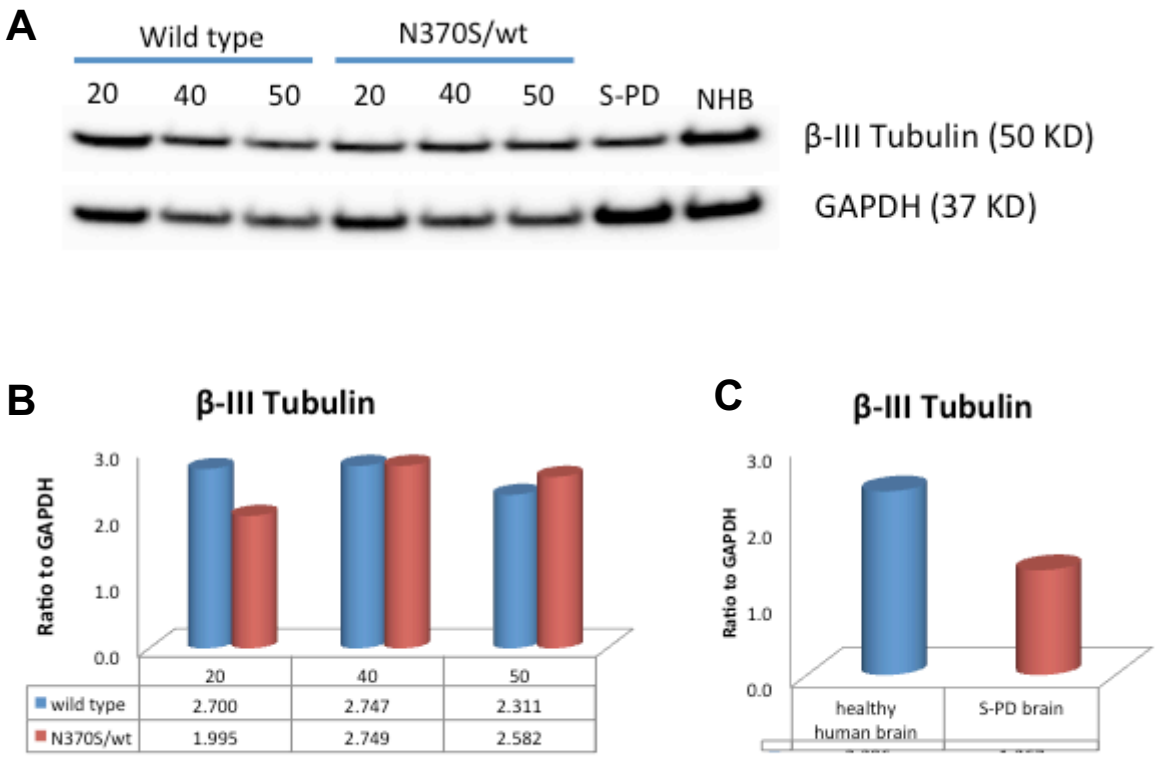
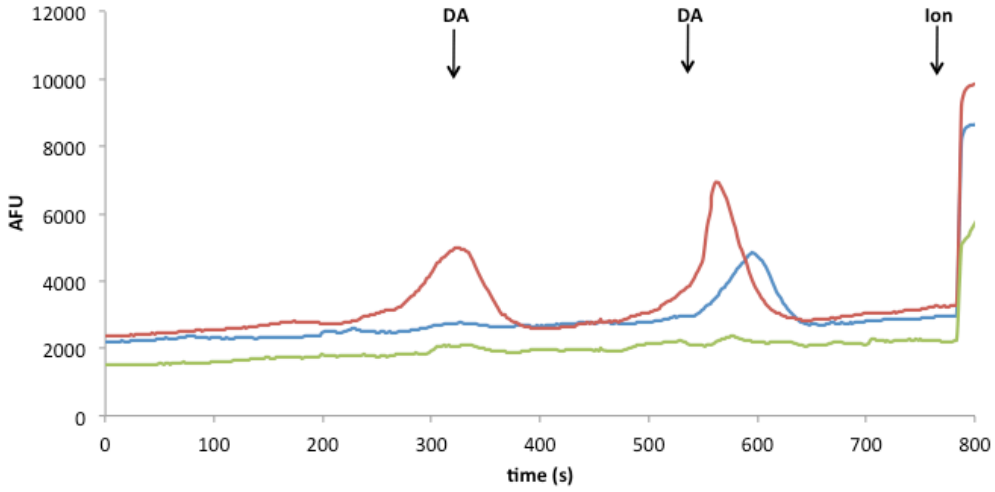


Figure 7.15. Optimization of adipose NCSC neuronal differentiation

Immunostaining of neuronal cultures at day 40. Cells were stained for β-Tubulin III. Representative western blots of β-Tubulin III protein levels in control and heterozygous *GBA* mutation positive carriers at different stages of neuronal differentiation (A). Quantification of β-Tubulin III protein normalized to GAPDH (B). Data are represented as mean + SEM; experiments were independently repeated for n=2. Levels of β-Tubulin III were optimal at 40 days neuronal differentiation (B) and consistent with levels in human brain (C).

Response to a dopamine flux in NCSC-derived neurons differentiated for 40 days as measured by the level of fluorescence (arbiturary fluorescence units, AFU) on live cell imaging.



40 days differentiation

	No. cells responded	total	%
Glutatmate	39	182	21%
NMDA	21	135	16%
Dopamine	33	59	56%
KCl	1	61	2%
Ossillating	46	281	16%
Ossillating inhibite by NMDA	33	38	87%

Figure 7.16. Functional analysis of differentiated neuronal cells

Functional analysis of wild type neuronal cultures at day 40. At day 40, 56% neuronal cells were responding to dopamine.

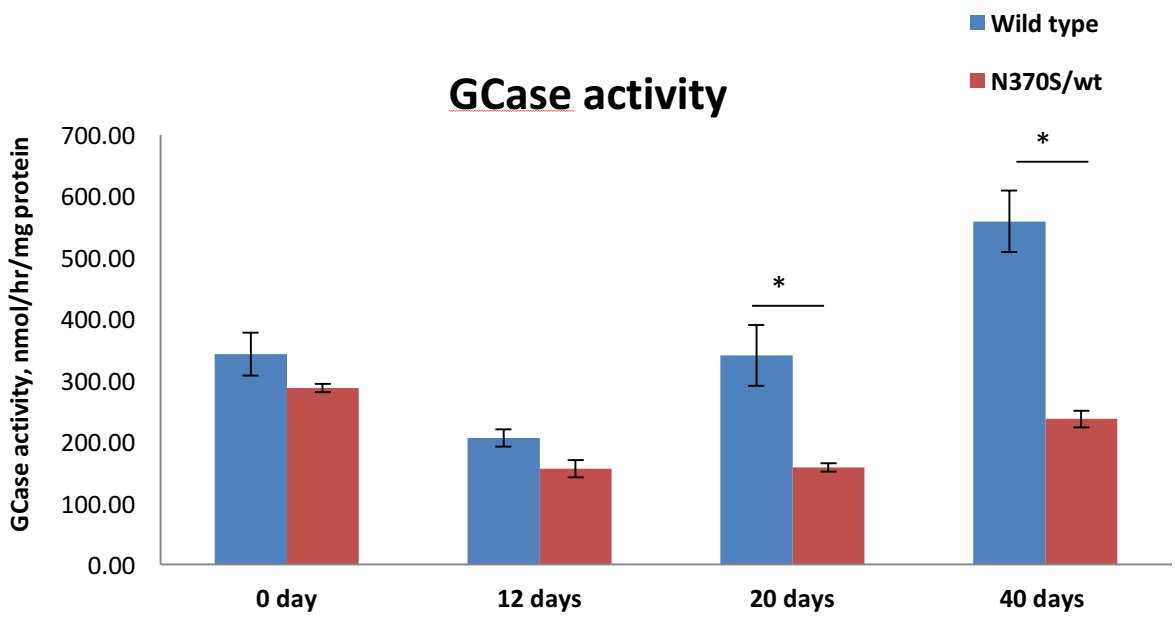


Figure 7.17. *GBA* mutant NCSC-derived neurons show decreased glucocerebrosidase activity at different stages of differentiation.

GCase activity was measured in neurons from control and heterozygous *GBA* mutation positive carrier NCSC-derived neurons at differentiation day 0, 12, 20 and 40. Enzyme activities are expressed as nmol/hr/mg. Data are represented as mean + SEM.; experiments were independently repeated three times in triplicate. *p<0.05; one-way ANOVA and Student's t-test.

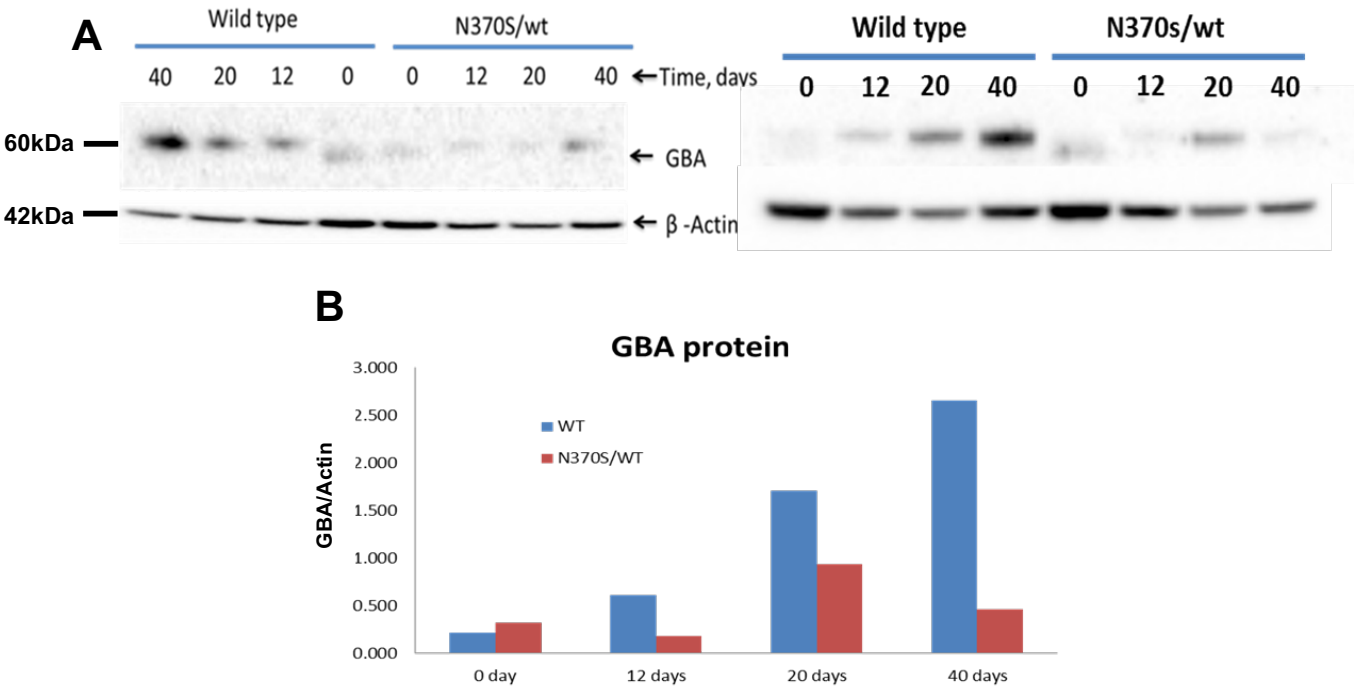


Figure 7.18. *GBA* mutant NCSC-derived neurons show decreased glucocerebrosidase protein levels at different stages of differentiation.

Representative western blots of GCase protein levels in control and heterozygous *GBA* mutation positive carriers at different stages of neuronal differentiation (A). There were significant reductions of GBA protein in *GBA* mutation carriers compared to controls. Quantification of GBA protein normalized to β-actin (B). Data are represented as mean + SEM; experiments were independently repeated for n=2.

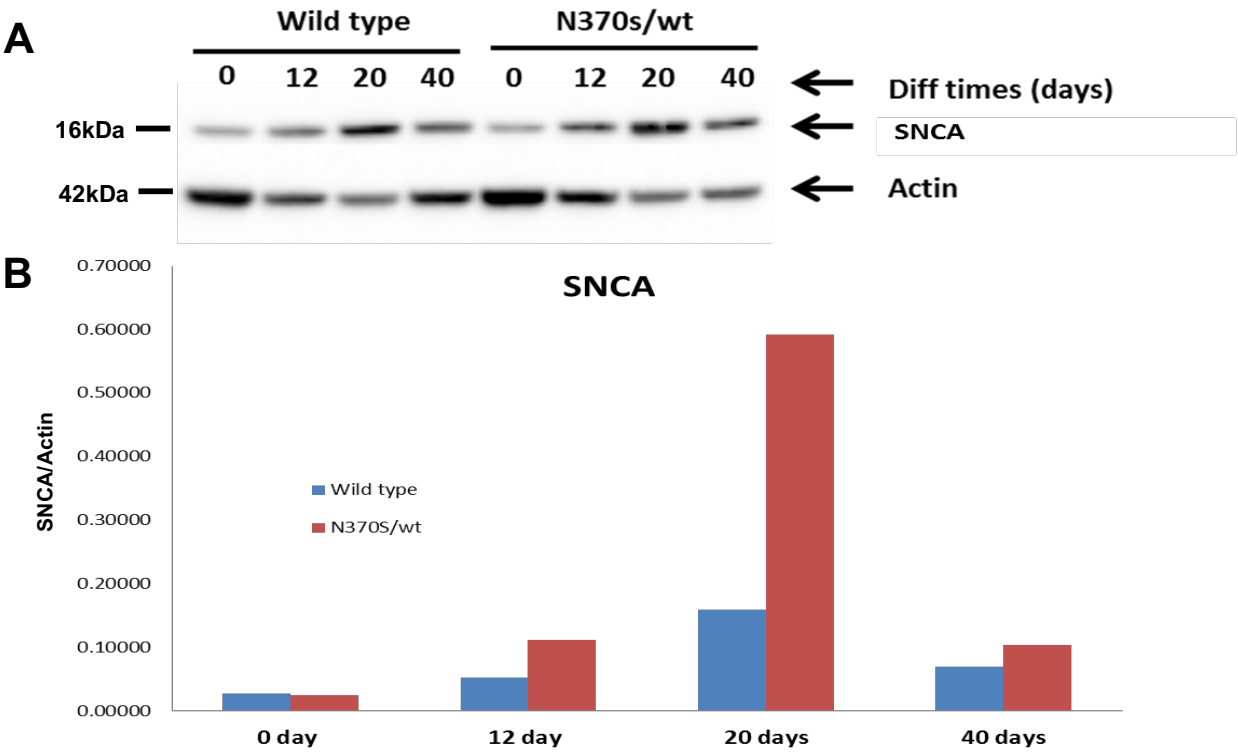


Figure 7.19. *GBA* mutant NCSC-derived neurons show increased α-synuclein (SNCA) protein levels at different stages of differentiation.

Representative western blots of SNCA protein levels in control and heterozygous *GBA* mutation positive carriers at different stages of neuronal differentiation (A). There were significant increases of SNCA protein in *GBA* mutation carriers compared to controls. Quantification of SNCA protein normalized to β-actin (B). Data are represented as mean + SEM; experiments were independently repeated for n=2.

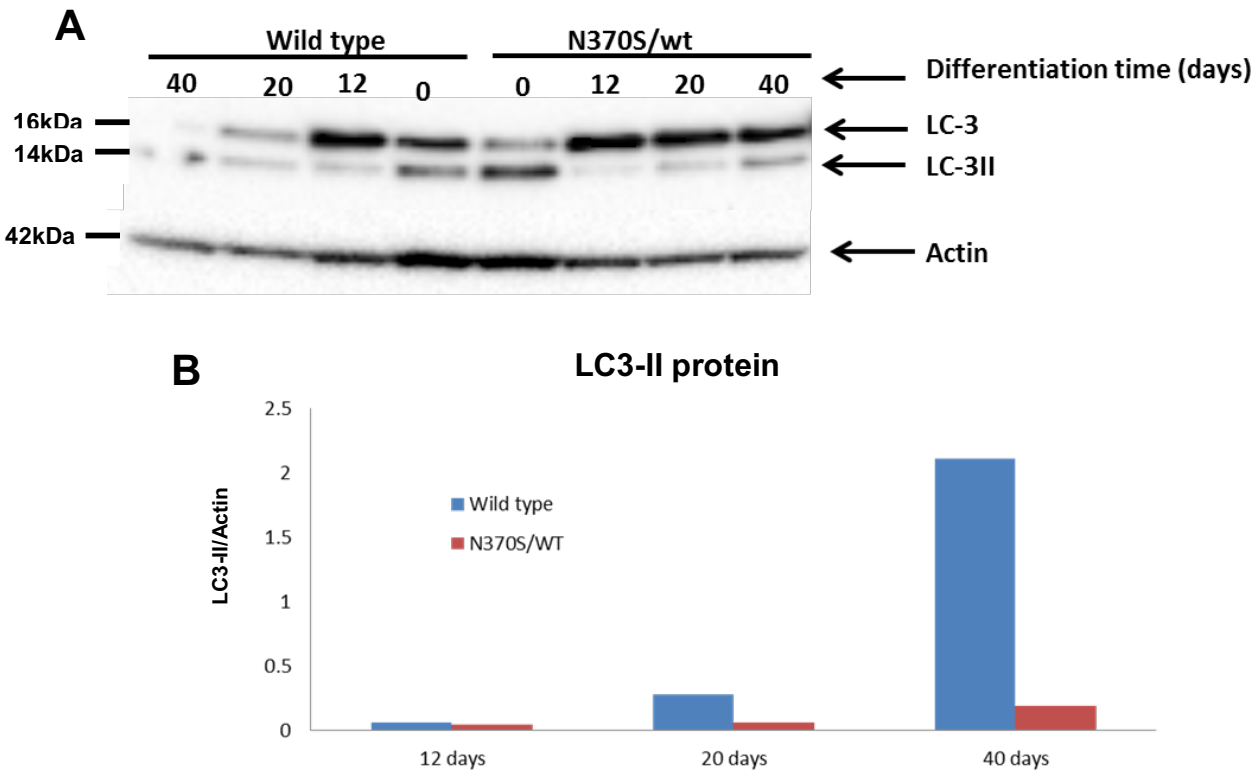


Figure 7.20. Changes in autophagy in *GBA* mutant NCSC-derived neurons at different stages of differentiation.

