

**The Role of Neutral Sphingolipids in the Pathogenesis of Parkinson Disease  
and Dementia with Lewy Bodies**

By

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## ABSTRACT

The molecular mechanisms underlying the association between mutations in *GBA1* and risk of developing the 'synucleinopathy' disorders Parkinson's disease (PD) and dementia with Lewy bodies (DLB) remain elusive. To better understand the precise molecular cascade that connects *GBA1* mutations with  $\alpha$ -synuclein dysregulation, a modified lipid extraction and HPTLC protocol was optimized to detect changes in levels of neutral sphingolipids (SLs) from neural cells and tissue expressing wild-type (WT) *GBA1*, mutant *GBA1*, or both. We demonstrate that mutant *GBA1* does not confer a dominant-negative effect on WT *GBA1*-mediated activity; however, *bona fide* loss-of-enzymatic function mutation events led to the accumulation of lipid substrates in neural cells and tissue, and enhance  $\alpha$ -synuclein/ubiquitin reactivity in brain tissue of mutant *gba1* mice. Our HPLC-MS/MS data are consistent with other studies demonstrating that heterozygous *GBA1* mutations do not lead to lipid accumulation, but may alter  $\alpha$ -synuclein degradation through a yet-to-be defined novel gain-of-toxic function event.

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## LIST OF ABBREVIATIONS

AAV	Adeno-associated virus
ACA	Anterior part of anterior commissure
ALP	Autophagy-lysosomal pathway
AD	Alzheimer's disease
CATHD	Cathepsin D
CBE	Conduritol $\beta$ -epoxide
CC	Corpus Callosum
CER	Ceramide
CG	Cingulum
CHOL	Cholesterol
CMA	Chaperone-mediated autophagy
CSF	Cerebrospinal fluid
CPU	Striatum
DLB	Dementia with Lewy bodies
DMEM	Dulbecco's modified Eagle's medium
EC	External capsule
FBS	Fetal bovine serum
FTD	Fronto-Temporal Dementia
GCase	Enzymatic activity of the glucocerebrosidase protein
GBA1/Gba1	Glucocerebrosidase protein in human/mouse
<i>GBA1/Gba1</i>	Gene encoding glucocerebrosidase in human/mouse
GD	Gaucher disease
GLUCER	Glucosylceramide
GLUSPH	Glucosylsphingosine
GSL	Glycosphingolipid

HPLC	High-performance liquid chromatography
HPTLC	High-performance thin layer chromatography
HRP	Horseradish peroxidase
IFG	Isofagomine
IHC	Immunohistochemistry
iPS	Induced pluripotent stem cells
LACCER	Lactosylceramide
LC-MS/MS	Liquid chromatography-tandem mass spectrometry
LBs	Lewy bodies
LFB	Luxol Fast Blue
LNs	Lewy neurites
LRRK2	Leucine-rich repeat kinase 2
LV	Lateral ventricle
MRM	Multiple reaction monitoring
ORO	Oil Red O
PC	Phosphatidylcholine
PE	Phosphatidylethanolamine
PINK1	<i>Pten</i> -induced putative kinase 1
PD	Parkinson's disease
SDS/PAGE	Sodium dodecyl sulfate-based polyacrylamide gel electrophoresis
SL	Sphingolipid
SM	Sphingomyelin
SN	Substantia Nigra
SNCA	Gene encoding $\alpha$ -synuclein in human
TLC	Thin layer chromatography

UPS Ubiquitin-proteasome system  
WT Wild-type

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## 1. INTRODUCTION

### 1.1 Parkinson's Disease and Parkinsonism

#### 1.1.1 Numerous Etiologies Can Lead to Parkinsonism

Parkinsonism is a motor syndrome characterized by the presence of bradykinesia, tremor, rigidity, and postural instability (Schapira, 2010). Numerous etiologies can lead to parkinsonism and only rare cases can be explained by a single genetic or environmental cause (Horvath et al., 2013; Kitada et al., 2012; Klein and Schlossmacher, 2007). Parkinsonism due to a single environmental trigger, such as exposure to a neurotoxin, drug, or virus, is referred to as secondary parkinsonism (Caudle et al., 2012; Dick et al., 2007; Kitada et al., 2012). Alternatively, parkinsonism that is caused by a single genetic event is referred to as monogenic Parkinson's disease (PD) (Valente et al., 2012). Autosomal recessive mutations in genes such as *parkin*, *pten*-induced putative kinase 1 (*PINK1*), and *DJ-1* are individually linked to early-onset familial forms of PD (Cookson, 2012; Dodson and Guo, 2007; Xiong et al., 2009). On the other hand, point mutations in genes such as *SNCA* and leucine-rich repeat kinase 2 (*LRRK2*) are linked to autosomal dominant forms of PD, but at variable penetrance rates (Valente et al., 2012). Apart from these rare forms, a combination of genetic and environmental factors are thought to contribute in the majority of parkinsonism cases (Kitada et al., 2012; Klein and Schlossmacher, 2006, 2007).

#### 1.1.2 Parkinson's Disease – A Complex Disorder

The most common form of parkinsonism is sporadic (idiopathic) PD, constituting greater than 85% of cases (Horvath et al., 2013; Kitada et al.,

2012). PD is considered a complex disease, with a multifactorial etiology that involves the complicated and mostly elusive interaction between an aging brain and several susceptibility genes and environmental risk factors (Kitada et al., 2012; Klein and Schlossmacher, 2006, 2007).

Biochemical and *in vivo* studies involving PD-linked gene products, along with clinical studies of patients with sporadic PD, have implicated three main molecular pathways that can initiate and sustain neurodegeneration and PD pathogenesis by: (1) accumulation of misfolded and aggregated proteins; (2) impairment of clearance systems such as the autophagy-lysosomal pathway (ALP) and ubiquitin-proteasome system (UPS); and (3) oxidative stress and mitochondrial dysfunction (Burbulla and Kruger, 2011; Burke, 2002; Cookson, 2012; Cookson and Bandmann, 2010; Dawson and Dawson, 2003; Valente et al., 2012).

Aging also plays a crucial role in the neurodegeneration observed in PD (Valente et al., 2012). There is an age-dependent decline in the efficiency of most cell protection pathways such as the UPS, ALS, and the surveillance and regulation of mitochondrial integrity and function (Naoi and Maruyama, 1999; Navarro and Boveris, 2010; Necchi et al., 2011; Rajawat and Bossis, 2008). Combined with the progressive accumulation of somatic mutations and DNA damage, this contributes to cell death, especially of vulnerable cell populations such as the dopaminergic cells of the *Substantia Nigra* (SN) (Park and Larsson, 2011; Van Houten and Kow, 1992).

Intriguingly, recent studies have also implicated chronic inflammation and microglia activation as playing an important role in the neurodegenerative process (Gao et al., 2011; McGeer and McGeer, 2004; Phani et al., 2012). This idea has

been supported by immunohistochemical studies that show activated microglia in PD-affected brain regions (Langston et al., 1999; McGeer et al., 1988) and studies on animal models of PD that demonstrate that anti-inflammatory agents can inhibit dopaminergic cell death (Liu et al., 2012; Salama et al., 2012).

### 1.1.3 Motor and Non-Motor Symptoms of PD

PD is the second most common neurodegenerative disorder after Alzheimer's disease (AD) (Sulzer, 2007). Clinically, it presents with a progressive motor dysfunction that includes bradykinesia, tremor, rigidity, and postural instability. The most consistent and prominent neuropathological feature of PD is a greater than 80% loss of melanin-containing dopaminergic cells in the *Substantia nigra pars compacta* (SNpc) at autopsy (Sulzer, 2007). However, there is great neuropathological heterogeneity between patients, and changes also include axonal degeneration, astrocytic gliosis, and the formation of proteinaceous Lewy inclusions in the brainstem and other brain regions (Braak et al., 2003).

Non-motor PD symptoms precede motor changes and tend to become progressively worse over time. These non-motor symptoms include, but are not limited to, dementia (Baker, 1999; Bouchard et al., 2008; Rana et al., 2012), insomnia (Caap-Ahlgren and Dehlin, 2001; Gjerstad et al., 2007), autonomic and gastrointestinal dysfunction (Zhu et al., 2012), and mood disorders such as depression (McDonald et al., 2003; Tan, 2012; van der Hoek et al., 2011). Some non-motor symptoms such as gastrointestinal dysfunction (Jost, 2010) and the impaired sense of smell (Morley and Duda, 2010; Postuma and Gagnon, 2010) may precede the clinical diagnosis of PD by decades. The

recognition of sensory, autonomic, and behavioral non-motor symptoms, along with evidence of chronic inflammation in patients, has changed the view of PD as simply a motor disorder to a more systemic illness, affecting various organs and tissues (McGeer and McGeer, 2004; Valente et al., 2012).

## **1.2 The Relationship between $\alpha$ -Synuclein and Parkinson's Disease**

$\alpha$ -Synuclein is a small, natively unfolded, 140-amino acid protein (Breydo et al., 2012). It is encoded by the *SNCA* gene, which contains 7 exons and is located on chromosome 4 (Breydo et al., 2012).  $\alpha$ -Synuclein was first described as a neuron-specific protein localized in the nucleus and presynaptic nerve terminals (Maroteaux et al., 1988).  $\alpha$ -Synuclein is abundant in both the soluble (cytosolic) and membrane-associated fractions of the brain, but is also found in other tissues, such as red blood cells (Barbour et al., 2008; George et al., 1995; Lee et al., 2002; McLean et al., 2000). Although the exact function of  $\alpha$ -synuclein is still unknown, it is thought to play a role in synaptic vesicle trafficking and release by promoting SNARE-complex assembly (Auluck et al., 2010; Burre et al., 2010; Kjaer et al., 2009).

Mounting evidence links  $\alpha$ -synuclein to PD and other related neurodegenerative conditions, termed synucleinopathies. Firstly, rare autosomal dominant forms of PD are associated with point mutations such as A53T, A30P or E46K in the *SNCA* gene, or a rise in the levels of wild-type (WT)  $\alpha$ -synuclein due to gene amplification (duplications or triplications) (Kruger et al., 1998; Oueslati et al., 2010; Polymeropoulos et al., 1997; Singleton et al., 2003; Xilouri et al., 2012; Xilouri et al., 2009). In addition,  $\alpha$ -synuclein is the major component of Lewy bodies (LBs) and Lewy neurites (LNs), characteristic intra-cytoplasmic inclusions

that form in different brain regions of patients with familial and sporadic PD, as well as other synucleinopathies such as dementia with Lewy bodies (DLB) (Goker-Alpan et al., 2006; Hruska et al., 2006). It is estimated that at autopsy, nigral LBs are present in over 80% of patients with a clinical diagnosis of PD (Hughes et al., 1992). Furthermore,  $\alpha$ -synuclein misprocessing promotes the formation of these insoluble LBs and LNs, reinforcing its importance in PD pathogenesis (Desplats et al., 2009; Lee et al., 2008; Liani et al., 2004; Rochet et al., 2000).

$\alpha$ -Synuclein aggregation is a process whereby monomeric forms of the protein associate to form dimers and oligomers, which gradually lead to the formation of mature amyloid-like fibrils through a process of “templating” (Hardy and Revesz, 2012; Marques and Outeiro, 2012). Both *in vitro* and *in vivo* studies demonstrate that WT  $\alpha$ -synuclein and disease-related variants initially form soluble oligomeric species, which appear as spherical, string-, or ring-like structures (Conway et al., 2000a; Conway et al., 2000b; Karpinar et al., 2009; Winner et al., 2011). These intermediary forms, termed protofibrils, can eventually culminate into the insoluble fibrils deposited within LBs and LNs. Some studies implicate the soluble oligomeric  $\alpha$ -synuclein species as being responsible for cell toxicity and death (Colla et al., 2012; Conway et al., 2000b; Karpinar et al., 2009; Mazzulli et al., 2011; Winner et al., 2011). On the other hand, others have demonstrated the spread of Lewy pathology via cell-to-cell transmission of the insoluble  $\alpha$ -synuclein fibrils in cultured primary cortical mouse neurons and WT, nontransgenic mice (Luk et al., 2012).

Several factors can lead to the misprocessing and aggregation of  $\alpha$ -synuclein (Xilouri et al., 2012). Chaperone-mediated autophagy (CMA) and macroautophagy

(both components of ALP), as well as the UPS are thought to be responsible for the majority of  $\alpha$ -synuclein degradation (Batelli et al., 2011; Bennett et al., 1999; Ebrahimi-Fakhari et al., 2011; Xilouri et al., 2012). *In vitro* and *in vivo* evidence suggests that impairment of these proteolytic pathways contributes to the abnormal accumulation, oligomerization, and aggregation of WT and mutant  $\alpha$ -synuclein (Batelli et al., 2011; Kim et al., 2011; Klucken et al., 2012; Miwa et al., 2006; Sawada et al., 2004; Vali et al., 2008; Vogiatzi et al., 2008; Wills et al., 2012). Furthermore, a rise in the protein burden of WT  $\alpha$ -synuclein or the expression of disease-related mutants can lead to additional UPS and ALP dysfunction, causing further impairment of  $\alpha$ -synuclein clearance (Bennett et al., 1999; Cuervo et al., 2004; Lee et al., 2008; Xilouri et al., 2009; Yang et al., 2009). Other determinants of  $\alpha$ -synuclein aggregation include post-translational modifications of the protein, its interaction with lipid membranes and various other proteins, and several environmental factors (Auluck et al., 2010; Breydo et al., 2012; Oueslati et al., 2010; Stefanis, 2012; Wills et al., 2012).

### **1.3 The *GBA1* Gene and Synucleinopathy Risk**

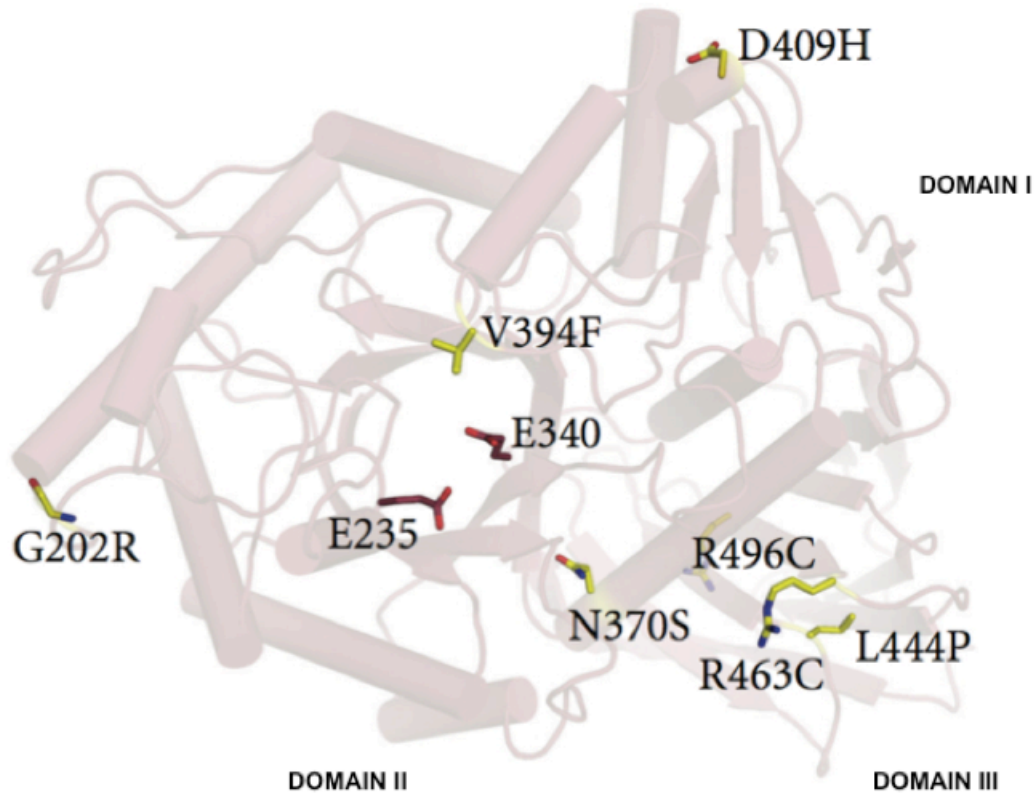
Recently, several lines of evidence have converged suggesting an association between *GBA1* mutations and risk of developing the synucleinopathies PD and DLB (DePaolo et al., 2009). It should be mentioned here that DLB is the second most frequent dementia after AD (Rampello et al., 2004). Clinical manifestations include parkinsonian features and memory impairment as seen in AD (Simard et al., 2000). The neuropathological hallmark of DLB is widespread cortical and brainstem  $\alpha$ -synuclein-positive neuronal inclusions, as well as formation of  $\beta$ -