Representative western blots of LC3 protein levels in control and heterozygous *GBA* mutation positive carriers at different stages of neuronal differentiation (A). There were significant reductions of LC3-II protein in *GBA* mutation carriers compared to controls for all four time points studied. Quantification of LC3-II protein normalized to β-actin (B). Data are represented as mean + SEM; experiments were independently repeated for n=2.

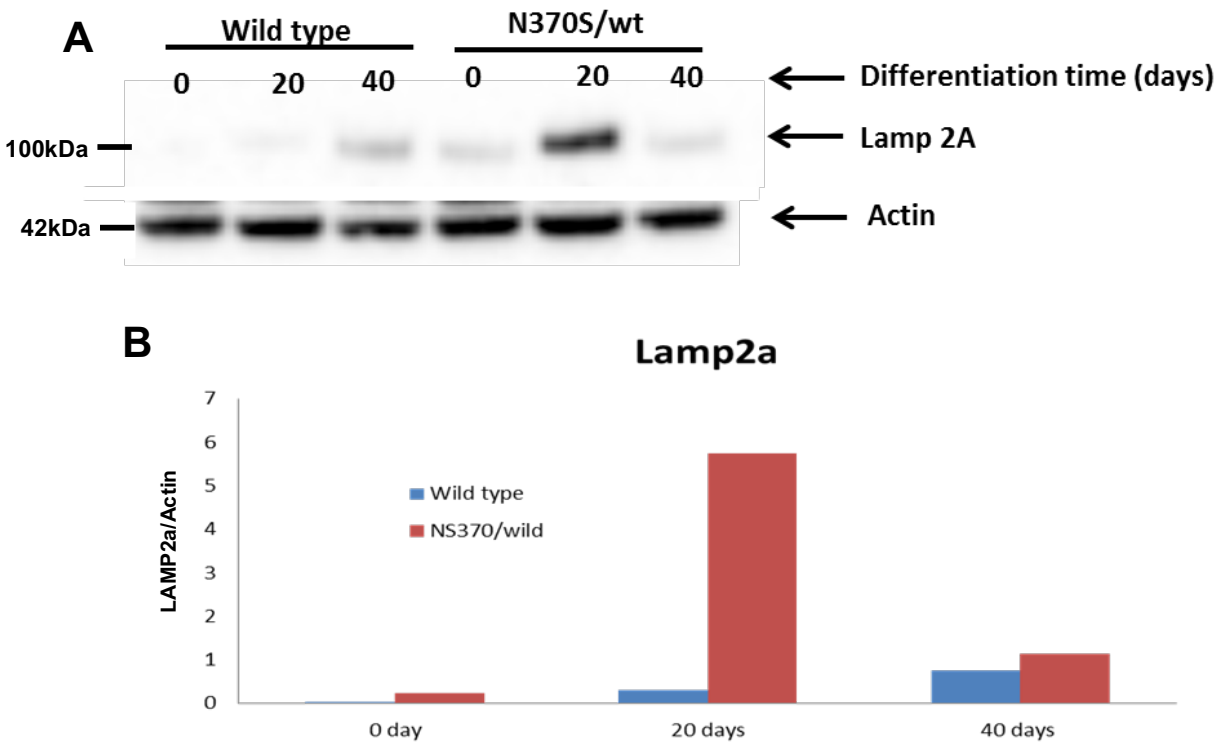


Figure 7.21. Changes in autophagy in *GBA* mutant NCSC-derived neurons at different stages of differentiation.

Representative western blots of LAMP2a protein levels in control and heterozygous *GBA* mutation positive carriers at different stages of neuronal differentiation (A). Quantification of LAMP2a protein normalized to β-actin (B). Data are represented as mean + SEM; experiments were independently repeated for n=2.

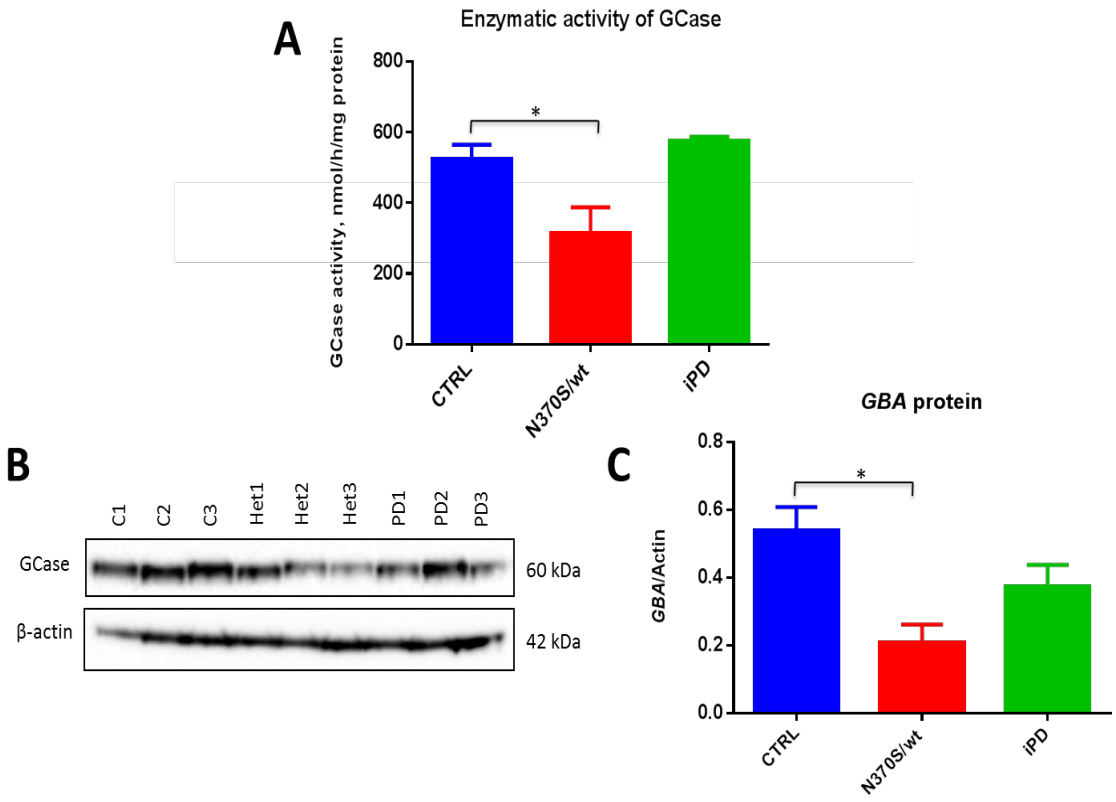


Figure 7.22. *GBA* mutant NCSC-derived neurons show decreased glucocerebrosidase enzyme activities and protein levels.

Glucocerebrosidase enzyme activity was measured in neurons from control, *GBA* heterozygous, and IPD NCSC-derived neurons at differentiation day 40 (A). Enzyme activities are expressed as nmol/hr/mg. Representative western blots of GBA protein levels in control, heterozygous *GBA* mutation positive carriers, and PD NCSC-derived neurons (B). There were significant reductions of GBA protein in *GBA* mutation carriers compared to controls (C). Data are represented as mean + SEM.; experiments were independently repeated three times. *$p<0.05$; one-way ANOVA and Student's *t*-test.

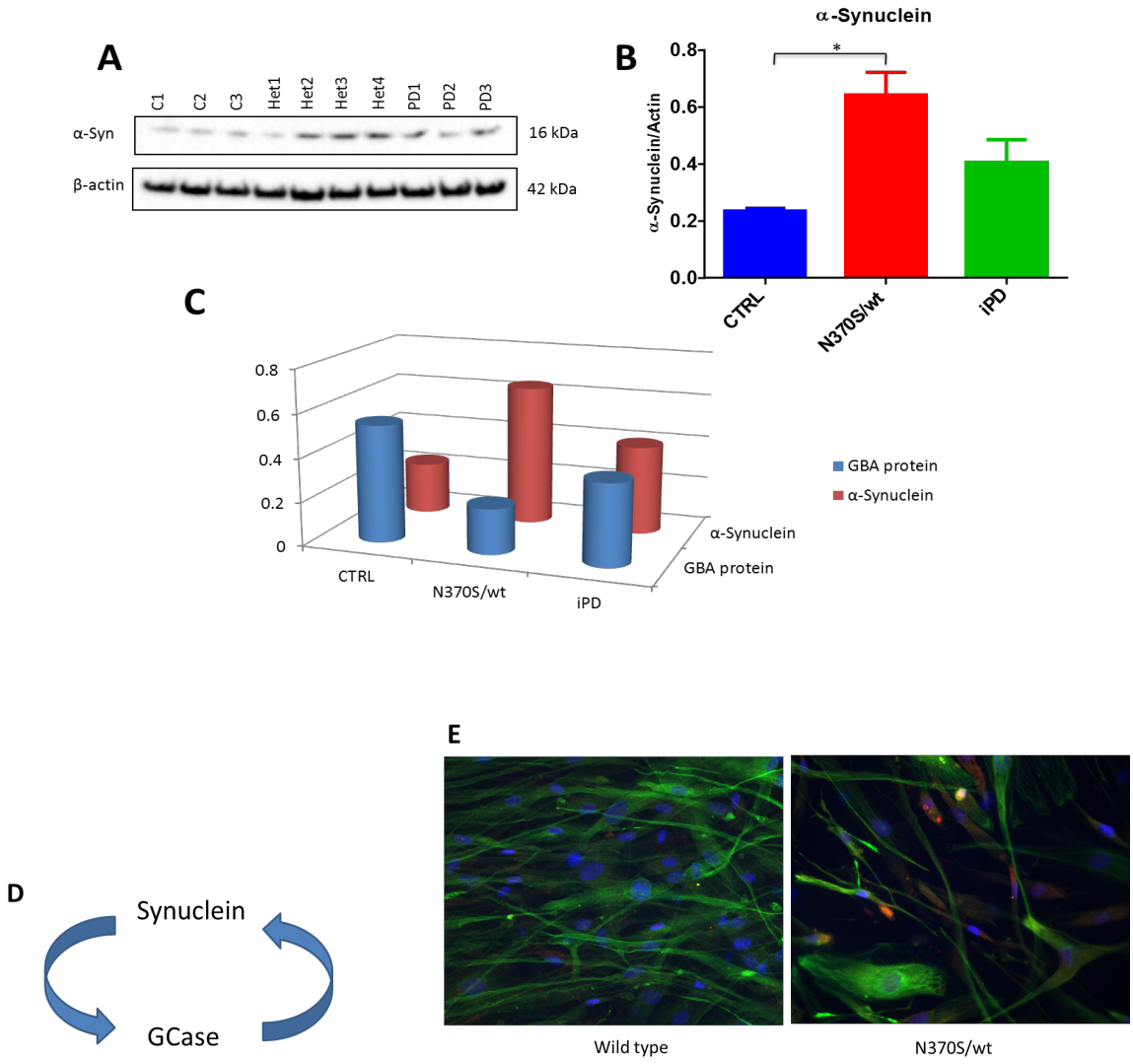


Figure 7.23. α-Synuclein levels are increased in *GBA* mutant NCSC-derived neurons.

Representative western blots showing the protein levels of SNCA in control, heterozygous *GBA* mutation positive carriers and PD NCSC-derived neurons at differentiation day 40 (A). Optical densities of SNCA bands were normalized by the averaged value of β-actin and expressed as a percentage of the control (CTRL) line value (B). Data are represented as mean + SEM.; experiments were independently repeated three times. *$p<0.05$, one-way ANOVA and Student's t-test. The reciprocal relationship between GCase and SNCA (C) (D). Immunocytochemistry of neuronal cultures at day 40 (E). Cells were stained for β-Tubulin III (TUB) (green), and SNCA (red). Nuclei were counterstained with DAPI (blue).

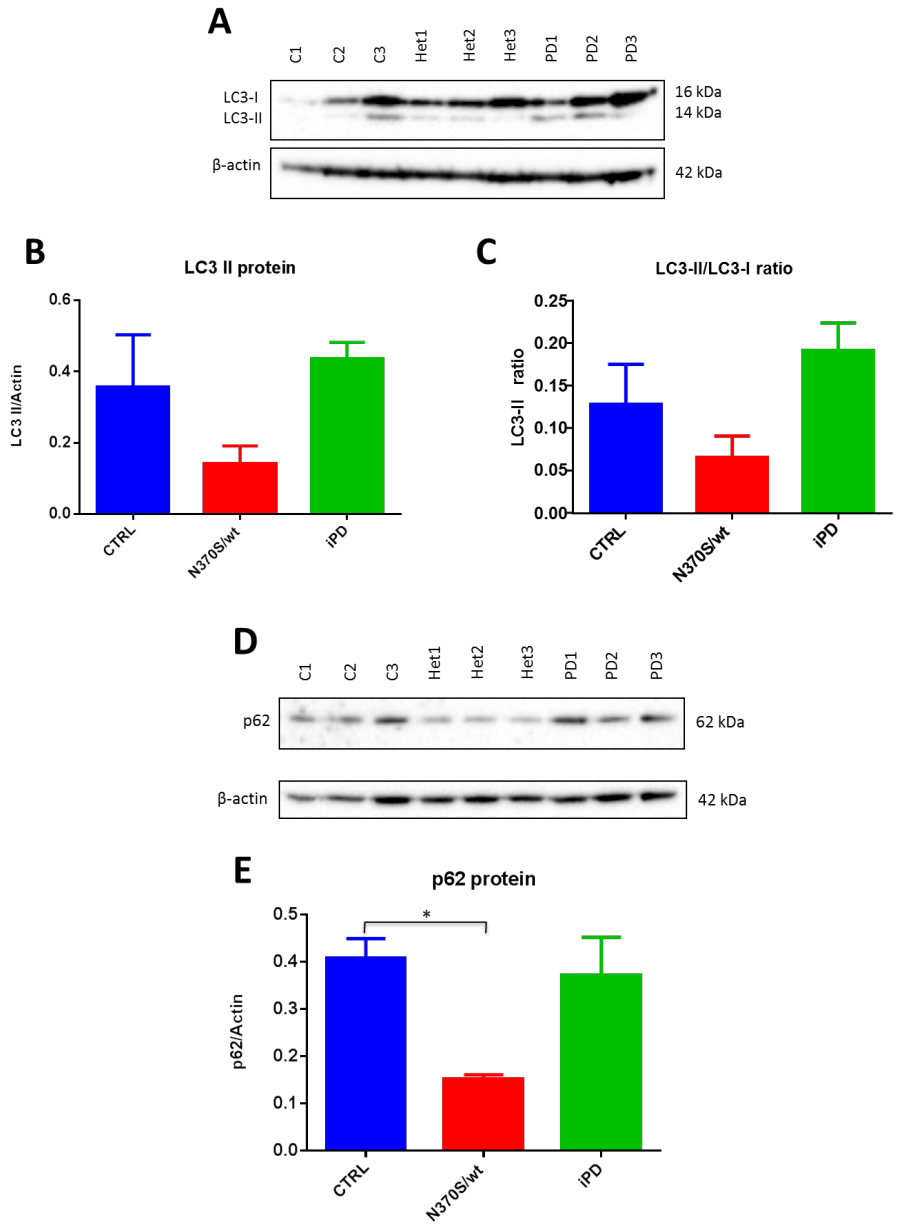


Figure 7.24. *GBA* mutant NCSC-derived neurons show changes in the autophagic/lysosomal system.

Representative western blots showing the protein levels of LC3 in NCSC-derived neuronal cultures at differentiation day 40 (A). Quantification of the basal levels of LC3-II relative to β-actin (B). Quantification of the ratio of LC3-II to LC3-I normalized to β-actin (C). Representative western blots showing the protein levels of p62 in NCSC-derived neuronal cultures at day 40 (D). Quantification of the basal levels of p62 relative to β-actin (E). Data are represented as mean + SEM.; experiments were independently repeated three times. *p<0.05; one-way ANOVA and Student's t-test.

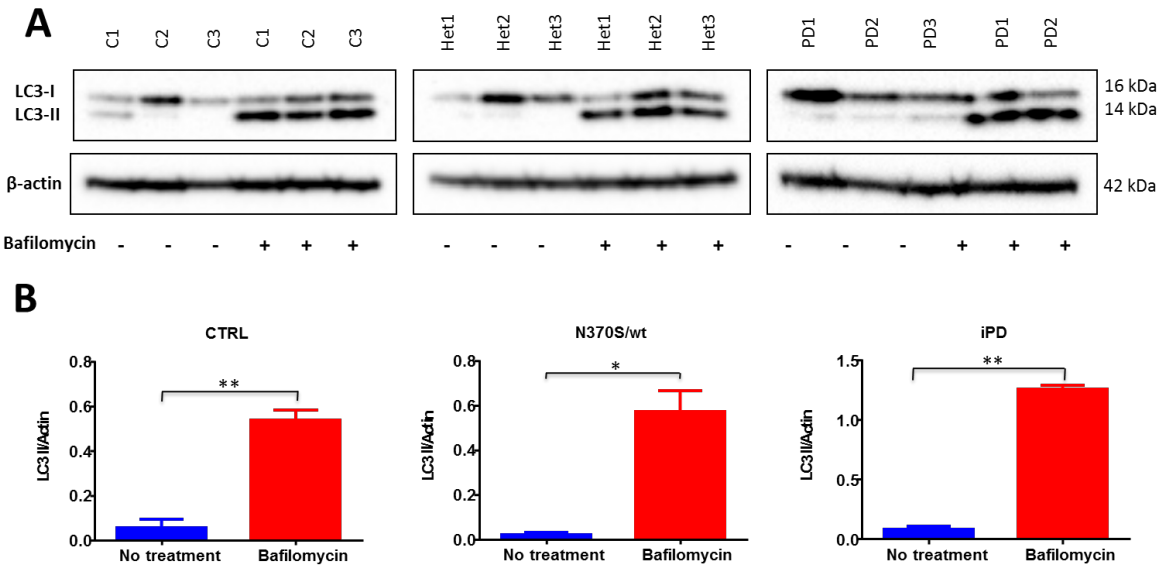


Figure 7.25. Bafilomycin increases LC3-II in *GBA* mutant NCSC-derived neurons

Western blot analysis for LC3 in NCSC-derived neuronal cultures at differentiation day 40, untreated (−) or treated with 0.2μM bafilomycin for 6 h (+) (A). Quantification of the basal levels of LC3-II relative to β-actin (B). Data are represented as mean + SEM; experiments were independently repeated three times in triplicate. *p<0.05; **p<0.01; one-way ANOVA and Student's t-test.

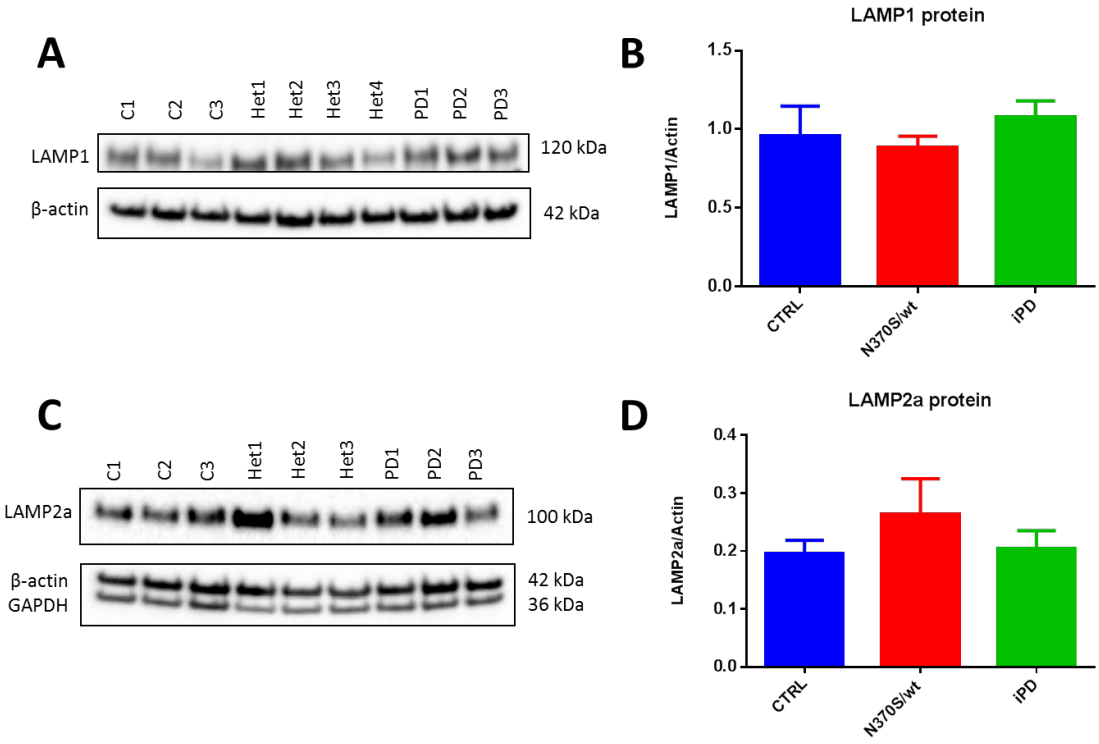


Figure 7.26. LAMP1 and LAMP2a levels are unaffected by the *GBA* mutation in NCSC-derived neurons.

Immunoblotting of differentiated NCSC cultures at differentiation day 40 under basal conditions. Western blot analysis for LAMP1 in NCSC-derived neuronal cultures (A). Quantification of the basal levels of Lamp1 relative to β-actin (B). Western blot analysis for LAMP2a in NCSC-derived neuronal cultures (C). Quantification of the basal levels of Lamp2a relative to β-actin (D). Data are represented as mean + SEM; experiments were independently repeated three times.

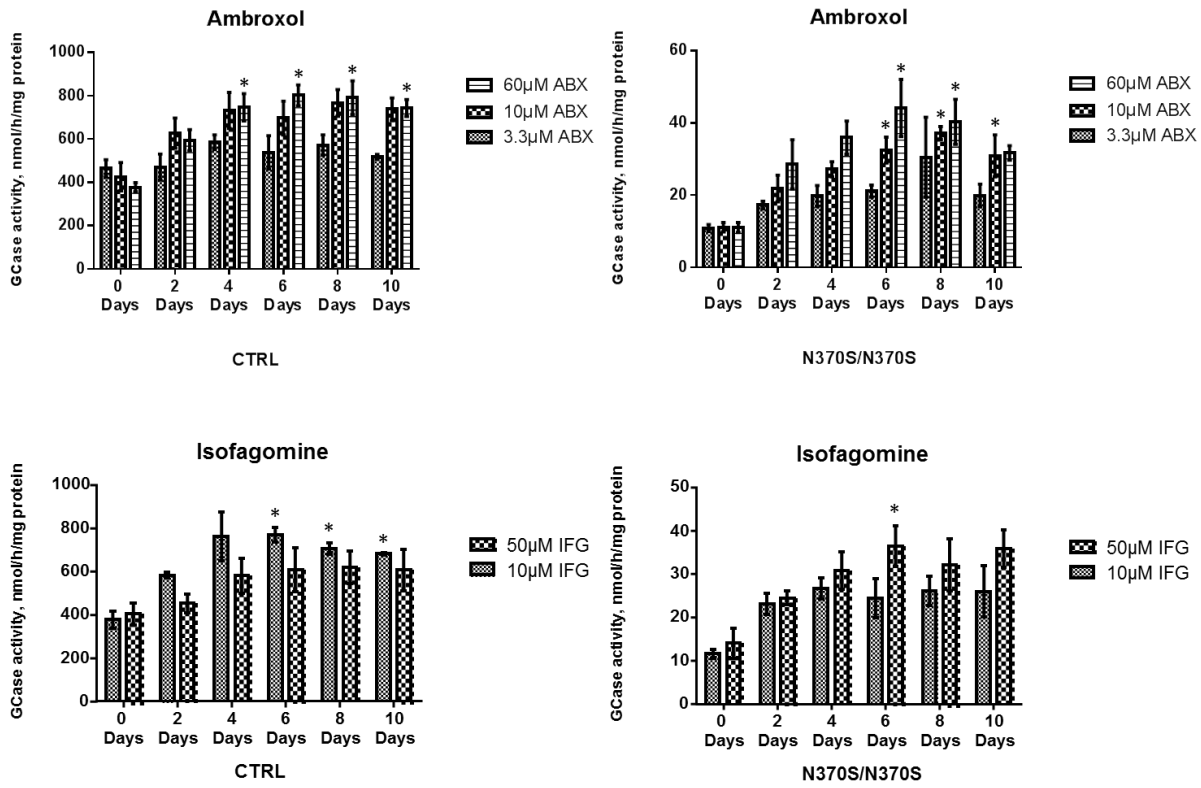


Figure 7.27. Effect of ambroxol and isofagomine on enzymatic GCase activity

Skin fibroblast cultures from a control (CTRL) and a GD patient (N370S/N370S) were treated with selected concentrations of ambroxol (ABX) and isofagomine (IFG), over an increasing number of days at 37°C. Samples containing 20µg protein were tested for GCase activity using 5mM of 4-methylumbelliferyl-β-D-glucopyranoside substrate. Data are expressed as the fold increase in GCase activity in the presence of ABX and IFG, in comparison to untreated cells (0 days of treatment). The results represent the mean ± SEM, of three independent experiments. Data were analysed using analysis of variance (One-way ANOVA). *, $P = < 0.05$.

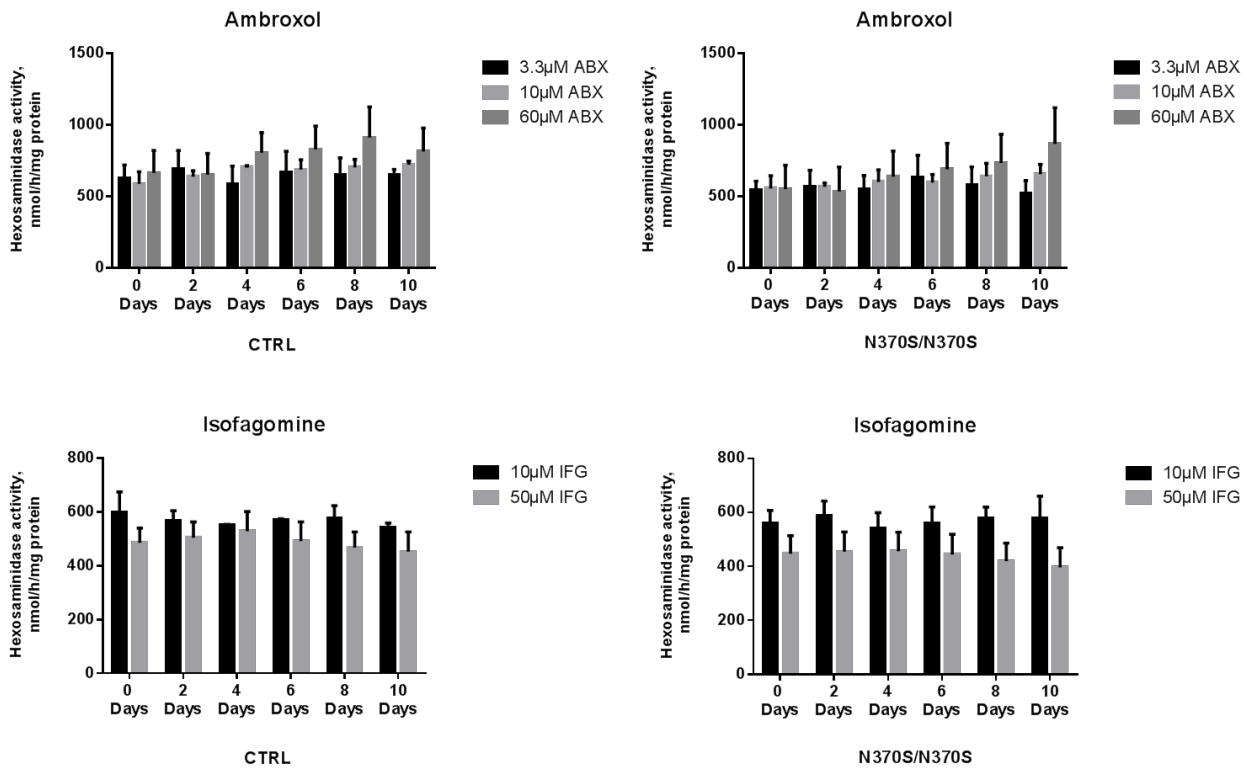


Figure 7.28. Effect of ambroxol and isofagomine on hexosaminidase activity.

Skin fibroblast cultures from a control and a GD patient (N370S/N370S) were treated with selected concentrations of ambroxol (ABX) and isofagomine (IFG), over an increasing number of days at 37°C. Samples containing 20μg protein were tested for hexosaminidase activity using 4-methylumbelliferyl-N-acetyl-glucosamide substrate. Data are expressed as the fold increase in activity in the presence of ABX and IFG, in comparison to untreated cells (0 days of treatment). The results represent the mean ± SEM, of three independent experiments. Data were analysed using analysis of variance (One-way ANOVA).

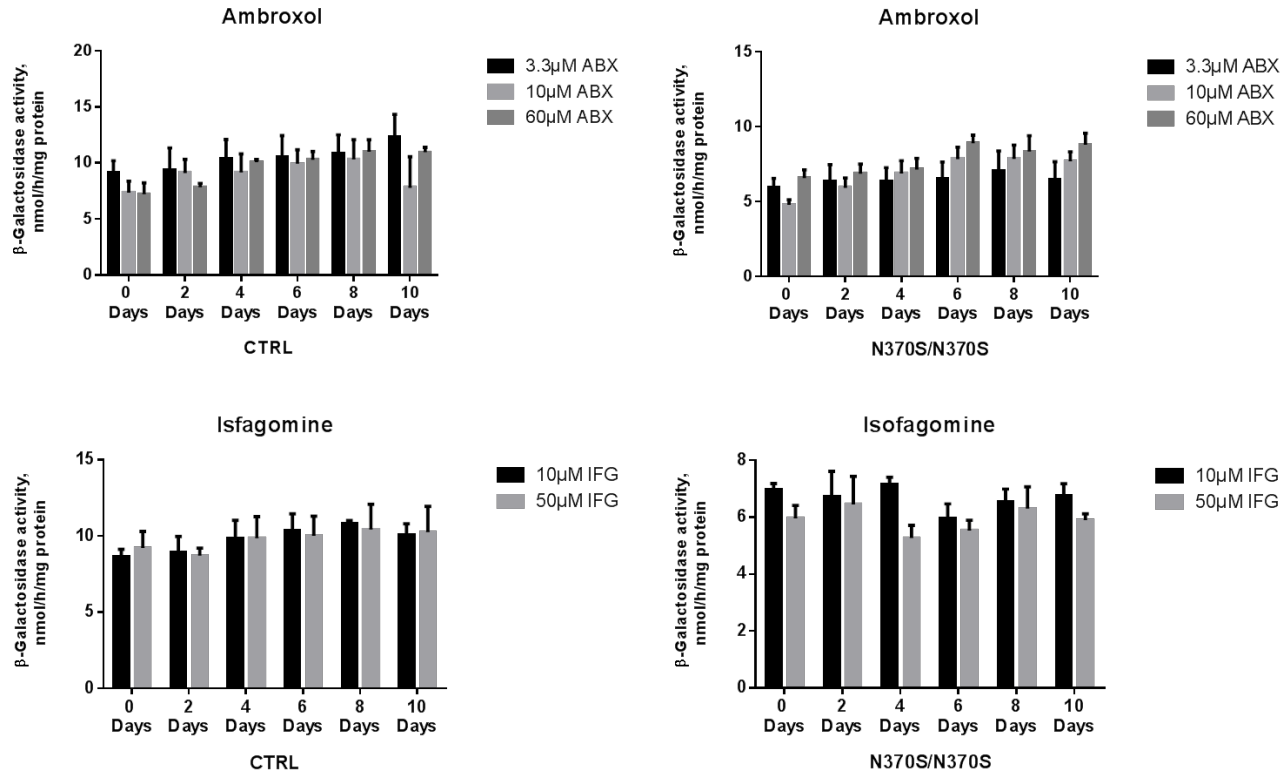


Figure 7.29. Effect of ambroxol and isofagomine on β-galactosidase activity.

Skin fibroblast cultures from a control and a GD patient (N370S/N370S) were treated with selected concentrations of ambroxol (ABX) and isofagomine (IFG), over an increasing number of days at 37°C. Samples containing 20μg protein were tested for β-Galactosidase activity using 4-methylumbelliferyl-β-D-galactopyranoside substrate. Data are expressed as the fold increase in activity in the presence of ABX and IFG, in comparison to untreated cells (0 days of treatment). The results represent the mean ± SEM, of three independent experiments. Data were analysed using analysis of variance (One-way ANOVA).

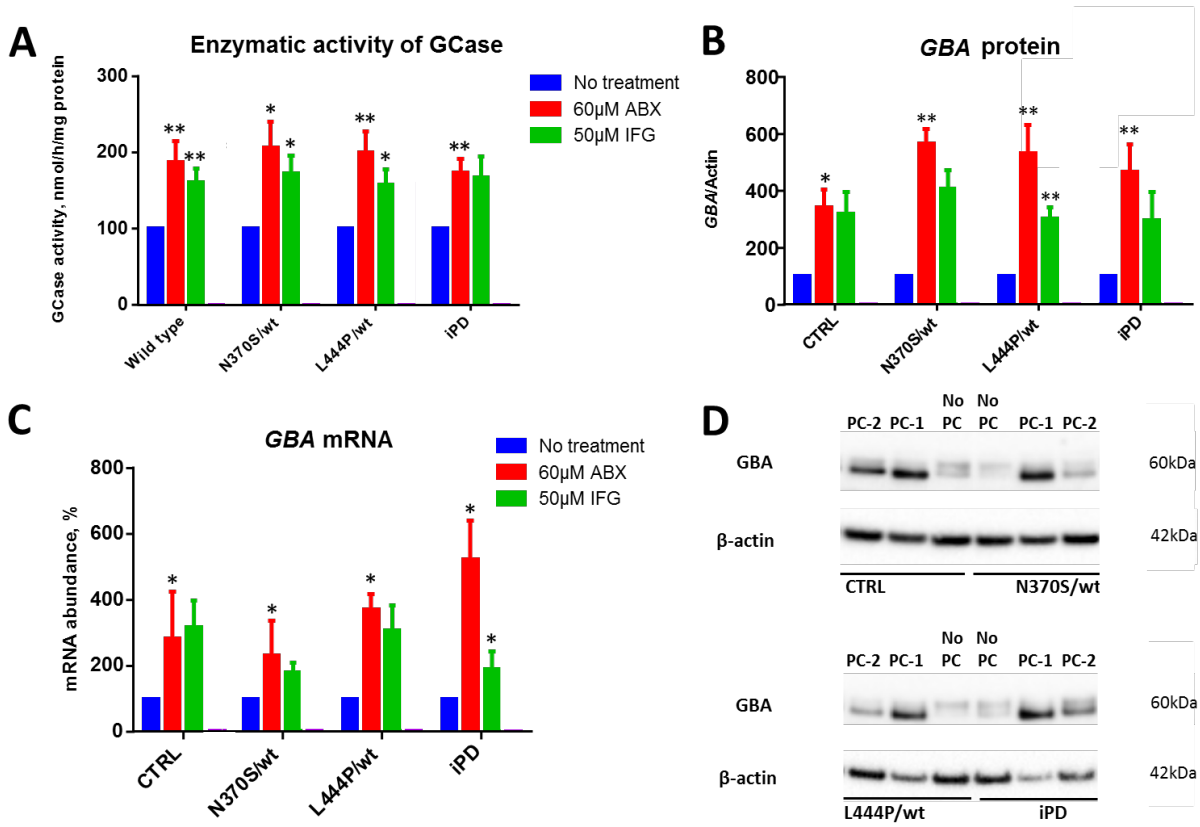


Figure 7.30. Significant restoration of GCase enzyme activity, protein and gene expression in *GBA* mutation carriers

Fibroblasts from controls, heterozygous *GBA* mutation carriers, and IPD patients were treated with 60μM ambroxol or 50μM isofagomine alternate daily for 6 days at 37 °C. Data are expressed as the percentage increase of GCase activity in the presence of each pharmacological chaperone in comparison to untreated cells (A). Quantification of GBA protein in each variant was determined by western blot with human-specific antibodies. All PC-treated cells showed increases in GBA protein levels. These are presented as the percentage change relative to the untreated cells (B). PC treatment significantly increased *GBA* messenger RNA levels. Bar chart demonstrating significant increases in *GBA* messenger RNA levels after PC treatment in controls, heterozygous *GBA* carriers and IPD (C). Representative western blots of GBA protein levels in control, *GBA* mutation carriers with and without PD, and idiopathic PD fibroblasts (D). The results represent the mean ± SEM, of three independent experiments. *p<0.05; **p<0.01; one-way ANOVA and Student's t-test. PC, pharmacological chaperone; PC-1, ambroxol; PC-2, isofagomine.