amyloid plaques and neurofibrillary tangles in the forebrain (Lowe and Dickson, 1997; Mata et al., 2008). The penetrance of PD and DLB in *GBA1* mutation carriers is currently unknown. However, all patients with *GBA1*-linked PD have thus far demonstrated  $\alpha$ -synuclein-positive LBs and LNs at autopsy, suggesting a close association between mutations in *GBA1* and  $\alpha$ -synuclein misprocessing and aggregation (Eblan et al., 2005; Wong et al., 2004).

The functional *GBA1* gene is located in the q21 region of chromosome 1 and is comprised of 11 exons (Barneveld et al., 1983; Germain et al., 1998; Stratil et al., 2004). The gene encodes a 497-residue protein called GBA1 (glucocerebrosidase, acid  $\beta$ -glucosidase, GCase, glucosylceramidase, EC.3.2.1.45). GBA1 is translated on endoplasmic reticulum (ER)-bound polyribosomes and translocated to the lumen of the ER as a 56 kDa polypeptide (Ron and Horowitz, 2005). Once in the ER, GBA1 undergoes N-linked glycosylation on four out of the five available asparagine residues (Aerts et al., 1986; Bergmann and Grabowski, 1989). GBA1 is then transported to the Golgi apparatus, where its high mannose sugar moieties are further modified (Aerts et al., 1986). GBA1 is finally trafficked to the lysosome as a mature 63 kDa protein (**Figure 1A**) (Bergmann and Grabowski, 1989; Ron and Horowitz, 2005).

Crystal structures of GBA1 show that it is comprised of three discontinuous domains: Domain I, an antiparallel  $\beta$ -sheet; Domain II, a triose phosphate isomerase (TIM) barrel harboring the active site; and Domain III, an eight-stranded  $\beta$ -barrel (**Figure 2**) (Atrian et al., 2008; Durand et al., 1997; Dvir et al., 2003; Lieberman, 2011). GBA1 falls into a large family of glycoside hydrolases, which use catalytic





Modified from Lieberman (2011).

**Figure 2. Overall GBA1 Structure Including Three Domains and the Active Site.**

GBA1 is composed of three discontinuous domains. Domain II harbors glutamate at active site residues 235 and 340, shown in red. The location of six amino acid substitution mutations are shown in yellow. Included are the prevalent and mild N370S missense mutation, as well as the common and more severe L444P mutation.

aspartate or glutamate for general acid/base reactions (Durand et al., 1997; Sinclair et al., 2006). Specifically, GBA1 uses glutamic acid at residues 235 and 340 as the catalytic acid/base and nucleophile, respectively (Dvir et al., 2003; Lieberman, 2011; Lieberman et al., 2007; Miao et al., 1994).

GBA1 catalyzes the hydrolysis of its lipid substrates glucosylceramide (GluCer) and glucosylsphingosine (GluSph) into glucose, ceramide (Cer), and sphingosine via cleavage of the  $\beta$ -glycosidic bond (**Figure 1B**) (Atrian et al., 2008). Optimal enzymatic activity of GBA1 (GCCase activity) depends on the protein's association with anionic phospholipid-containing membranes, the activator protein saposin C (SapC), and a lysosomal pH of 3-5 (Atrian et al., 2008; Sinclair et al., 2006; Vaccaro et al., 1994; van Weely et al., 1993; Wilkening et al., 1998).

Cer, GluCer, and GluSph are sphingolipids (SLs), a class of lipids that contain sphingoid bases, such as sphingosine, as the backbone (Messner and Cabot, 2010). The most common SLs in mammalian cells are sphingomyelin (SM) and glycosphingolipids (GSLs), both synthesized from the hydrophobic Cer (Ichikawa and Hirabayashi, 1998). GSLs consist of three components: (1) a sphingoid base; (2) a long chain fatty acid that is amide linked at the 2-amino position; and (3) one or more sugar residues joined through a  $\beta$ -glycosidic bond to the sphingoid backbone at the 1-hydroxyl position (**Figure 1B**) (Messner and Cabot, 2010). Cer is composed of the first two components mentioned above and its sphingoid base is sphingosine, an (usually) 18-carbon amino alcohol with an unsaturated hydrocarbon chain (Yamaguchi et al., 1994).

De novo synthesis of Cer occurs in the ER and it is subsequently transported to

the cytosolic side of the trans-Golgi by the ceramide transport protein CERT (Messner and Cabot, 2010). At the cytosolic leaf, Cer is converted to GluCer by the enzyme GluCer synthase (Ichikawa and Hirabayashi, 1998). GluCer can then be pumped or flipped from the cytosolic side of the Golgi to the lumen, where it may be converted to lactosylceramide (LacCer) and downstream, higher order GSLs (Messner and Cabot, 2010).

Sections of the plasma membrane containing GSLs destined for degradation are endocytosed and trafficked to the lysosome (Kolter and Sandhoff, 1998). Hydrolysing lysosomal enzymes cleave the sugar residues in a sequential manner to produce Cer (Kolter and Sandhoff, 1998). Deacylation of Cer generates sphingosine, which can re-enter the biosynthetic pathway or be further degraded (Kolter and Sandhoff, 1998). GluSph is synthesized via glucosylation of sphingosine, and may also be generated through deacylation of GluCer (Sasagasako et al., 1994; Yamaguchi et al., 1994).

### **1.1 Homozygous and Compound Heterozygous *GBA1* Mutations Cause Gaucher Disease**

Gaucher disease (GD) is the most common lysosomal storage disorder and has an autosomal recessive inheritance pattern (Chen and Wang, 2008; Grabowski, 2012; Sidransky, 2004). Homozygous and compound heterozygous mutations in the *GBA1* gene lead to insufficient GCCase activity (Brady et al., 1966; Cox, 2001; Grabowski, 2012; Sidransky, 2004). This in turn results in the accumulation of the enzyme's lipid substrates GluCer and GluSph in the lysosomes of macrophages (Brady et al., 1966; Cabrera-Salazar et al., 2010; Cox, 2001;

Nilsson and Svennerholm, 1982). Expansion of these engorged macrophages, or Gaucher cells, in visceral tissues causes the primary disease manifestations of hepatosplenomegaly, anemia, thrombocytopenia, weakening of bones (due to rearrangement of bone matrix), and in severe cases neurological dysfunction (Chen and Wang, 2008; Grabowski, 2005, 2012; Sidransky et al., 1992a).