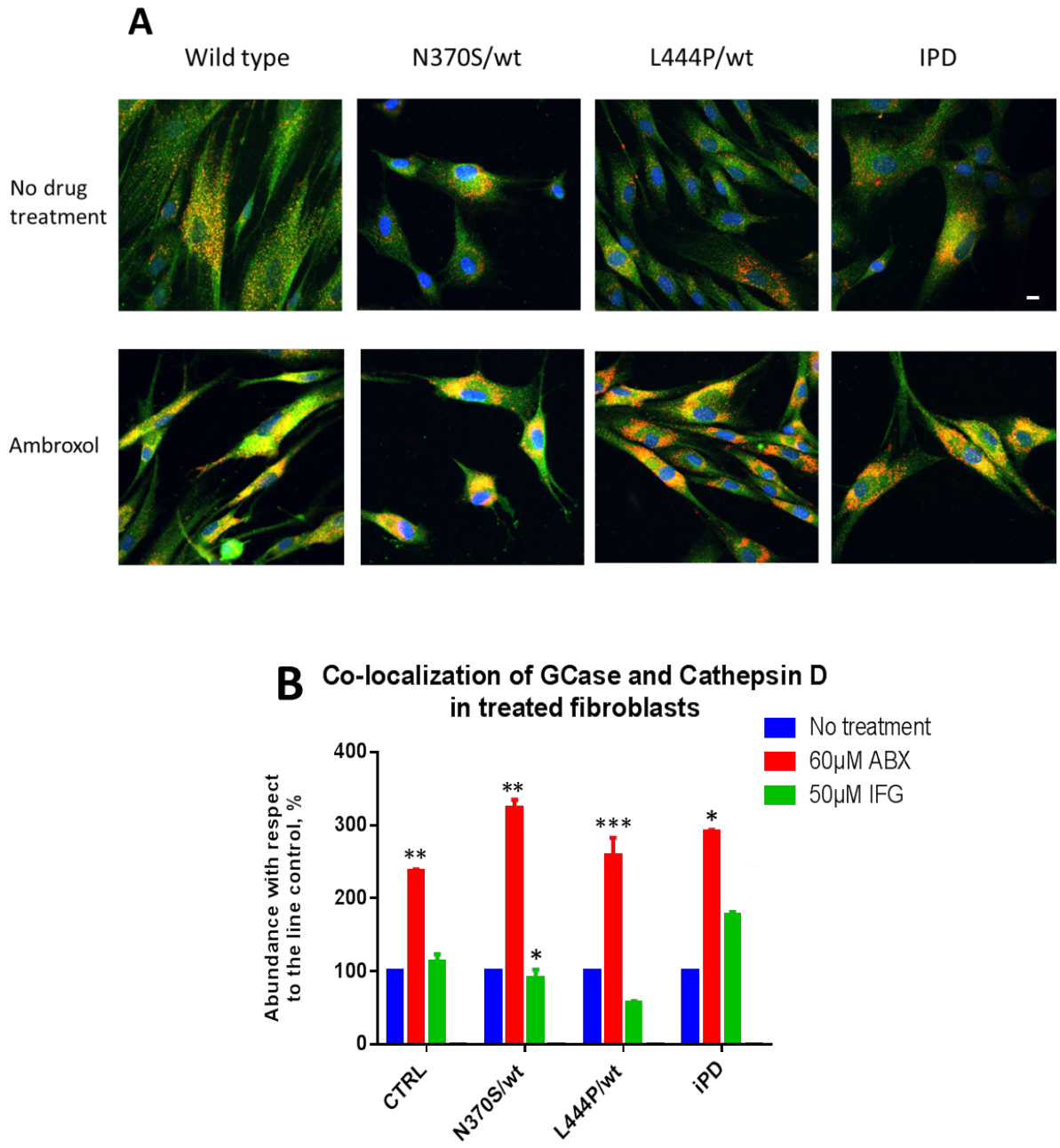


Figure 7.31. Correction of GBA localization in *GBA* mutation carriers

Representative slices (depth 3μM) showing colocalization (*yellow)* of GBA (*green*) with lysosomal marker cathepsin D (*red*), conducted by immunofluorescence staining (A). Nuclei were counterstained with DAPI (*blue*). Quantification of the percentage increase of colocalization in the presence of each pharmacological chaperone in comparison to untreated cells (B). Data are represented as the mean ± SEM, of three independent experiments. **p*<0.05; ***p*<0.01*;* ****p*<0.001; one-way ANOVA and Student's *t*-test. *Scale bar*, 10 μm.

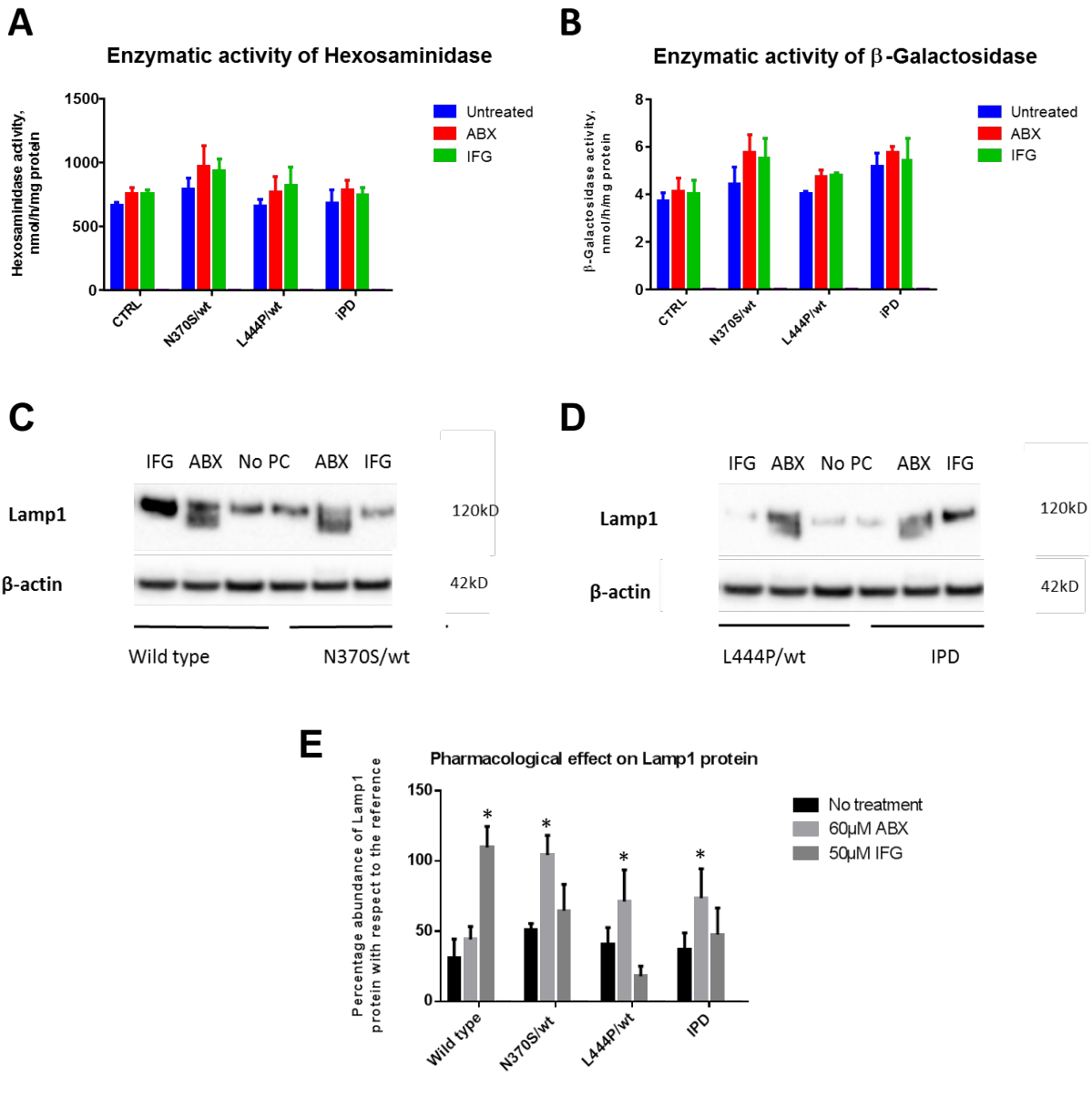


Figure 7.32. Expansion of the lysosomal compartment in *GBA* mutation carriers

Hexosaminidase and β-galactosidase activity were unchanged in heterozygous *GBA* mutation carrier fibroblasts compared with healthy control cells (A) (B). Representative western blots for LAMP1 protein in human fibroblasts (C) (D). Quantification of the basal levels of LAMP1 relative to β-actin (E). Data are represented as the mean ± SEM, of three independent experiments. *p<0.05; one-way ANOVA and Student's *t*-test. PC, pharmacological chaperone; ABX, ambroxol; IFG, isofagomine.

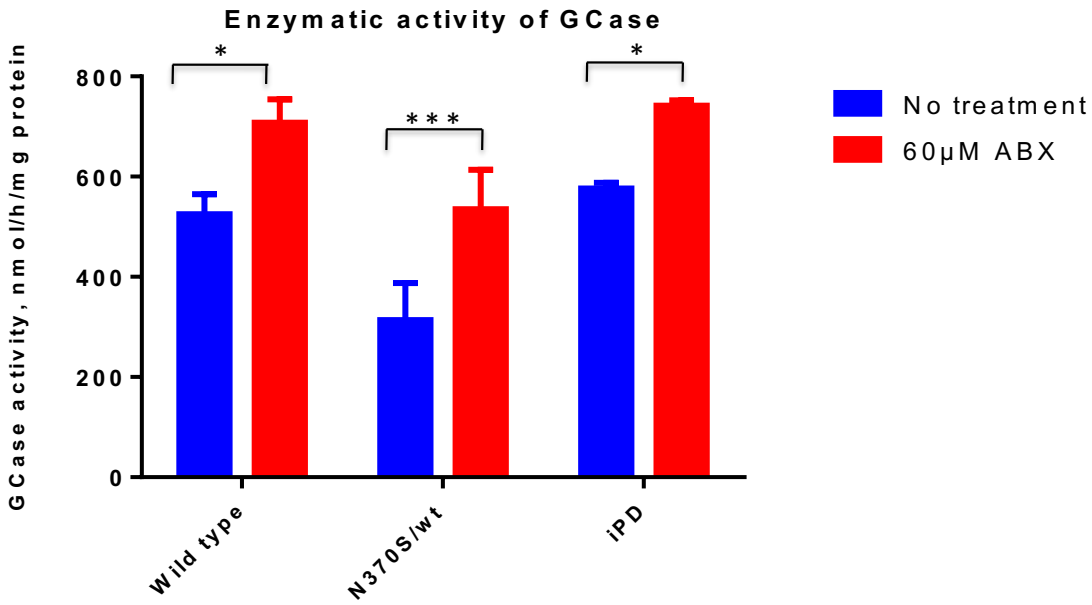


Figure 7.33. Glucocerebrosidase enzyme activities are enhanced by ambroxol in *GBA* mutant NCSC-derived neurons.

NCSC-derived neurons from controls, heterozygous *GBA* mutation carriers, and IPD patients were treated with 60µM ambroxol alternate daily for 6 days at 37 °C. Samples containing 20µg of protein were tested for GCase activity as described. Data are expressed as the increase of GCase activity in the presence of ambroxol in comparison to untreated cells. The results represent the mean ± SEM, of three independent experiments. *p<0.05; ***p<0.001; one-way ANOVA and Student's t-test.

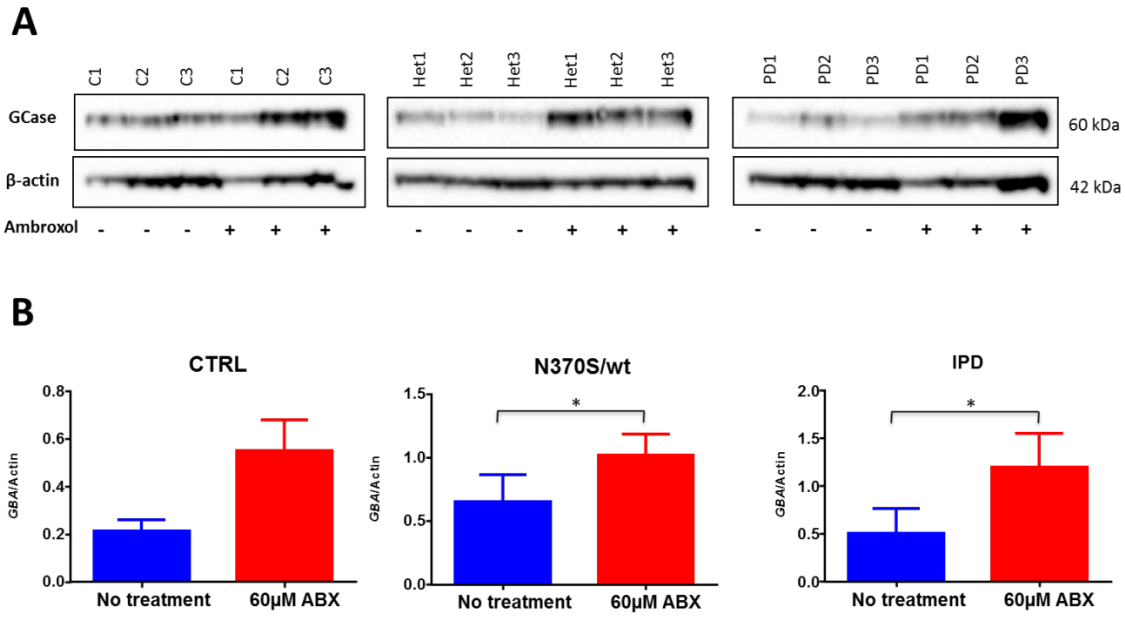


Figure 7.34. Glucocerebrosidase protein levels are increased by ambroxol in *GBA* mutant NCSC-derived neurons.

NCSC from controls, heterozygous *GBA* mutation carriers, and IPD patients were differentiated for 40 days and treated with 60μM ambroxol alternate daily for 6 days at 37 °C. Representative western blots of GCase protein levels in control, heterozygous *GBA* mutation positive carriers, and PD NCSC-derived neurons (A). Ambroxol treatment significantly increased GBA protein levels. Bar chart demonstrating significant increases in GBA protein levels after ABX treatment in heterozygous *GBA* carriers and IPD (B). The results represent the mean ± SEM, of three independent experiments. *p<0.05, one-way ANOVA and Student's t-test.

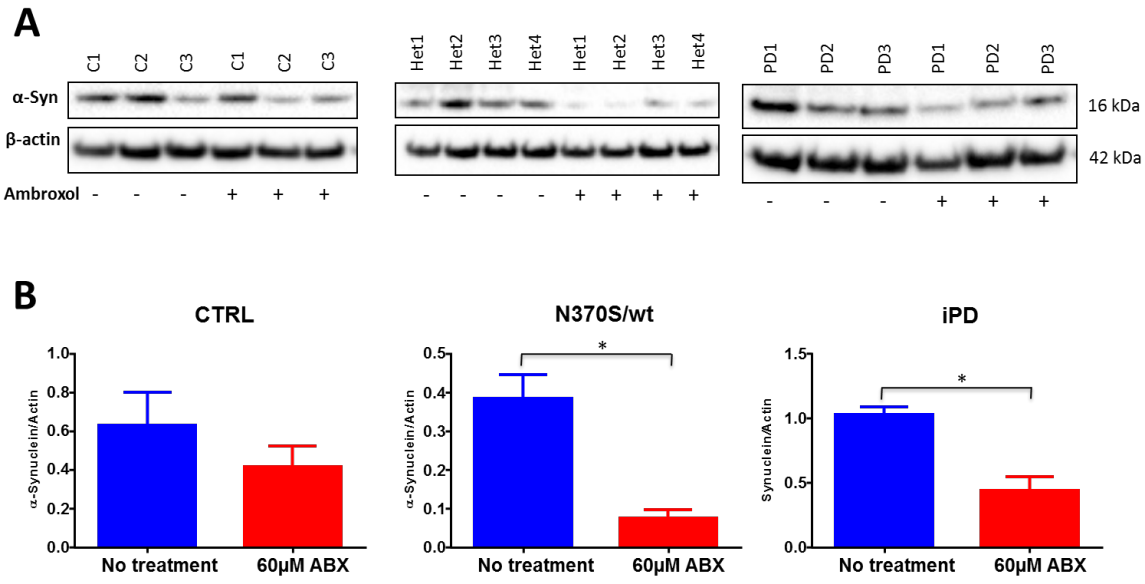


Figure 7.35. α-Synuclein protein levels are reduced by ambroxol in *GBA* mutant NCSC-derived neurons.