GD has been divided into three clinical phenotypes based on the presence (neuronopathic) or absence (non-neuronopathic) of central nervous system (CNS) involvement (Goker-Alpan et al., 2005; Grabowski, 2012). Type I (non-neuronopathic) GD is the most prevalent variant and involves visceral organs. Neuronopathic GD is sub-divided into type II (acute) and type III (sub-acute) GD based on the age of onset and the rate of progression of CNS deterioration (Aviner et al., 2009; Sidransky et al., 1994; Tayebi et al., 1998). Therapies exist only for non-neuronopathic GD, and include enzyme replacement therapy with a glycan-modified recombinant enzyme that does not penetrate the blood brain barrier (Cerezyme®), or substrate reduction therapy using chemical inhibitors of GluCer synthase (Grabowski, 2005; Sidransky et al., 2007).

Over 300 mutations have been identified in the *GBA1* gene, most of them being missense mutations (Hruska et al., 2008). The most prevalent mutations reported in GD patients are the N370S and L444P missense mutations (**Figure 2**) (Goker-Alpan et al., 2005; Sidransky et al., 1992b; Tayebi et al., 1998). The N370S mutation is most common among GD type 1 patients, while the more severe L444P mutation is associated with type 3 GD. This mutation is also found in compound heterozygous patients with type 1 or type 2 GD (Aviner et al., 2009; Goker-Alpan et al., 2005;

Sidransky et al., 1994; Tayebi et al., 1998). Although some generalizations can be made about particular mutations occurring more commonly in certain forms of GD, no consistent genotype-to-phenotype correlation has been identified as yet (Goker-Alpan et al., 2005; Sidransky et al., 1994). In fact, patients with the same genotype can exhibit great phenotypic variation and the confounding variables that modify the phenotypic expression of *GBA1* genotypes remain unknown (Cox, 2001; Germain et al., 1998; Goker-Alpan et al., 2005; Sidransky et al., 1994; Sidransky et al., 1992b).

## **1.2 *GBA1* Mutations Increase the Risk of Synucleinopathy**

### **1.2.1 A Clinical Description of Parkinsonism in GD Patients**

A possible link between GD and the ‘synucleinopathy’ disorders of the brain began to emerge with the clinical observation of parkinsonism in a subset of GD patients. Neudorfer et al. (1996) reported the co-occurrence of parkinsonism in six patients with type I GD. The authors noted that patients developed an early-onset, atypical parkinsonian disorder that presented by the fourth to sixth decades of life, had a relatively severe and progressive clinical course, and was refractory to L-DOPA treatment (Neudorfer et al., 1996). Subsequent case reports and studies with larger patient cohorts corroborated these findings (Tayebi et al., 2001; Tayebi et al., 2003; Varkonyi et al., 2003). Additionally, pedigree studies demonstrated that GD patients often had relatives with early-onset atypical parkinsonism who were either known or obligate mutation carriers (Goker-Alpan et al., 2004; Halperin et al., 2006). This finding indicated that even heterozygous *GBA1* mutations might increase the risk for developing parkinsonism.

### 1.2.2 Neuropathology of Patients with GD and Parkinsonism

Neuropathological analyses of patients with GD and parkinsonism revealed the presence of  $\alpha$ -synuclein-positive LBs in various brain regions (Tayebi et al., 2003; Wong et al., 2004). Importantly, these LBs were not present in GD patients that did not exhibit the parkinsonian phenotype. GD patients with parkinsonism demonstrated hippocampal CA2-4 gliosis that is consistent with GD pathology, depletion of pigmented SN neurons, SN gliosis, and nigral brainstem-type LBs (Tayebi et al., 2003; Wong et al., 2004). In addition, some patients had numerous Lewy body-like inclusions in the CA2-4 regions of the hippocampus, while others had cortical-type LBs in the temporal lobe, entorhinal cortex, and cingulate gyrus, congruent with a more diffuse DLB-type neuropathology (Tayebi et al., 2003; Wong et al., 2004).

### 1.2.3 Genetic Association Studies

Multiple clinics and centers around the world subsequently began conducting genetic studies to investigate the frequency of *GBA1* mutations in patients with PD. In one such study, brain samples demonstrated *GBA1* mutations in as many as 21% of subjects diagnosed with PD (Lwin et al., 2004). Among the high-risk Ashkenazi Jewish subset of ethnic groups, *GBA1* mutations were present in 31.3% of patients with idiopathic PD (Aharon-Peretz et al., 2004). In contrast, only 4.1% of patients with AD and 6.2% of control subjects had *GBA1* mutations (Aharon-Peretz et al., 2004). In 2009, Sidransky and colleagues published a landmark study on this topic - a collective analysis of 5691 patients with PD and 4898 controls from 16 centers across 12 countries. When the entire *GBA1* coding region was

sequenced in 1883 non-Ashkenazi Jewish patients and 1611 controls, mutations were observed in 6.9% of the PD patients and 1.3% of the control subjects (odds ratio 5.4; 95% CI, 3.9-7.6). Among the Ashkenazi Jewish subjects, higher mutation frequencies were seen; 19.3% of PD patients and 4.1% of controls had *GBA1* mutations (Sidransky et al., 2009). These findings have revealed that mutations in *GBA1* are the most common known genetic risk factor for developing PD (Sidransky et al., 2009).

Similarly, *GBA1* mutations have also been associated with DLB. When all eleven exons and flanking intronic regions of *GBA1* were sequenced in autopsy-confirmed DLB patients, mutations were found in 23% (8 of 35) of cases (Goker-Alpan et al., 2006). In another study, when the *GBA1* coding region was sequenced in brain tissue of autopsied patients with a neuropathological diagnosis of DLB (95 cases), AD (60 cases), and controls (32 cases), mutations were found in 28% of DLB patients, 10% of AD patients, and 3% of control subjects (odds ratio 6.48; 95% CI, 2.45-17.16) (Clark et al., 2009).

### **1.3 Mechanisms Underlying the *GBA1*-Synucleinopathy Association**

The molecular mechanisms underlying the association between mutations in *GBA1* and risk of developing PD and DLB remain elusive. The loss-of-enzymatic function (LOF) and novel gain-of-toxic function (GOF) models have both been proposed as possible explanations for this association. Intriguingly, growing evidence supports the existence of a bidirectional pathogenic relationship between  $\alpha$ -synuclein aggregation and GCase activity (DePaolo et al., 2009; Mazzulli et al.,

2011; Sardi et al., 2012; Velayati et al., 2010).

### 1.3.1 A Loss-of-Enzymatic Function and Abnormal Lipid Metabolism

In support of the LOF model, pharmacological inhibition of GCase activity was shown to cause an accumulation of  $\alpha$ -synuclein in human neuroblastoma and mouse SN cells (Manning-Bog et al., 2009). In primary neuronal cultures and human GD neurons derived from induced pluripotent stem (iPS) cells, a reduction in GCase activity compromised lysosomal protein degradation, which led to  $\alpha$ -synuclein accumulation and aggregation followed by neurotoxicity (Mazzulli et al., 2011). In this study, a reduction in GCase activity led to the accumulation of GluCer, which *in vitro* stabilized soluble  $\alpha$ -synuclein oligomers - the presumed toxic species (Mazzulli et al., 2011). Importantly,  $\alpha$ -synuclein overexpression in primary cortical neurons inhibited the ER maturation/trafficking of GBA1, leading to a further reduction in lysosomal GCase activity (Mazzulli et al., 2011). These findings implicate a pathogenic feed-forward loop whereby reduced GCase activity enhances  $\alpha$ -synuclein oligomerization and aggregation, which further inhibits lysosomal GCase activity in a reciprocal manner (Mazzulli et al., 2011).