NCSC from controls, heterozygous *GBA* mutation carriers, and IPD patients were differentiated for 40 days and treated with 60μM ambroxol alternate daily for 6 days at 37 °C. Representative western blots of SNCA protein levels in control, heterozygous *GBA* mutation positive carriers, and PD NCSC-derived neurons (A). Ambroxol treatment significantly reduced SNCA protein levels. Bar chart demonstrating significant reductions in SNCA protein levels after ABX treatment in heterozygous *GBA* mutation carriers and IPD (B). The results represent the mean ± SEM, of three independent experiments. *p<0.05, one-way ANOVA and Student's t-test.

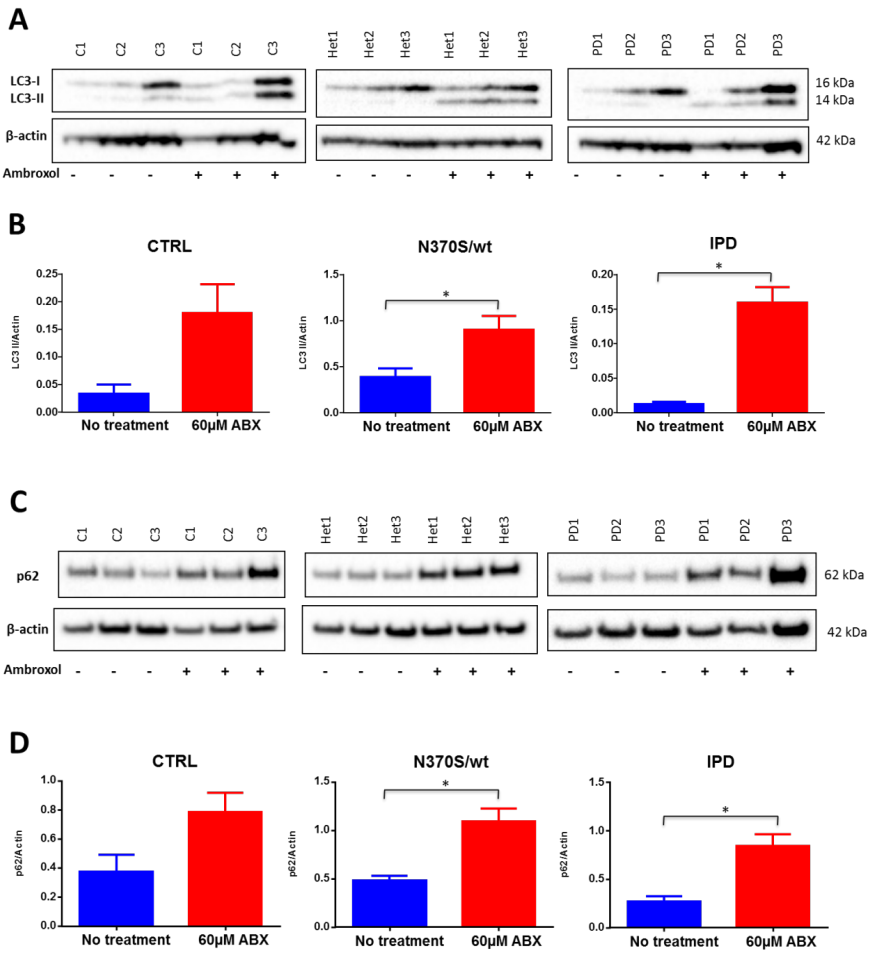


Figure 7.36. Markers of macroautophagy are increased by ambroxol in *GBA* mutant NCSC-derived neurons.

NCSC from controls, heterozygous *GBA* mutation carriers, and IPD patients were differentiated for 40 days and treated with 60μM ambroxol alternate daily for 6 days at 37 °C. Representative western blots of LC3 protein levels (A). Ambroxol treatment significantly increased LC3-II protein levels. Bar chart demonstrating significant increases in LC3-II protein levels after ABX treatment in heterozygous *GBA* carriers and IPD (B). Representative western blots of p62 protein levels in control, heterozygous *GBA* mutation positive carriers, and PD NCSC-derived neurons (C). Ambroxol treatment significantly increased p62 protein levels. Bar chart demonstrating significant increases in p62 protein levels after ABX treatment in heterozygous *GBA* mutation carriers and IPD (D). The results represent the mean ± SEM, of three independent experiments. *p<0.05; one-way ANOVA and Student's t-test.

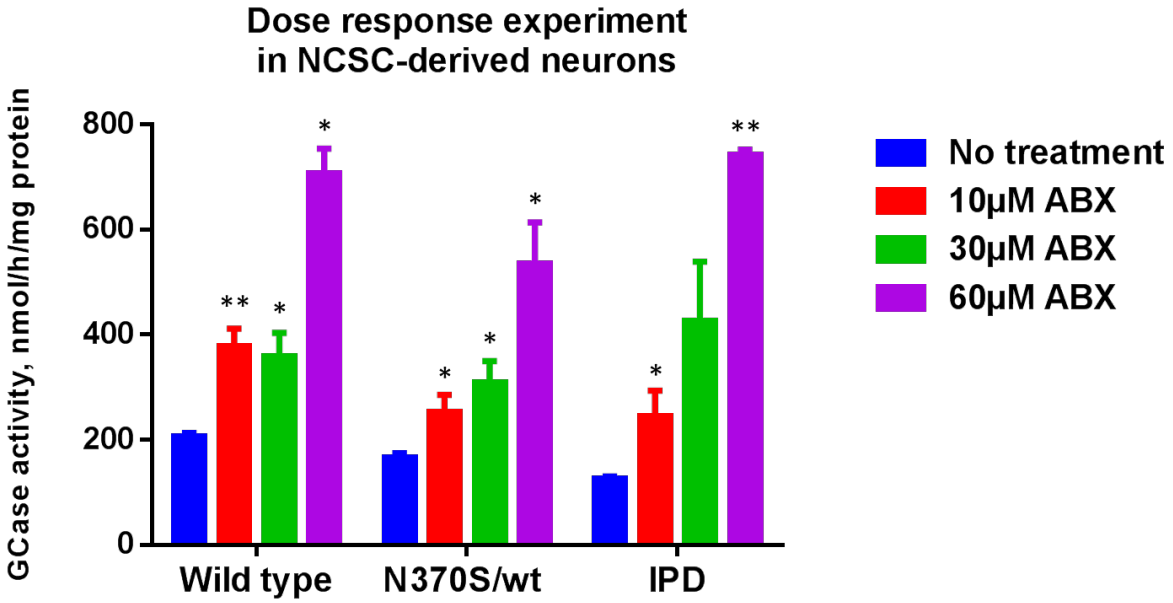


Figure 7.37. Glucocerebrosidase enzyme activities are enhanced by 10μM and 30μM concentrations of ambroxol in *GBA* mutant NCSC-derived neurons.

NCSC cultures from controls, heterozygous *GBA* mutation carriers, and IPD patients were differentiated for 40 days and treated with selected concentrations of ambroxol (ABX), over 6 days at 37°C. Samples containing 20μg protein were tested for GCase activity using 5mM of 4-methylumbelliferyl-β-D-glucopyranoside substrate. Data are expressed as the fold increase in GCase activity in the presence of ABX, in comparison to untreated cells (0 days of treatment). The results represent the mean ± SEM, of three independent experiments. *p<0.05; **p<0.01; one-way ANOVA and Student's t-test.

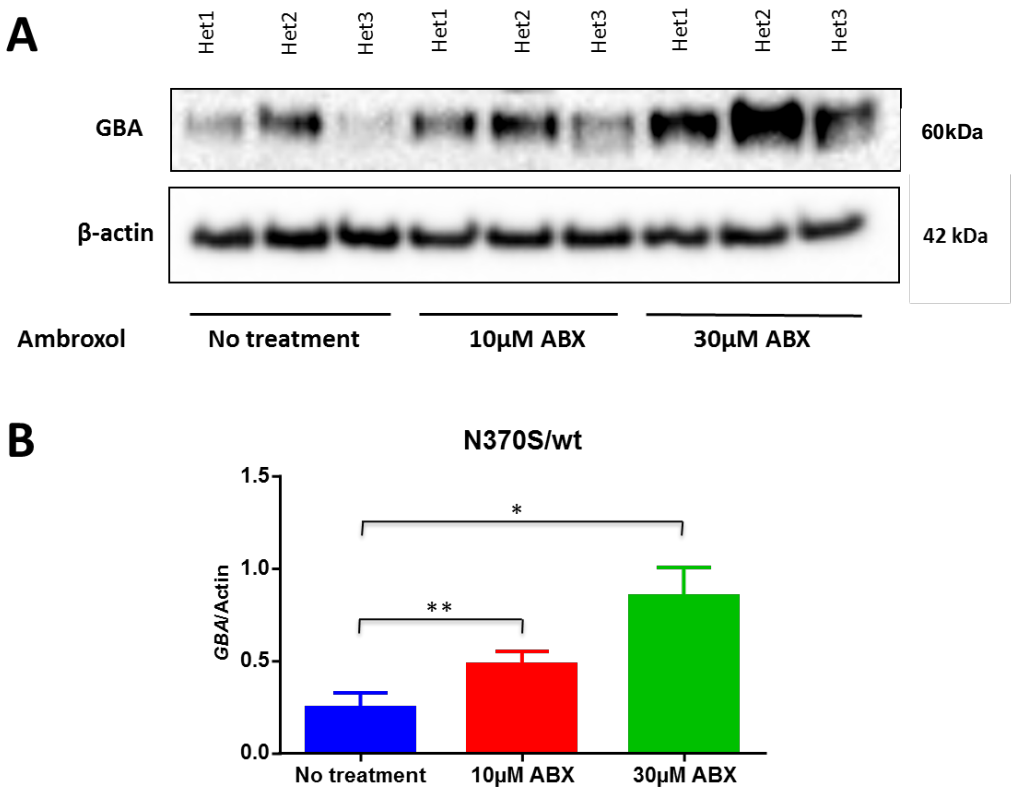


Figure 7.38. GCase protein levels are increased by 10µM and 30µM concentrations of ambroxol in *GBA* mutant NCSC-derived neurons.

NCSC from heterozygous *GBA* mutation carriers were differentiated for 40 days and treated with selected concentrations of ambroxol (ABX), over 6 days at 37°C. Representative western blots of GBA protein levels in heterozygous *GBA* mutation positive carriers NCSC-derived neurons (A). Both 10µM and 30µM ambroxol significantly increased GBA protein levels. Bar chart demonstrating significant increases in GBA protein levels after ABX treatment in heterozygous *GBA* mutation carriers (B). The results represent the mean ± SEM, of three independent experiments. *p<0.05; **p<0.01; one-way ANOVA and Student's t-test.

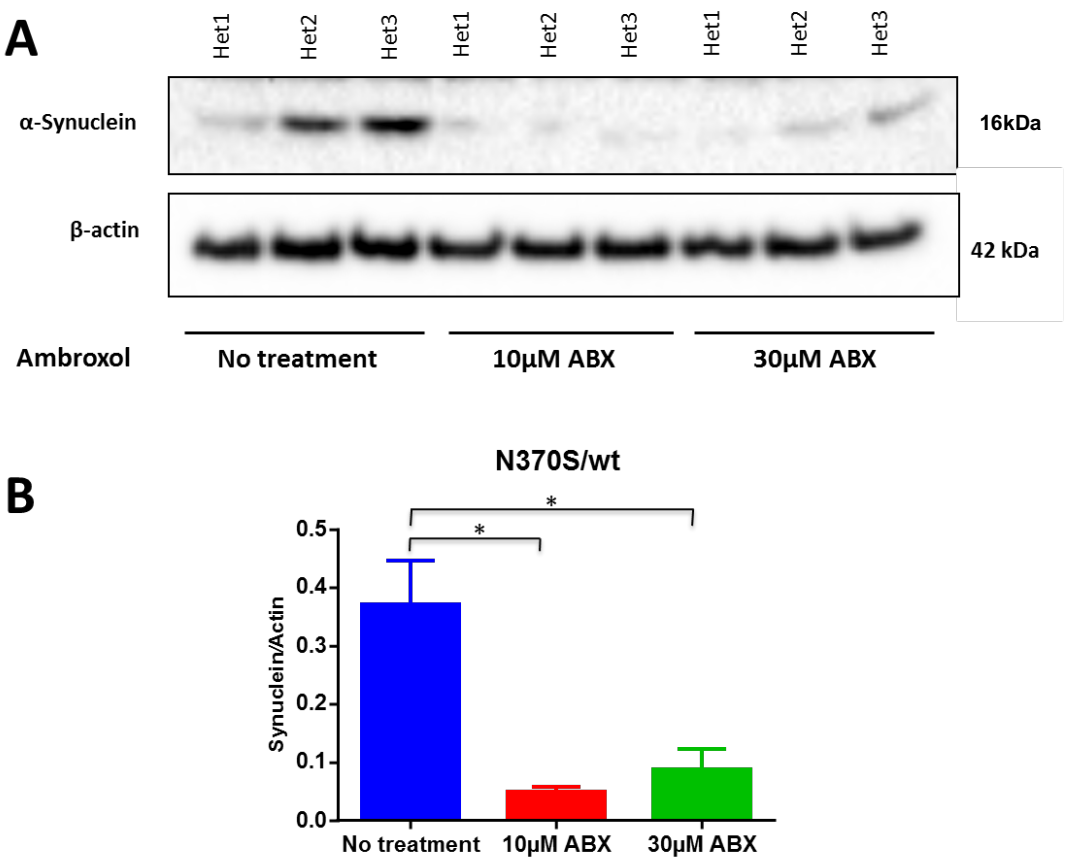


Figure 7.39. α-Synuclein protein levels are reduced by 10μM and 30μM concentrations of ambroxol in GBA mutant NCSC-derived neurons.

NCSC from heterozygous *GBA* mutation carriers were differentiated for 40 days and treated with selected concentrations of ambroxol (ABX), over 6 days at 37°C. Representative western blots of SNCA protein levels in heterozygous *GBA* mutation positive carriers NCSC-derived neurons (A). Both 10μM and 30μM ambroxol significantly reduced SNCA protein levels. Bar chart demonstrating significant reductions in SNCA protein levels after ABX treatment in heterozygous *GBA* mutation carriers (B). The results represent the mean ± SEM, of three independent experiments. *p<0.05; one-way ANOVA and Student's t-test.

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Chapter 9: Appendix

9.1 Validation of the BCA assay

The BCA assay was validated by the use of 8 samples of serially diluted fibroblast cell lysates. I calculated that one 80% confluent cell plate = ~1mg/ml on BCA assay. Loading double the lysate concentration gave double the protein concentration. This tested the pipetting accuracy as well as the linearity of the BCA assay. Standards measured in duplicate improved the accuracy of the readings. This is shown in **Figure 9.1**.

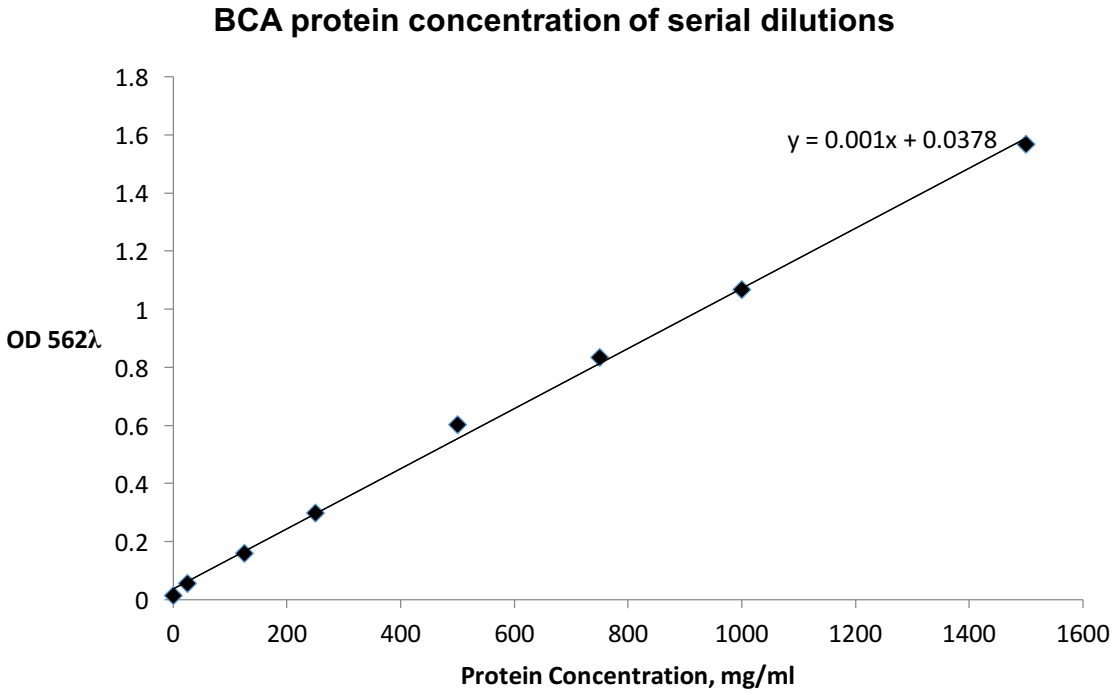


Figure 9.1. Validation of BCA assay with varying volumes of cell lysate

9.2 Validations of the GCase assay

9.2.1 Testing the GCase assay conditions

To test the accuracy of the assay, I measured GCase activity for a range of conditions (**Table 9.1**). The GCase activity followed the predicted pattern for the 8 conditions (**Table 9.2**). Sodium taurocholate enhances the GCase activity 5-6 fold when compared to the measurement in the presence of H_20 without sodium taurocholate. The sum of the GCase activity measured in condition 7 (measurement of lysosomal GCase in the presence of 1μM deoxynojirimycin (DNJ), a *GBA*2 inhibitor) and condition 8 (conduritol β epoxide inhibition on *GBA1*) should be equal to that measured in condition 6 (total GCase activity measured in the presence of H_20). This is not the case which questions the specificity of the inhibitors: DNJ and CBE. CBE inhibition appears complete (minimal *GBA* activity seen in conditions 4 and 8). DNJ inhibition on *GBA2* may not be complete. The mutant GCase activity measured is higher in the presence of H_20 than in the presence of sodium taurocholate. This would be consistent with a compensatory increase in *GBA* activity in the presence of a *GBA* mutation. Secondly, in the absence of sodium taurocholate, there is no inhibition on *GBA* activity and therefore, the total GCase activity measured is higher. This is shown in **Figure 9.2** and **Figure 9.3**.

Table 9.1. A range of GCase assay conditions

	Condition							
	1	**2**	**3**	**4**	**5**	**6**	**7**	**8**
NaT	+	+	+	+	-	-	-	-
H_20	-	-	-	-	+	+	+	+
Lysate	-	+	+	+	-	+	+	+
DNJ	-	-	+	-	-	-	+	-
CBE	-	-	-	+	-	-	-	+
Buffer	+	+	+	+	+	+	+	+

NaT: sodium taurocholate; DNJ: deoxynojirimycin; CBE: conduritol B epoxide; H_20: water.

Table 9.2. The predicted GCase activity for a range of assay conditions

Condition	Predicted GCase activity, nmol/h/mg
1	Minimal
2	~400
3	~300
4	~30
5	Minimal
6	~300
7	~200
8	~40

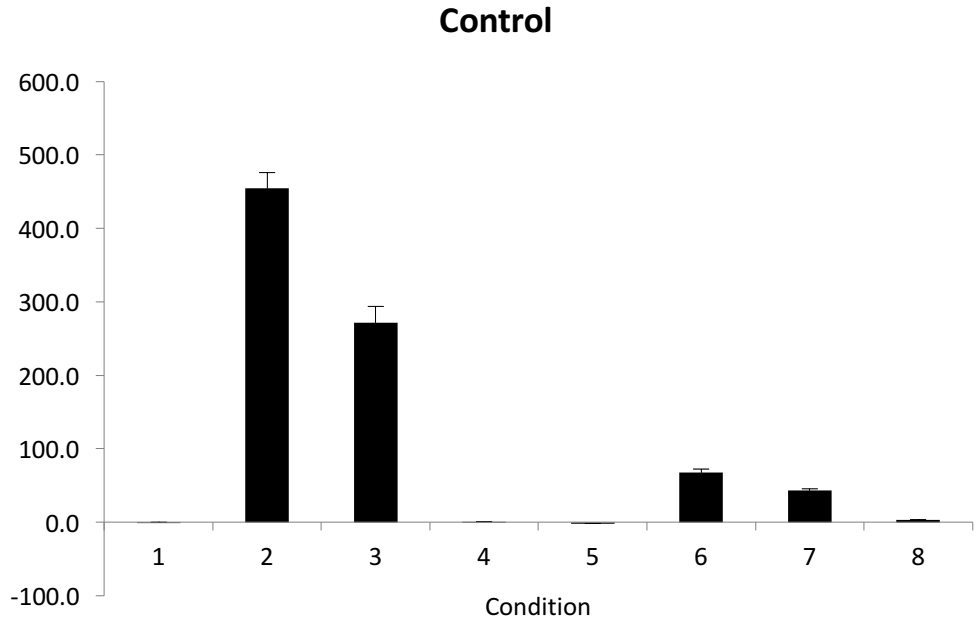


Figure 9.2. Measured GCase activity upon different assay conditions in a control line

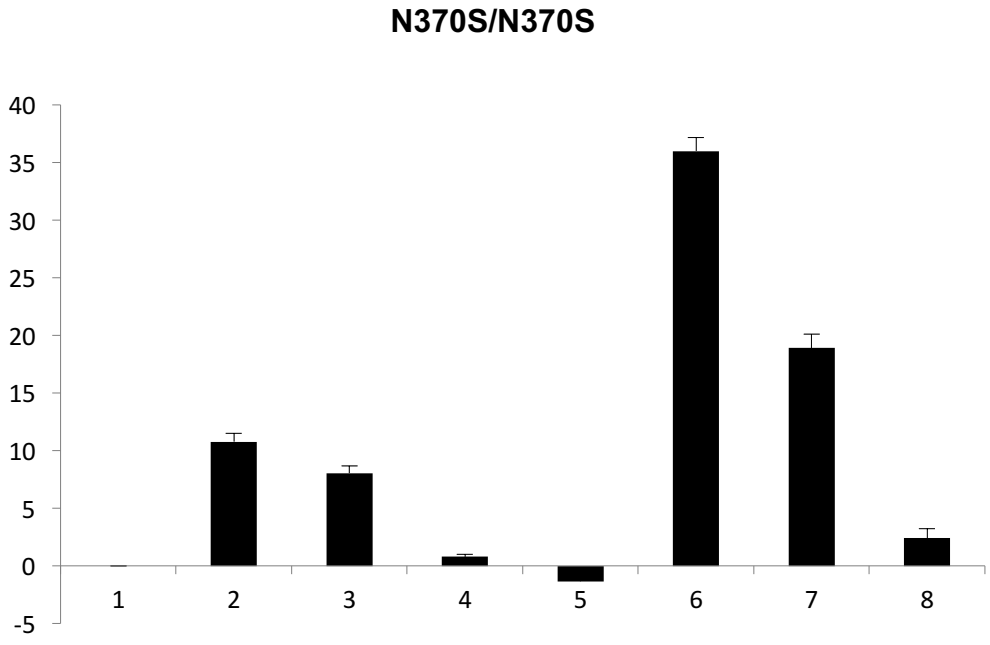


Figure 9.3. Measured GCase activity upon different assay conditions in a mutant line

9.2.2 The activity of GCase against the amount of loaded protein

The GCase assay was validated with fibroblast cell lines. The cell lysates were serially diluted to obtain samples of decreasing protein concentration (**Table 9.3**). By plotting the GCase activity of the cell lysate samples against its known protein amounts, calculated using the BCA assay, the linear range for the GCase assay can be isolated to be within 0.5 to 2mg/ml for fibroblast cells. Therefore, for a normal GCase assay, I would not want to go above 2mg/ml or below 0.5mg/ml, as the GCase activity measured will be outside the linear range of normal. This is shown in **Figure 9.4**.

Table 9.3. Calculated protein concentration for serially diluted cell lysate

Loadings	Expected protein concentration, mg/ml	Actual protein calculation, mg/ml
1	6	3.57
2	5	3.47
3	4	2.75
4	3	1.91
5	2	1.37
6	1.3	0.89
7	0.7	0.31
8	0.0	0.00

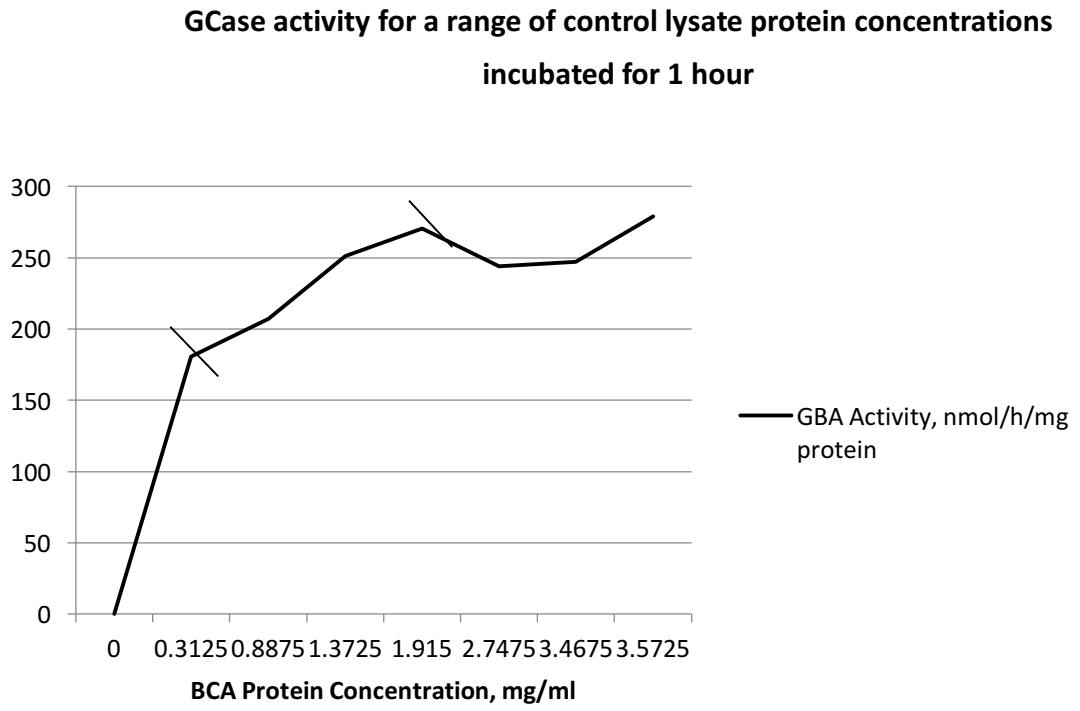


Figure 9.4. Validation of the GCase assay with serially diluted fibroblast cell samples

9.2.3 The activity of GCase against the amount of loaded protein and the incubation time

Fibroblast cell lysates were serially diluted to obtain samples of decreasing protein concentration. GCase activity was measured in the presence of different protein concentrations of lysate and different incubation times. GCase activity enhanced with longer incubations but not with increasing protein concentrations (corrected for by the BCA calculation). GCase activity actually decreased at high lysate protein concentrations, suggesting an inhibitory effect on the GCase assay. Greater error was seen at 30 minute incubations. Readings were more linear at the 1 hour incubation. When incubating for 2 hours compared to 1 hour for 1mg/ml protein concentration, the activity was not dissimilar. This is shown in **Figure 9.5**.

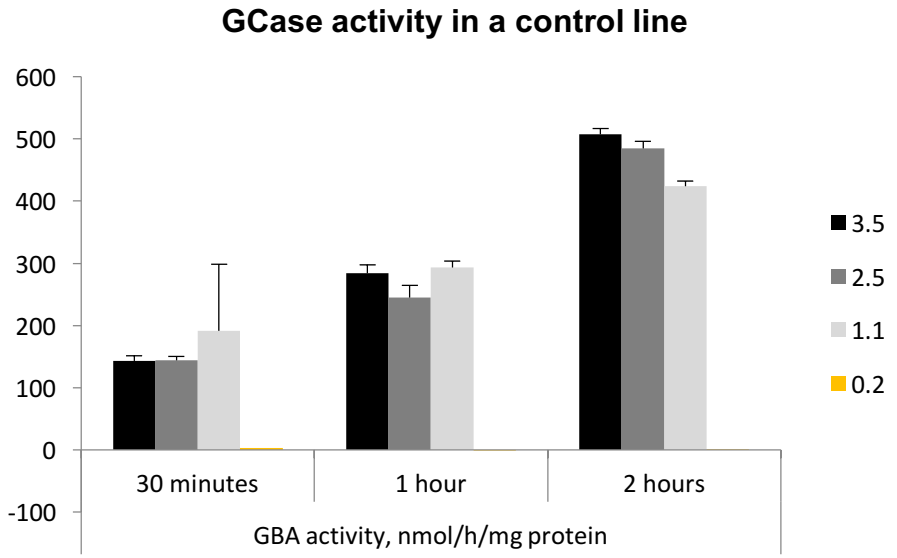


Figure 9.5. Validation of the GCase assay for increasing protein concentrations and incubation time

9.2.4 CβE inhibition of GCase

The conduritol-β-epoxide (CβE) fraction of GCase activity was validated with the fibroblast cell lines. Samples containing 20µg protein were tested for GCase activity using 5mM of 4-methylumbelliferyl-β-D-glucopyranoside substrate. 1µM of CBE was added to cell lysate of a constant protein concentration. Data are expressed as the CβE-sensitive or CβE-negative (residual) GCase activity. The results represent the mean ± SEM, of three independent experiments. CβE inhibition led to 90% GCase inhibition. This is shown in **Figure 9.6** and **Figure 9.7**.

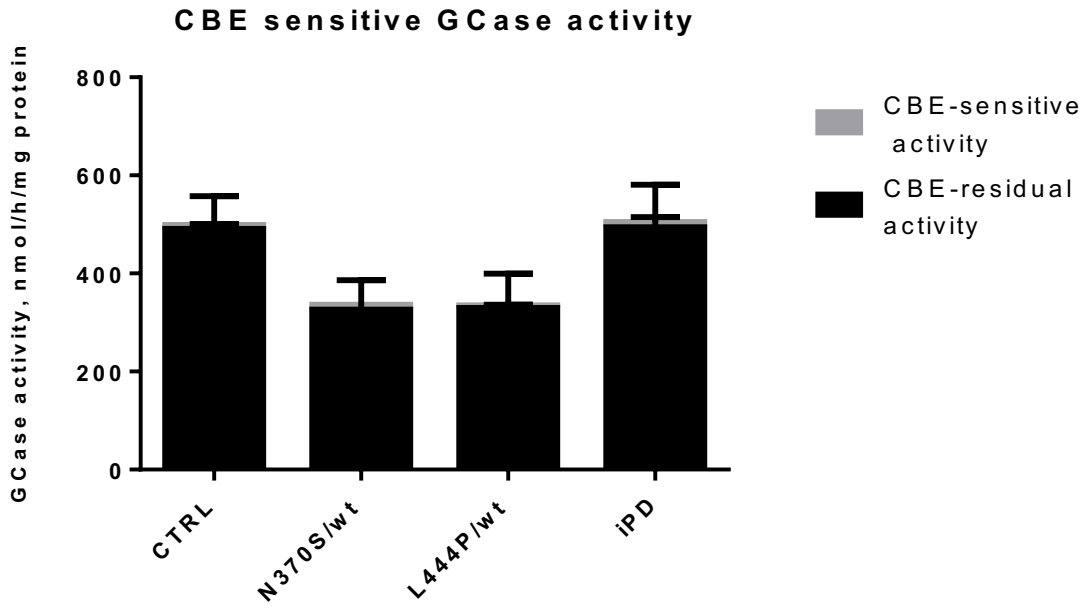


Figure 9.6. Validation of CβE inhibition of GCase in fibroblast cells.

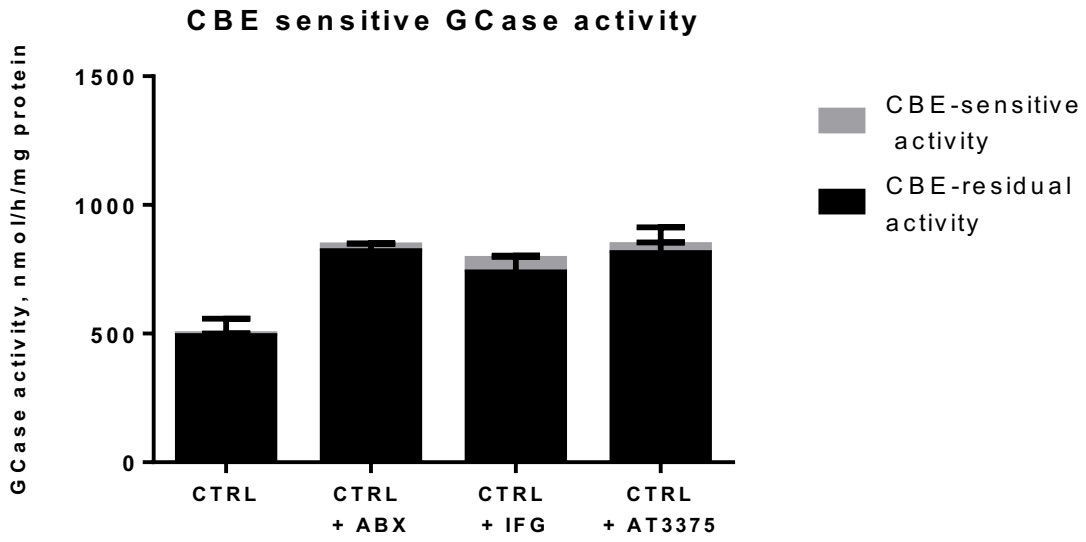


Figure 9.7. Validation of CβE inhibition of PC enhanced GCase in fibroblast cells.

9.3 Validation of the β-Hexosaminidase assay

The β-Hexosaminidase assay was validated with fibroblasts in the manner described in the GCase assay validation. By plotting the activity of the cell lysate samples against its known protein amounts, calculated using the BCA assay, Protein concentrations over 1mg/ml are within the normal linear range of β-Hexosaminidase. This is shown in **Figure 9.8**.

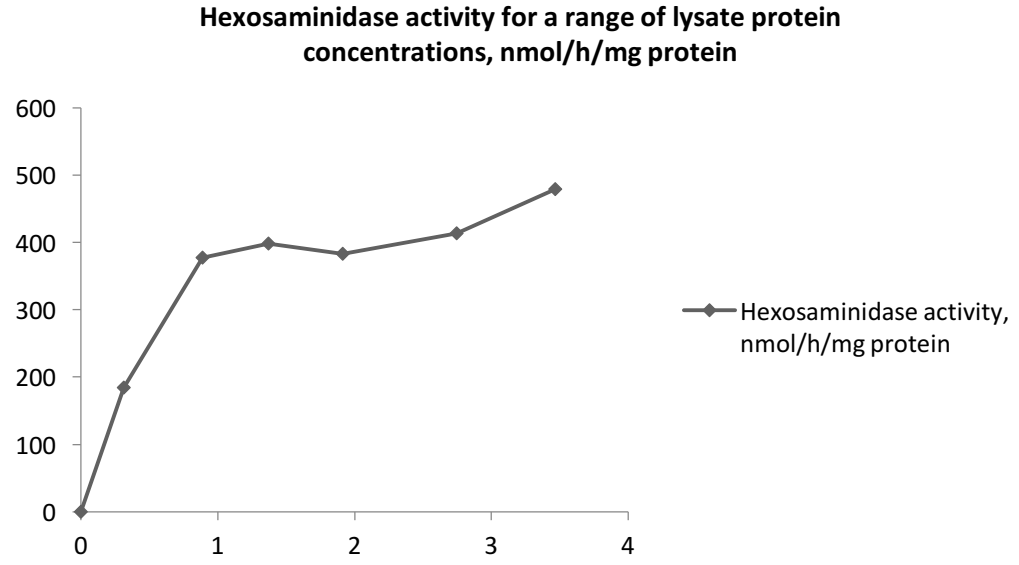


Figure 9.8. Validation of the β-Hexosaminidase assay with serially diluted fibroblast cell samples.