In homozygous D409V/D409V *gba1* mice, an age-dependent rise in proteinase K-resistant, endogenous  $\alpha$ -synuclein (as well as ubiquitin-positive) aggregates was observed in the hippocampus, a region affected in patients with DLB, advanced PD, and GD with parkinsonism (Sardi et al., 2011). These D409V/D409V *gba1* mice also demonstrated an age-dependent increase in the lipid substrate GluSph, as well as memory deficits. Of therapeutic importance, adeno-associated virus (AAV) – mediated expression of WT GBA1 in the hippocampus of these

mice lowered GluSph levels, ameliorated  $\alpha$ -synuclein/ubiquitin neuropathology, and reversed memory impairment (Sardi et al., 2011). This study indicates that rescuing and enhancing GCase activity could provide a therapeutic target for the management of *GBA1*-linked synucleinopathies (Sardi et al., 2011; Sardi et al., 2013).

Analysis of the cerebrospinal fluid (CSF) of patients with PD and DLB demonstrated a significant reduction in GCase activity compared to control subjects (Balducci et al., 2007; Parnetti et al., 2009). Additionally, a significant decrease in GCase activity was found not only in the brains of patients with *GBA1*-linked PD, but also in patients with sporadic PD (Gegg et al., 2012). These findings suggest that GCase activity is impaired in synucleinopathies. However, it is not yet known whether a reduction in GCase activity that does not lead to GD, will nonetheless result in the accumulation of lipid substrates upstream of  $\alpha$ -synuclein accumulation/aggregation.

A caveat to the LOF model is that few *GBA1*-mutation carriers develop PD, with the lifetime risk and penetrance rate of PD still unknown. In addition, only a minority of GD patients develop parkinsonism despite having homozygous *GBA1* mutations that lead to insufficient GCase activity and the accumulation of lipid substrates (Gegg et al., 2012; Westbroek et al., 2011). A possible explanation is that several other factors also mediate  $\alpha$ -synuclein processing, and *GBA1* mutations exacerbate and accelerate, but do not necessarily initiate, PD pathology (Gegg et al., 2012; Westbroek et al., 2011). This would explain why patients with *GBA1*-linked PD have an earlier age of disease onset and a more progressive clinical course than PD

patients without mutations (Sidransky et al., 2009).

### 1.3.2 A Novel Gain-of-Toxic Function

In support of the GOF model, the exogenous expression of several GBA1 mutants (N370S, D409V, D409H, L444P, E340K, E235K) in two neural cell lines significantly raised human  $\alpha$ -synuclein levels in a *GBA1* cDNA dose-dependent manner (Cullen et al., 2011). Importantly, the over-expression of GBA1 mutants did not lead to a reduction in the endogenous GCase activity of neural cells (Cullen et al., 2011). In rat pheochromocytoma cells, treatment with rapamycin, an autophagy-inducer, reversed the mutant GBA1-induced accumulation of  $\alpha$ -synuclein, thereby correcting the observed GOF effect (Cullen et al., 2011). This study indicates that the expression of GBA1 variants is sufficient to raise  $\alpha$ -synuclein levels, and that these mutants do not act in a dominant-negative manner towards WT GBA1 (Cullen et al., 2011).

Yap et al. (2011) demonstrated that GBA1 and  $\alpha$ -synuclein interact selectively under lysosomal pH conditions using immunoprecipitation and fluorescence spectroscopy in human tissue and in *in vitro* studies, respectively (Yap et al., 2011). In support of this interaction, exogenous GBA1 and  $\alpha$ -synuclein colocalized within cathepsin D-positive lysosomal compartments in dopaminergic human neuroblastoma cells (Yap et al., 2011). Surprisingly,  $\alpha$ -synuclein exhibited reduced binding affinity for both the GD-related N370S variant and the inhibitor conduritol  $\beta$ -epoxide (CBE) - bound GBA1 protein (Yap et al., 2011). This finding indicates that the lysosomal interaction between WT GBA1 and  $\alpha$ -synuclein may help to maintain  $\alpha$ -synuclein homeostasis. *GBA1* mutations that result in

decreased  $\alpha$ -synuclein-GBA1 interaction may perturb this beneficial effect, leading to the aberrant accumulation and aggregation of  $\alpha$ -synuclein (Yap et al., 2011). However, the protease responsible for the degradation of  $\alpha$ -synuclein remains elusive.

A limitation of the GOF hypothesis is that in humans, nonsense mutations in *GBA1* such as 84GG or IVS2 +1 result in the absence of a mutant protein being expressed, yet seem to confer an even higher risk of developing synucleinopathy than mutations that result in the expression of a misfolded protein with residual enzymatic activity (Gan-Or et al., 2008; Sidransky et al., 2009). For example, the odds ratios for 84GG and IVS2 +1 were 14.0 (95% CI 4.8–40.6) and 42.0 (95% CI 4.7–377.2), respectively (Gan-Or et al., 2008). In contrast, the common and relatively mild N370S mutant, which retains the highest residual GCase activity, had an odds ratio of 2.2 (95% CI 1.5– 3.1) (Gan-Or et al., 2008; Sidransky et al., 2009).

#### **1.4 Conclusion**

The molecular mechanisms underlying the association between mutations in *GBA1* and risk of developing synucleinopathy remain intensely debated but unresolved. Both LOF function and GOF models have been proposed. Several lines of evidence support each model, and both mechanisms may co-conspire to fuel  $\alpha$ -synuclein accumulation and aggregation (Sardi et al., 2012). Further *in vitro* and *in vivo* studies are required to better understand the relationship between mutations in *GBA1* and the misprocessing of  $\alpha$ -synuclein, as well as to provide insights into possible therapeutic targets.

## OBJECTIVES AND STATEMENT OF HYPOTHESIS

### Objectives:

Discovery of the relationship between *GBA1* mutations and the risk of developing PD and DLB has generated the challenge to delineate the molecular cascade underlying this close association. It is unclear whether loss-of-enzymatic function and novel gain-of-toxic-function effects are mediated through independent molecular mechanisms, or if they share abnormal SL metabolism upstream of  $\alpha$ -synuclein misprocessing. The objective of this thesis is to determine what, if any, changes occur in the GluCer/GluSph/Cer lipid profile of neural cells and tissue expressing wild type *GBA1*, mutant *GBA1*, or both.

### Hypothesis:

Neural cells and tissue, which model a homozygous genotype, will show an accumulation of the lipid substrates GluCer and GluSph, while those modeling a heterozygous genotype will not show GluCer and GluSph accumulation, thereby indicating an independent mechanism underlying  $\alpha$ -synuclein dysregulation.

### Aims:

1. To optimize a lipid extraction and high-performance thin layer chromatography (HPTLC) protocol to assess changes in the GluCer/GluSph/Cer lipid profile of neural cells.
2. To use this lipid assay to determine if there are similar SL changes in a previously characterized mouse model containing two mutant D409V - encoding knock-in *gba1* alleles (Xu et al., 2003).

## **2. MATERIALS AND METHODS**

### **2.1 Chemical Reagents**

All lipid standards used for the lipid extraction and HPTLC experiments including Cer (18:1/18:0), Chol (plant-derived), GalCer (d18:1/16:0), GluCer (d18:1/18:0), GluSph (d18:1), LacCer (d18:1/16:0), PC (porcine brain-derived), PE (porcine brain-derived), and SM (porcine brain-derived) were purchased from Avanti Polar Lipids, Inc. (Alabaster, AL). All lipid extraction and HPTLC solvents including chloroform, methanol, and glacial acetic acid were HPLC grade and were supplied by Thermo Fisher Scientific Inc. (Waltham, MA).

### **2.2 Cell Culture**

The untagged human WT *GBA1* cDNA-containing plasmid was purchased from Origene Inc. (Rockville, MD) and the human WT *SNCA* cDNA-containing plasmid was prepared previously in the Schlossmacher laboratory (Cullen et al., 2009). PCR site directed mutagenesis was previously used to introduce the D409V and D409H point mutations to the WT *GBA1* gene, and the sequence was previously confirmed by DNA sequencing (Cullen et al., 2011). DMEM culture medium, FBS, and Trypsin were supplied by Thermo Fisher Scientific Inc. (Waltham, MA). The *GBA1* inhibitor conduritol  $\beta$ -epoxide (CBE) was purchased from Sigma-Aldrich (St. Louis, MO) and the *GBA1* chaperone / partial antagonist isofagomine (Ifg) was obtained from Cedarlane (Burlington, ON). All cell culture experiments were carried out in the human dopaminergic M17 neuroblastoma cell line, a kind gift from Dr. L Petrucelli.

M17 cells were cultured in T-75 (75 cm<sup>2</sup>) flasks in DMEM culture medium supplemented with 10% FBS and 1% of penicillin-streptomycin.

Cells were incubated at 37°C and 5.0% CO<sub>2</sub> atmosphere. The DMEM media was renewed every 2 days and cells were passaged every 3-4 days.

Twenty-four hours prior to transfection, 1x10<sup>6</sup> cells were seeded per well (3.5 cm<sup>2</sup>, 6-well plates). Transient expression of *GBA1*- and *SNCA*-containing cDNAs was carried out using 5.0 µg cDNA/well and Lipofectamine 2000 (Invitrogen, Carlsbad, CA). For experiments involving the inhibition or augmentation of *GBA1* function, cells were treated twenty-four hours after transfection with 100 µM CBE or 10 µM Irg, respectively. Forty-eight hours post-transfection, cells were washed with PBS and harvested by incubation with 0.25% Trypsin for 10 seconds. The cell suspension was collected and pelleted by centrifugation at 1000 x g for 3 min. The supernatant was removed and cells were washed twice with PBS. Cell pellets were snap frozen and stored at -20°C until further processing.

### **2.3 Lipid Extraction**

The following lipid extraction protocol was modified from Farwanah et al. (2009). Cell pellets were suspended in 0.8 mL of water and homogenized using an ultrasound bath. For analysis of biological samples (e.g., mouse brain), 10 mg of tissue was mechanically homogenized in 0.8 mL of water. For lipid extraction, 3 mL of chloroform and methanol (1:2; v/v) were added to the suspension. After vortexing, the homogenous solution was incubated at 40°C in a water bath overnight. This extraction procedure was repeated three times and the supernatants were pooled. Cell debris was removed by centrifugation at 800 x g for 2 min and the solvents were evaporated under a stream of nitrogen gas.

Lipid extracts were re-dissolved in 2 mL of chloroform and methanol (1:1; v/v). For alkaline base hydrolysis, 150  $\mu$ l of 1 M KOH was added to the solution. After vortexing, the extract was incubated for 2 hours at 40°C in a water bath. The solution was cooled to room temperature and was neutralized with 6  $\mu$ l of glacial acetic acid. A solvent ratio of 1.8 water: 2 chloroform: 2 methanol (v/v/v) led to a phase separation and the bottom chloroform phase was taken. Neutral lipids were re-extracted by adding 2 ml of chloroform to the upper phase. The re-extraction step was repeated three times and the lower chloroform phase was pooled. After evaporation, the resulting lipid extracts were re-dissolved in a mixture of chloroform and methanol (2:1; v/v).

#### **2.4 Separation of Neutral SLs using HPTLC**

The HPTLC glass plates (Silica 60, 10 x 10 cm) were purchased from Merck (Darmstadt, Germany) and the TLC developing tank was obtained from Thermo Fisher Scientific Inc. (Waltham, MA). Lipid extracts were separated and visualized by HPTLC as previously described (Farwanah et al., 2009). Lipid standards (1  $\mu$ g/ $\mu$ l) and extracts (25  $\mu$ l unless otherwise stated) were dotted onto the HPTLC plate. The loaded plate was developed twice in a solvent system of chloroform, methanol, and acetic acid (70:30:8; v/v/v) to a distance of 6 cm from the bottom. A third development in 100 mL of chloroform was performed to the top of the plate. The plate was allowed to dry between each development. To visualize lipid-containing bands, plates were treated for 30 min in a Coomassie blue staining solution (0.03% in 30% MeOH in 100 mM NaCl). The plate was subsequently washed twice with a de-staining solution (30% MeOH in 100 mM NaCl). All HPTLC

experiments were performed in duplicates and representative plates are shown.

## **2.5 Separation and Profiling of Neutral SLs using LC-MS/MS**

For the quantitative analysis of neutral SLs, liquid chromatography coupled to tandem mass spectrometry (LC-MS/MS) was used, as previously described (Cabrera-Salazar et al., 2010). Briefly, brain tissue was homogenized in water for a final concentration of 100 mg/ml (w/v). For lipid extraction, 1 ml of acetonitrile, methanol, and water (97:2:1; v/v/v) was added to 10  $\mu$ l of brain homogenate at room temperature and shaken vigorously for 2-3 min. For the separation of GluCer and GluSph, extracts were injected into an Atlantis HILIC silica column (Waters Corp., Milford, MA). These GSLs were subsequently detected using MRM mode tandem mass spectrometry with an AB Sciex API-4000 mass spectrometer. For the separation and detection of Cer, extracts were injected onto an Acquity BEH C8 column (Waters Corp., Milford, MA), and MRM mode detection was performed using an AB Sciex API-5000 mass spectrometer. Analytes were quantified against standard curves. All SL standards were obtained from Matreya, LLC (Pleasant Gap, PA). Our collaborators at Genzyme Corporation performed the mass spectrometry experiments and analyzed the resulting data.

## **2.6 GCCase Activity Assay**

GCCase activity was determined employing a fluorometric assay measuring 4-methylumbelliferone, as previously described (Cabrera-Salazar et al., 2010). Briefly, the synthetic substrate 4-methylumbelliferyl- $\beta$ -D-glucopyranoside (Sigma-Aldrich, St. Louis, MO) was added to cell or tissue lysates. The reaction was

terminated after 1 hour by the addition of 1M Glycine (pH 12.5). Cleaved 4-methylumbelliferoone was measured by a fluorometer using Softmax pro software (Ex365/Em445). The resulting enzymatic activity was converted to the mass of cleaved substrate per milligram of protein per hour using the specific activity of the standard Cerezyme ® (Genzyme Corporation, A Sanofi Company, Cambridge, MA) and normalized to the amount of protein in each sample determined using the micro BCA Protein Assay Reagent Kit (Thermo Fisher Scientific Inc., Waltham, MA). GCase activity measured in vector control cells (transfected with the empty pcMV XL5 plasmid) was set at 100%. For each experimental arm, the GCase activity was determined in three independent trials (n=3), and each trial was carried out in triplicates. Cell and tissue lysates were sent to Genzyme Corporation, where Dr. Pablo Sardi kindly carried out the GCase activity experiments.

## **2.7 Animal Studies**

All animal procedures were approved by the University of Ottawa Animal Care Committee and were in accordance with the Guidelines of the Canadian Council on Animal Care. For lipid staining experiments, a previously characterized mouse model with mutant D409V - encoding knock-in *gba1* alleles and their age-matched, WT littermates were used (Xu et al., 2003). Homozygous D409VD409V *gba1* mice were crossed with C57BL/6 mice (representing the strain's background) to generate heterozygous WT/D409V - expressing *gba1* mice. Mouse tissue homogenates were genotyped using the same primers as previously described (Cullen et al., 2009; Xu et al., 2003). Dr. Julianna Tomlinson from the Schlossmacher laboratory carried out

breeding and genotyping of these D409V *gba1* mice.