9.4 Validation of the β-galactosidase assay

The β-galactosidase assay was validated with fibroblasts in the manner described in the GCase assay validation. By plotting the activity of the cell lysate samples against its known protein amounts, calculated using the BCA assay, all protein concentrations were within the linear range for β-galactosidase activity. This is shown in **Figure 9.9**.

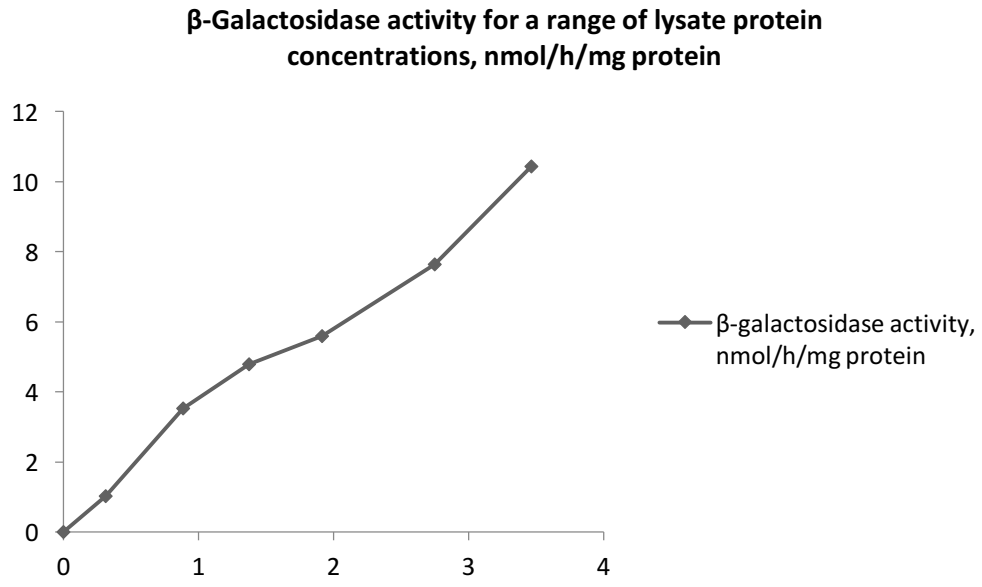


Figure 9.9. Validation of the β-galactosidase assay with serially diluted fibroblast cell samples.

9.5 Antibodies and primers used

Table 9.4. List of antibodies used for my studies

Antibody	Dilution	Application	Source	Cat. No.
GBA	1:1500	WB	Calbiochem	AP1140
β-ACTIN	1:5000	WB	Abcam	ab8227
BiP	1:1000	WB	Abcam	ab21685
LAMP1	1:1000	WB	Abcam	Ab24170
LAMP2a	1:500	WB	Abcam	ab18528
Hsc70	1:1000	WB	Abcam	ab51052
GAPDH	1:5000	WB	Abcam	ab9485
LC3	1:1000	WB	Cell Signaling	2775
p62	1:1500	WB	BD Biosciences	610832
Alpha-synuclein	1:500	WB/ICC	Abcam	ab1903
Beta III Tubulin	1:500	WB/ICC	Abcam	ab18207
Tyrosine Hydroxylase	1:1000	ICC	Abcam	ab6211
Nurr1	1:200	ICC	Abcam	ab55769
GBA	1:50	ICC	Sigma Aldrich	G4171
Cathepsin D	1:500	ICC	Abcam	ab6313

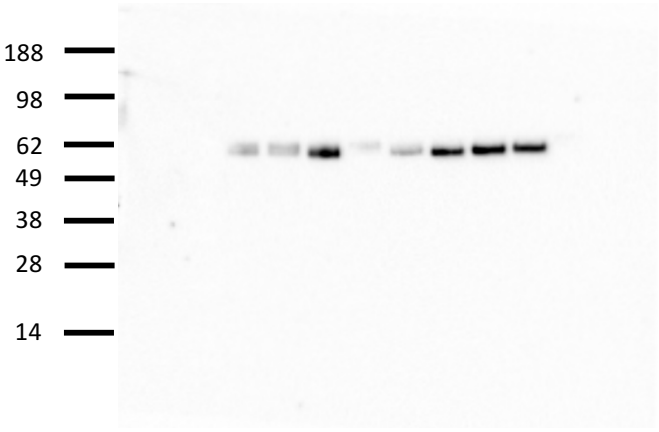
ICC, Immunocytochemistry; WB, Western Blot.

Table 9.5. List of primers used for my studies

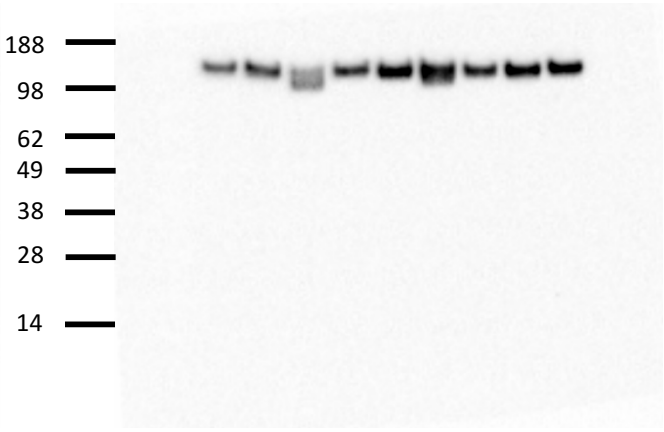
Gene	Source	Cat. No.
GBA	Life Technologies	Hs00986836_g1
PARD3	Life Technologies	Hs00969077_m1

9.6 Full-length images of immunoblots for fibroblast cells

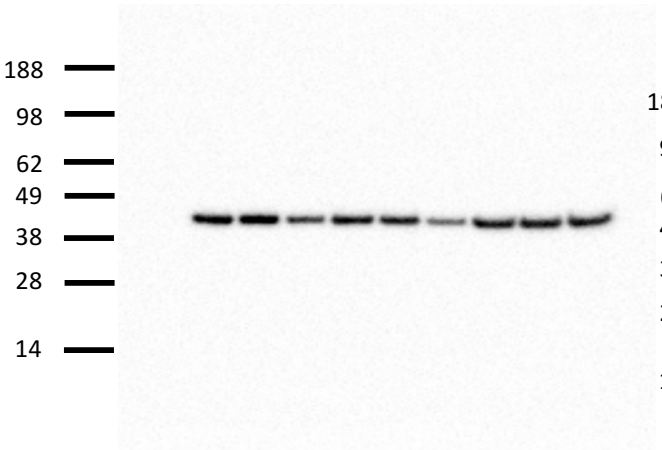
GBA



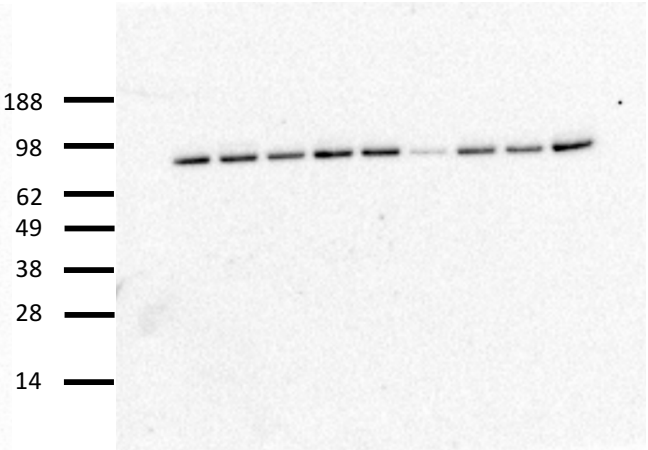
LAMP1



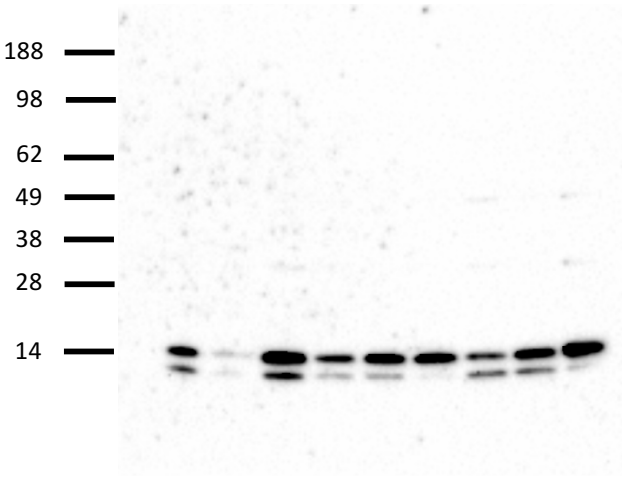
β-actin



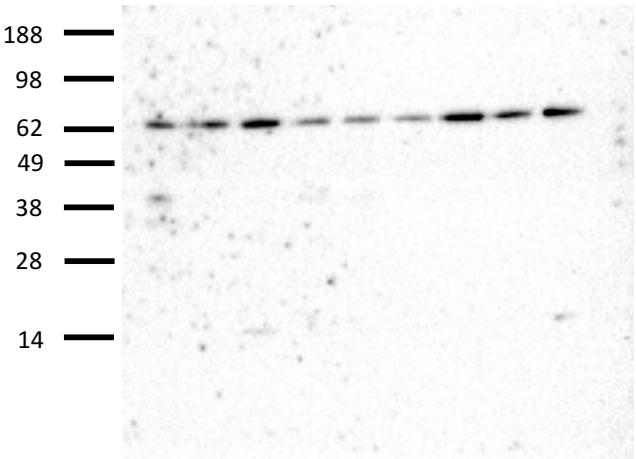
Hsc70



LC3



p62



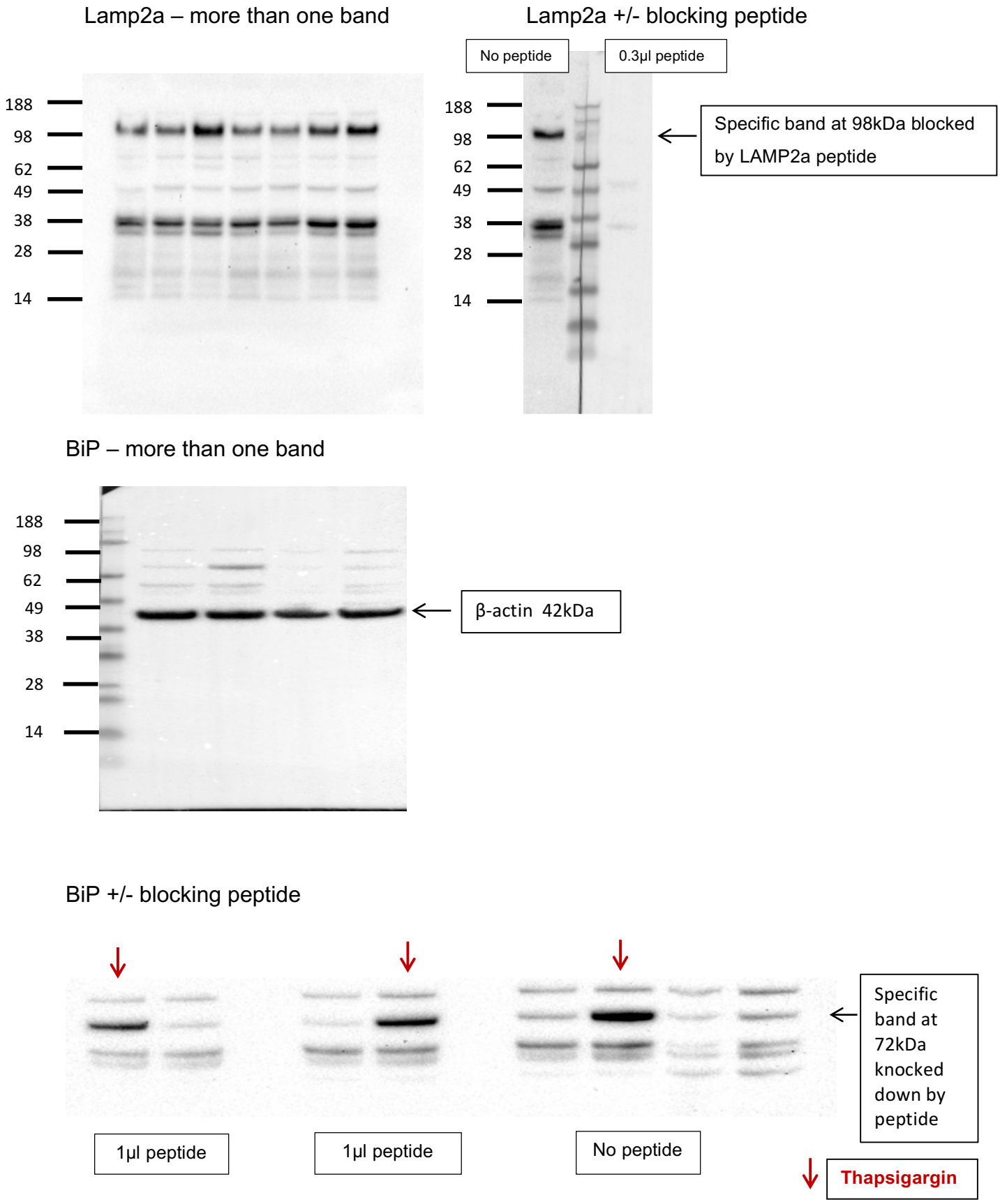
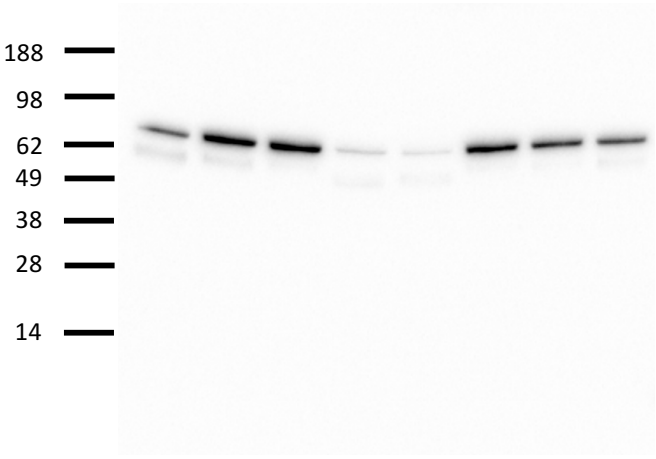


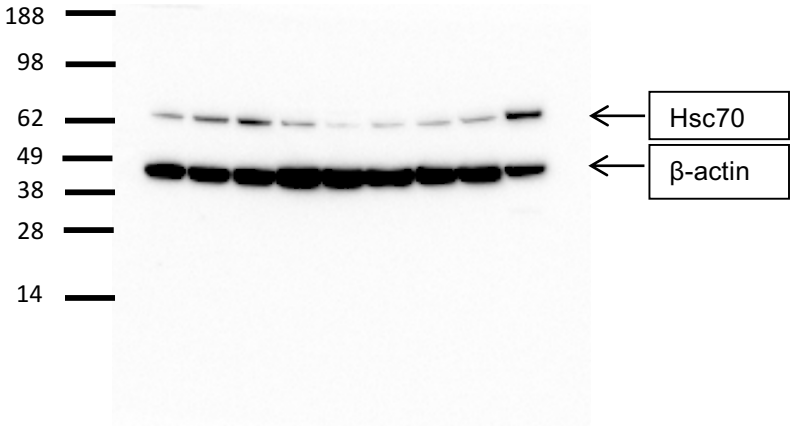
Figure 9.10. All western blots are shown in full scan. The corresponding figures and antibodies are indicated.