## **2.8 Oil Red O Staining of Neutral Lipids**

Fresh mouse brains from 3.5-month-old D409V *gba1* mice were fixed in 10% formalin. Serial coronal sections (10  $\mu\text{m}$ ) of fresh-frozen tissue were mounted on glass slides and stained for neutral lipids with Oil Red O, as previously described (Kruth, 1984). Sections were placed in absolute propylene glycol for 2 min and subsequently stained with filtered 0.5% Oil Red O in absolute propylene glycol solution for 16 hours. Slides were differentiated in an 85% propylene glycol solution for 1 min, rinsed in 2 changes of distilled water, and counterstained with Mayer's hematoxylin solution for 15-60 sec. After rinsing slides thoroughly in several changes of distilled water, sections were mounted in warmed glycerin jelly solution. Mouse brains were sectioned and stained by the Pathology Laboratory at the University of Ottawa.

## **2.9 Luxol Fast Blue Staining for Myelin and Nerve Cells**

Fresh-frozen brains from 3.5-month-old D409V *gba1* mice were serially sectioned (coronal) at a thickness of 10  $\mu\text{m}$  and incubated with a 0.1% Luxol Fast Blue in ethyl alcohol solution overnight in a 56°C oven (Kluver and Barrera, 1953). Excess stain was rinsed off with 95% ethyl alcohol. Slides were subsequently rinsed with distilled water and differentiated in 0.05% lithium carbonate solution for 30 sec. The differentiation was continued for another 30 sec in 70% ethyl alcohol. Slides were rinsed in distilled water and counterstained in filtered 0.1% cresyl echt violet solution for 6 min. Slides were rinsed in 2 changes of 95% ethyl alcohol. The

dehydration was continued with 2 changes of absolute ethyl alcohol for 2 min and 2 changes of xylene for 2 min. Sections were then mounted with Permount medium (Thermo Fisher Scientific Inc., Waltham, MA). Mouse brains were sectioned and stained by the Pathology Laboratory at the University of Ottawa.

### **2.10 Western Blotting**

Cells were lysed forty-eight hours post-transfection in 150  $\mu$ L of lysis buffer (50 mM Tris-HCL; 140 mM NaCl; 2 mM EDTA; 0.5% Triton X-100, 1X protease inhibitor). After centrifugation at 15, 000 x g for 15 min at room temperature, the supernatant was normalized to the amount of protein in each sample using the Pierce BCA Kit (Thermo Fisher Scientific Inc., Waltham, MA). Cell lysates (30  $\mu$ g protein per lane) were subjected to denaturing sodium dodecyl sulfate-based polyacrylamide gel electrophoresis (SDS/PAGE) under reducing conditions and transferred (electroblotted) to a nitrocellulose membrane. The membrane was blocked with 10% fat-free milk and probed with the following antibodies: mouse monoclonal anti-GBA1 (1:1500; AbCam, Cambridge, MA), mouse monoclonal anti- $\alpha$ -synuclein (1:1000; BD Transduction, San Jose, CA), and mouse monoclonal anti- $\beta$ -actin (1: 5000; Abcam). The membranes were subsequently incubated with anti-mouse secondary antibody conjugated to horseradish peroxidase (HRP) enzyme, and protein bands were visualized using SuperSignal West Pico Chemiluminescent Substrate (Thermo Fisher Scientific Inc., Waltham, MA).

### **2.11 Immunohistochemistry**

For immunohistochemistry (IHC), a previously characterized mouse model

containing two D409V mutant knock-in *gba1* alleles and their age-matched, WT littermates were used (Xu et al., 2003). Slides containing paraffin-embedded brain sections from these mice were a kind gift from Dr. Grabowski. Monoclonal anti- $\alpha$ -synuclein (Syn-1) antibody was from BD Transduction (San Jose, CA) and anti-ubiquitin antibody was from Dako Cytomation (Carpentaria, CA). To validate IHC studies, another well-characterized mouse model containing homozygous V394L - encoding knock-in *gba1* alleles was also used (Xu et al., 2003). All brain sections were from 12-month-old mice. Adel Farah from the Schlossmacher laboratory performed the IHC experiments, as previously described (Schlossmacher et al., 2002; Schlossmacher and Shimura, 2005).

### **2.12 Statistical Analysis**

Experimental data were analyzed using the student *t* test and one-way analysis of variance (ANOVA). Statistical analyses were performed using GraphPad Prism (GraphPad Software, Inc) and StatPlus®:mac LE (AnalystSoft Inc.). A *p*-value of less than 0.05 was considered significant.

### **2.13 Sources of Funding**

Funding for this project was provided through the following awards and scholarships: NSERC Alexander Graham Bell Canada Graduate Scholarship (CGS) (2010-2011); CIHR Training Program in Neurodegenerative Lipidomics Graduate Supplemental Award (2010-2012); University of Ottawa FGPS Excellence Scholarship (2010-2012); and the Ontario Graduate Scholarship (2011-2012).

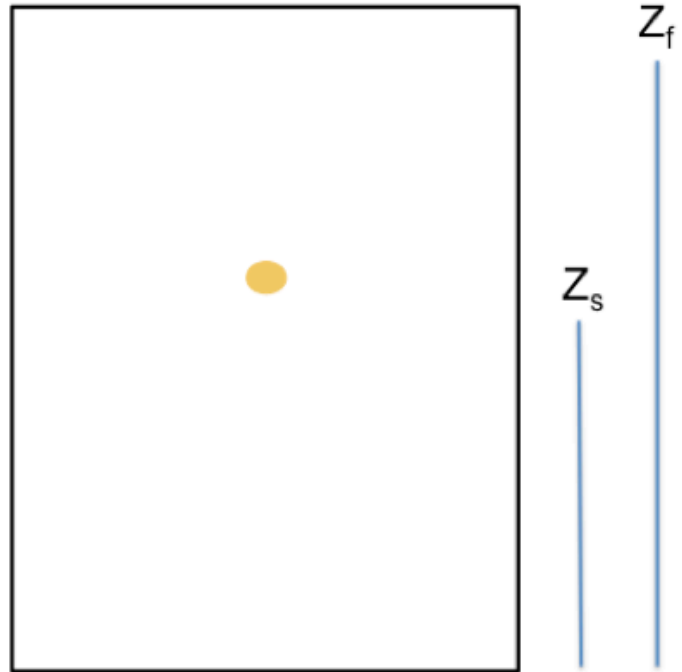
### 3. RESULTS

#### 3.1 An HPTLC Protocol for the Separation of Neutral SLs and Cholesterol

High-performance thin layer chromatography (HPTLC) can be used for the separation and partial characterization of SLs in mixtures (Meisen et al., 2004; van Echten-Deckert, 2000; Variyar, 2011). The HPTLC technique involves the application of lipid extracts onto a silica plate (stationary phase), which is subsequently developed in a solvent system (mobile phase). The solvent migrates along the plate in an ascending manner, carrying the analytes (e.g., lipid extracts) with it (van Echten-Deckert, 2000). The retention factor ( $R_f$ ) is a qualitative value that is expressed as the distance travelled by a particular analyte in a mixture ( $Z_s$ ) divided by the distance travelled by the solvent front ( $Z_f$ ) (**Figure 3**). Polar, high-molecular weight lipids interact to a larger extent with the stationary phase, and consequently have a lower  $R_f$  value (Variyar, 2011). In contrast, non-polar, low molecular weight lipids have a higher  $R_f$  value. The ability to separate lipid classes and subclasses by HPTLC depends in part on the types and proportions (v/v) of solvents that comprise the solvent system (van Echten-Deckert, 2000; Variyar, 2011).