9.7 Full-length images of immunoblots for adipose NCSC

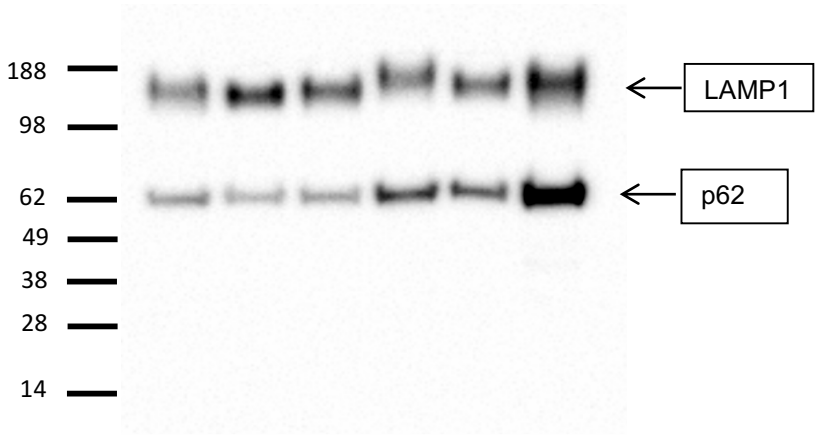
GBA



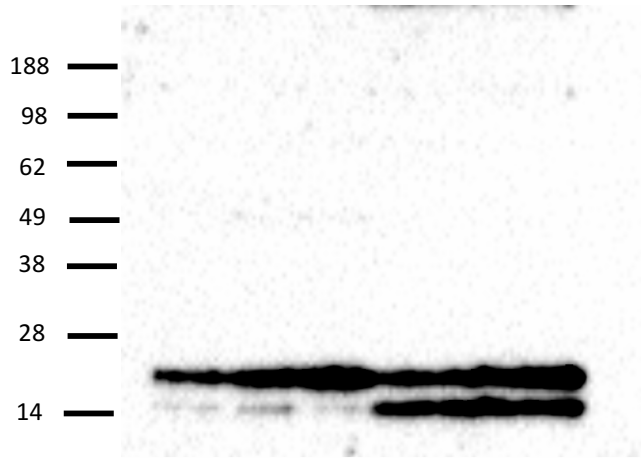
Hsc70 + β-actin



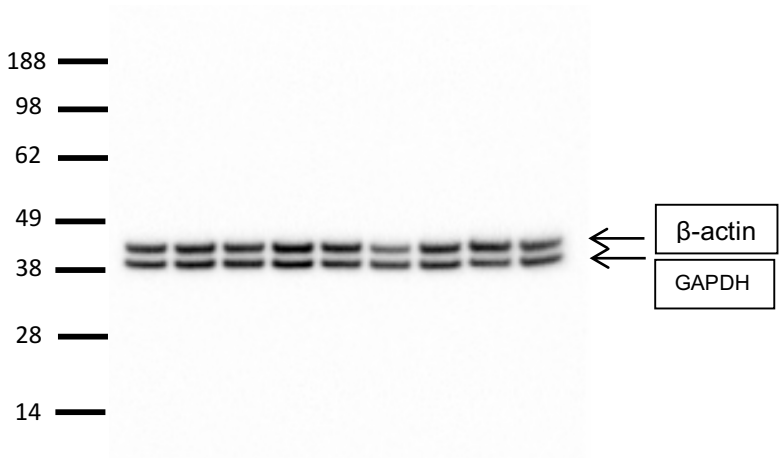
LAMP1 + p62

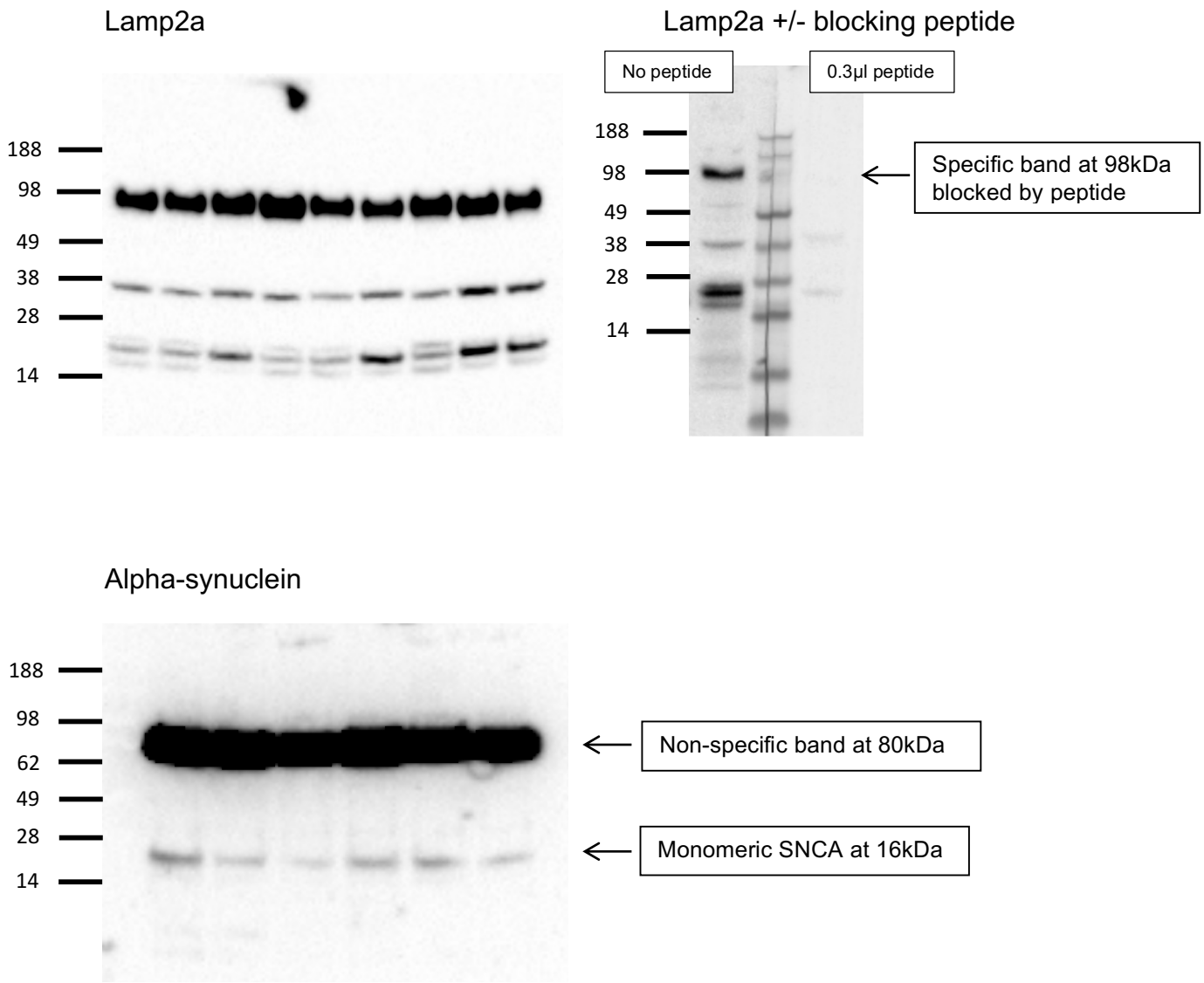


LC3



GAPDH





Predicted and observed band size of 16kDa. Additional band at: 80 kDa (possible post-translational modification) as per information from manufacturer:

http://www.abcam.com/alpha-synuclein-antibody-4d6-b1903.html#description_images_2

Figure 9.11. All western blots are shown in full scan. The corresponding figures and antibodies are indicated.

9.8 Viability of fibroblasts is unchanged with 48 hour drug treatment

Cytotoxicity assay

To evaluate the cytotoxicity of ABX and IFG, I cultured control fibroblasts in the presence of every day vs. alternate day PC treatment for 5 days, and then assayed the cell viability. After 5 days *in vitro*, a sample of medium was collected. The release of the cytosolic enzyme glucose-6-phosphate dehydrogenase (G6PD) was measured from damaged cells into the media. The G6PD assay was performed using the Vybrant Cytotoxicity Assay Kit (Life Technologies) as per the manufacturer's instructions. The results showed that the viability of fibroblasts was unchanged with alternate day drug treatment, but cytotoxicity increased two-fold upon every day treatment for both PC at concentrations of 60μM ABX and 50μM IFG. This is shown in **Figure 9.12**.

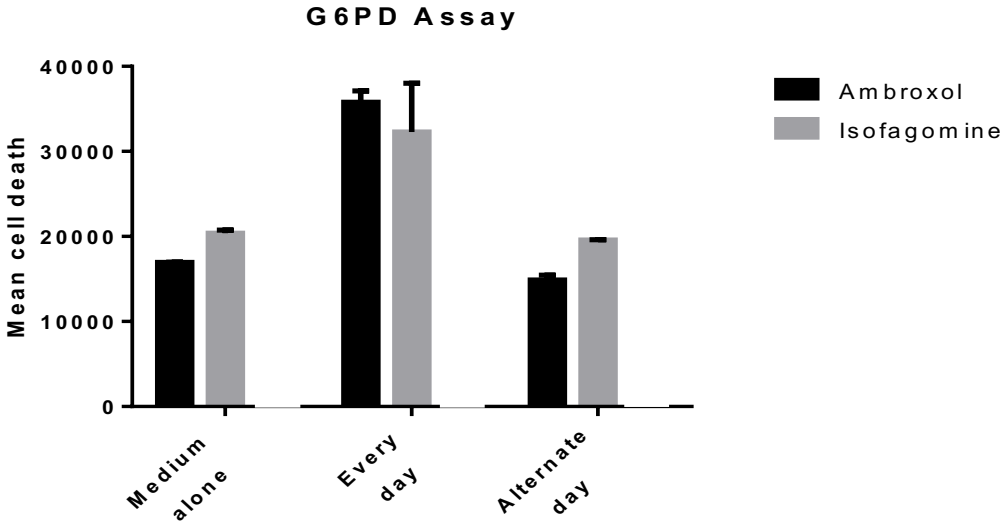


Figure 9.12. Cytoxicity of every day vs. alternate day drug treatment.

9.9 Evaluating the effect of different drug lots

Skin fibroblast cultures from a control (CTRL) and a mutant line (N370S/N370S) were treated with different lots of 60μM ambroxol (ABX) and 50μM isofagomine (IFG) for 6 days at 37°C. The results represent a single experiment. GCase activity was maximal in both a control and mutant line for ABX from Sigma Aldrich. GCase activity was maximal in the mutant line for IFG from Santa Cruz Biotechnology. This is shown in **Figure 9.13** and **Figure 9.14**.

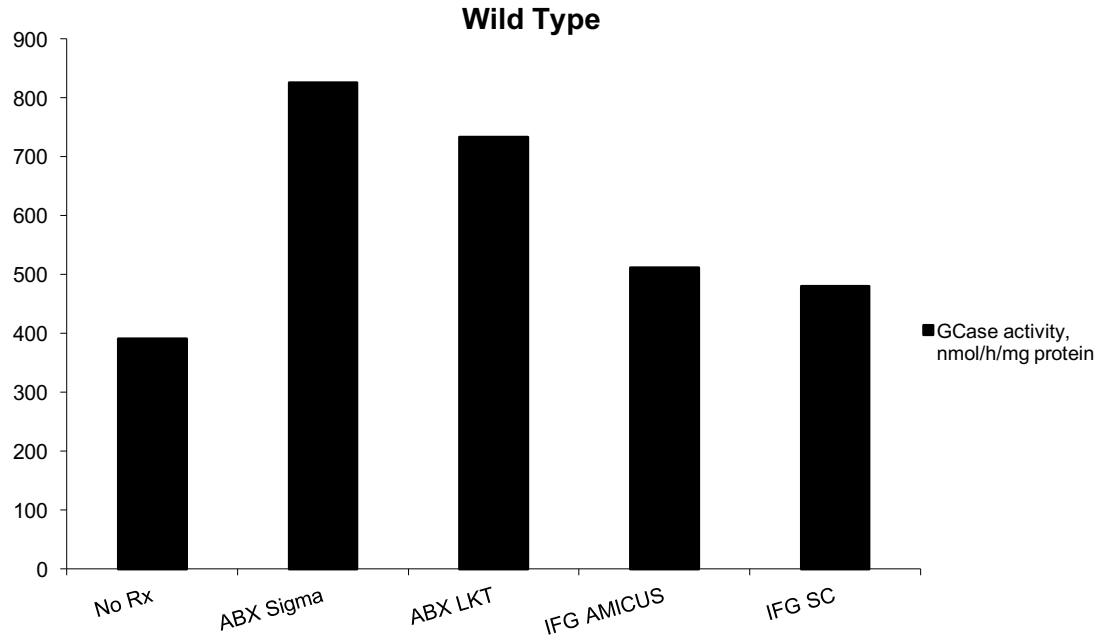


Figure 9.13. Measured GCase activity for different drug lots in a control line

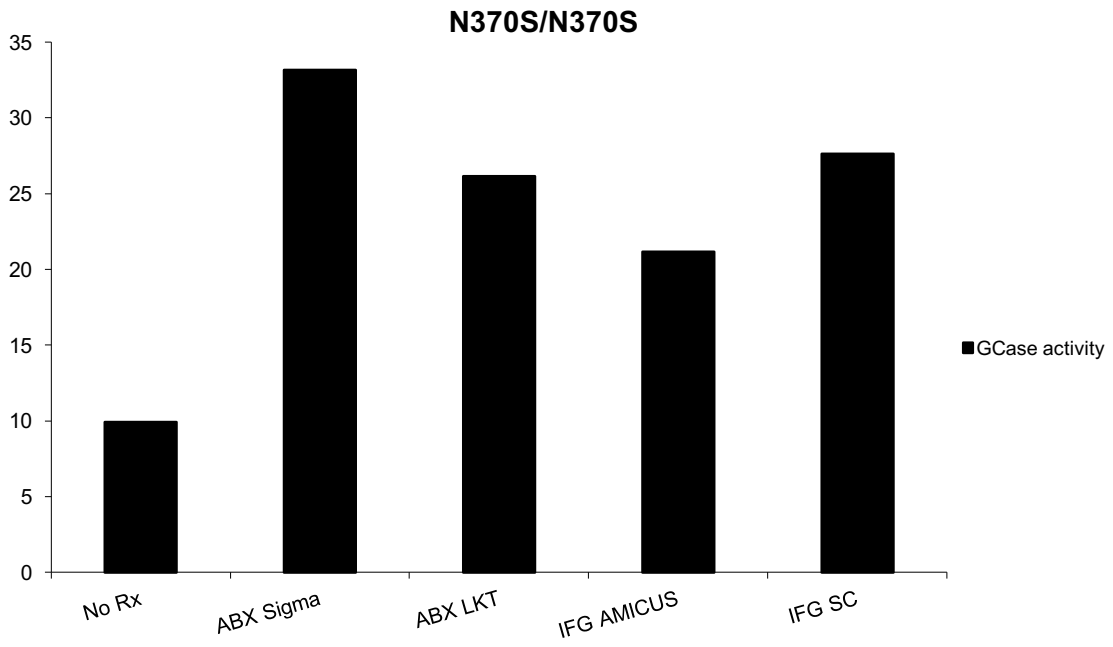


Figure 9.14. Measured GCase activity for different drug lots in a mutant line

9.10 Measurement of IFG enhanced GCase following a 24-hour washout period

I performed an experiment to determine whether any potential inhibition of GCase by IFG *in situ* could be minimised by a washout period. GCase levels in control fibroblasts were measured after 5 days incubation in the presence of 50μM IFG, followed by a 24-hour washout period to minimize potential GCase inhibition by IFG *in situ*. For comparison, the effect of 5 days incubation in the presence of 50μM IFG, followed by no washout period was also assessed. Samples containing 20μg protein were tested for GCase activity using 5mM of 4-methylumbelliferyl-β-D-glucopyranoside substrate. Data are expressed as the measured GCase activity in the presence of IFG. The experiment was repeated independently 5 times. There was no significant difference ($P > 0.05$) in GCase activity when the measurements were performed after a washout period. This is shown in **Figure 9.15**.

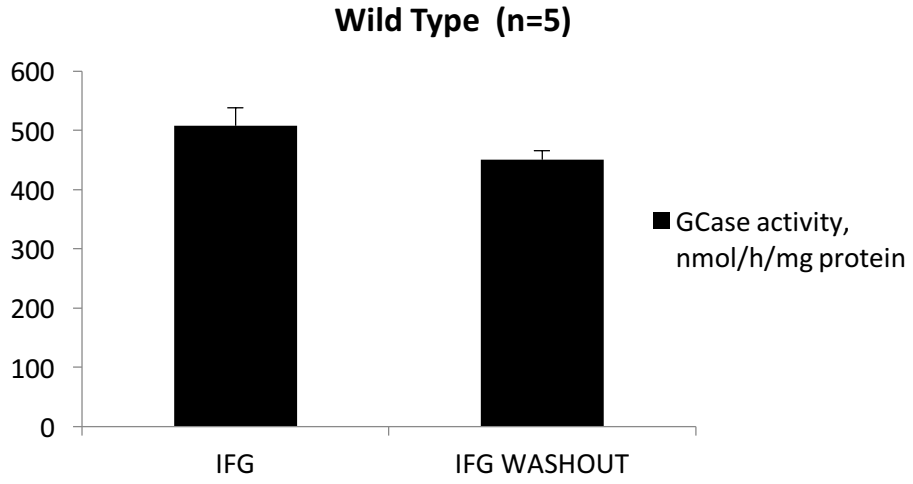


Figure 9.15. Comparison of the measured GCase activity following IFG treatment in the presence or absence of a 24-hour washout period.

9.11 Skin biopsies I obtained from the patient cohort

Table 9.6. List of skin biopsies (n=25) obtained from the patient cohort

Subject	Genotype	Gender	Diagnosis	Age at biopsy
C1	wt/wt	Female	CTRL	67
C2	wt/wt	Female	CTRL	70
C3	wt/wt	Male	CTRL	81
C4	wt/wt	Female	CTRL	82
C5	wt/wt	Male	CTRL	52
Het1	N370S/wt	Female	*GBA* Carrier	67
Het2	N370S/wt	Female	*GBA* Carrier	60
Het3	N370S/wt	Female	*GBA* Carrier	82
Het4	N370S/wt	Male	*GBA* Carrier	52
Het5	N370S/wt	Male	GBA-PD	80
Het6	N370S/wt	Female	GBA-PD	75
Het7	N370S/wt	Female	GBA-PD	53
Het8	L444P/wt	Male	*GBA* Carrier	62
Het9	L444P/wt	Female	*GBA* Carrier	45
Het10	L444P/wt	Male	*GBA* Carrier	67
Het11	L444P/wt	Male	GBA-PD	72
Het12	L444P/wt	Male	GBA-PD	85
GD1	N370S/N370S	Male	GD	70
PD1	wt/wt	Female	IPD	85
PD2	wt/wt	Male	IPD	79
PD3	wt/wt	Female	IPD	81
PD4	wt/wt	Female	IPD	72
PD5	wt/wt	Male	IPD	80
PD6	wt/wt	Female	IPD	75
PD7	wt/wt	Female	IPD	53

CTRL, healthy control; IPD, Idiopathic Parkinson's disease; *GBA* Carrier, heterozygous *GBA* mutation carrier; GBA-PD, heterozygous *GBA* mutation carrier with PD; GD, Gaucher disease.

Chapter 10: Publications

10.1 Glucocerebrosidase Mutations and the Pathogenesis of Parkinson's Disease.

Beavan M, Schapira AH (2013).

Ann Med 45:511-21. doi: 10.3109/07853890.2013.849003.

10.2 Visual short term memory deficits associated with *GBA* mutation and Parkinson's disease.

Zokaei N, McNeill A, Proukakis C, **Beavan M**, Jarman P, Korlipara P, Hughes D, Mehta A, Hu MT,Schapira AH, Husain M (2014).

Brain 137:2303-11. doi: 10.1093/brain/awu143.

10.3 Evolution of prodromal clinical markers of Parkinson's disease in a glucocerebrosidase mutation positive cohort.

Beavan M, McNeill A, Proukakis C, Hughes DA, Mehta A, Schapira AH (2015).

JAMA Neurol 72:201-8. doi: 10.1001/jamaneurol.2014.2950.

10.4 *GBA* mutation and preclinical markers of Parkinson's disease - reply.

Beavan M, Schapira AH (2015).

JAMA Neurol 1;72:724. doi: 10.1001/jamaneurol.2015.0484.