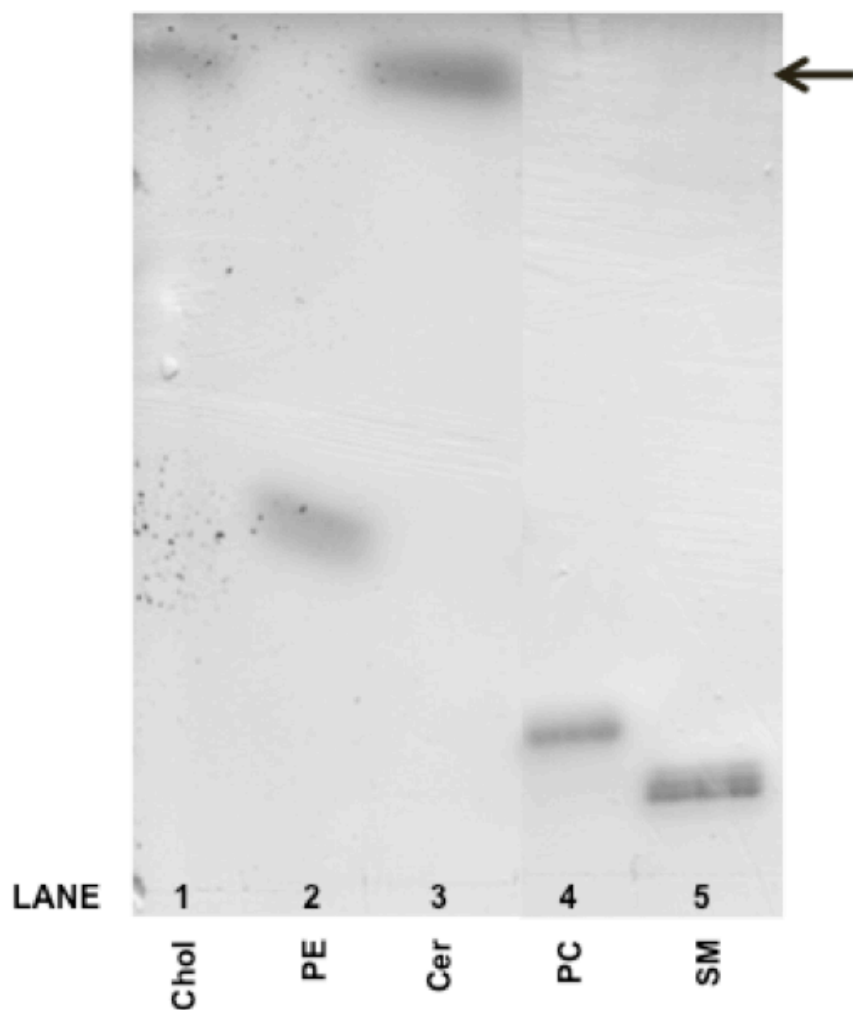
As shown in **Figure 4**, a solvent system composed of chloroform, methanol, and water (65:25:4; v/v/v), used for the general separation of phospholipids, can resolve several polar, phospholipid standards such as phosphatidylethanolamine (PE), phosphatidylcholine (PC), and sphingomyelin (SM). Since neutral, non-polar lipids such as ceramide (Cer) and cholesterol (Chol) co-migrate to the solvent front (arrow), this solvent system is not ideal to separate the neutral SLs of interest.

$$R_f = \frac{Z_s}{Z_f}$$



**Figure 3. Retention Factor.**

The retention factor ( $R_f$ ) is expressed as the distance travelled by a particular analyte in a mixture ( $Z_s$ ) divided by the distance travelled by the solvent front ( $Z_f$ ). The analyte is represented as a yellow dot.



**Figure 4. General HPTLC Separation of Phospholipids.**

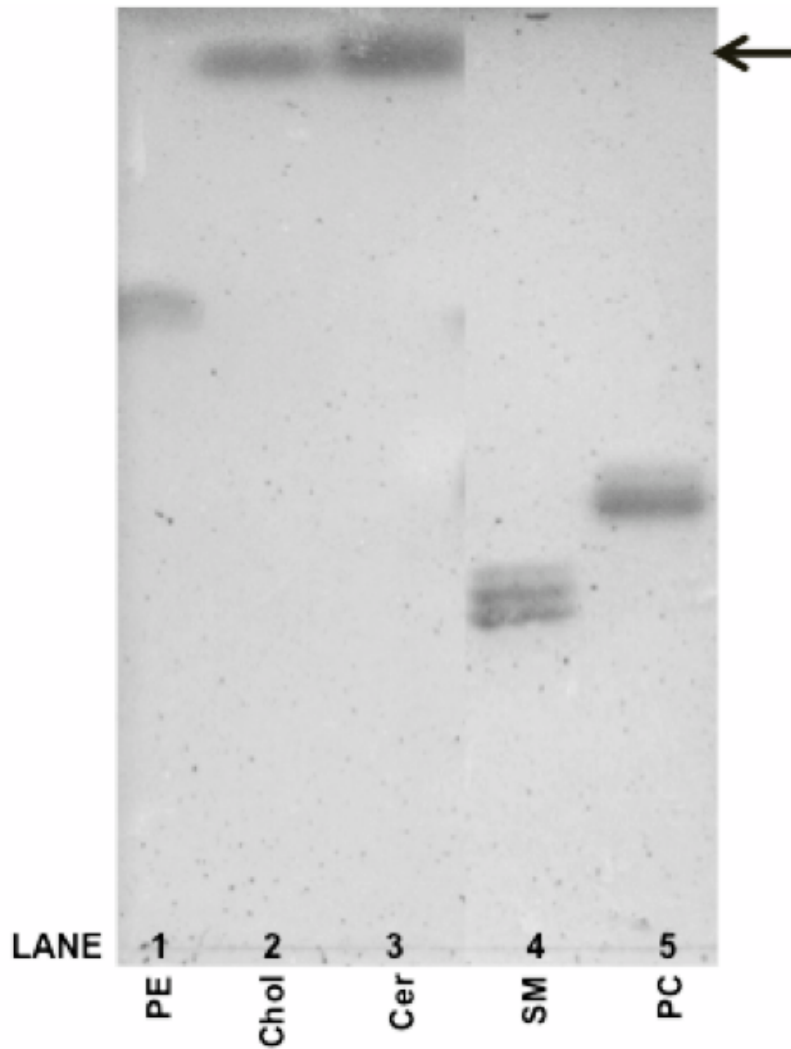
Lipid standards (3  $\mu$ l) were applied to the HPTLC plate and developed in a chloroform, methanol, and water (65:25:4; v/v/v) solvent system used for the general separation of phospholipids. Cer, ceramide standard; Chol, cholesterol standard; PC, phosphatidylcholine standard; PE: phosphatidylethanolamine standard; SM, sphingomyelin standard.

Next, an HPTLC protocol established for the separation of neutral SLs was used (Meisen et al., 2004). The HPTLC plate was developed to the top of the plate in chloroform, methanol, and water (120:70:17; v/v/v). **Figure 5** shows that this solvent system also results in the co-migration of Cer and Chol (arrow), and is therefore not ideal for the separation of neutral SLs from cholesterol.

Farwanah and colleagues (2009) have recently developed an HPTLC procedure for the specific separation of neutral SLs from Chol. The protocol involves two developments in chloroform, methanol, and acetic acid (70:30:8; v/v/v) to a distance of 6 cm from the bottom of the plate, followed by a third development to the top of the plate in pure chloroform. The first two steps are necessary for the chromatographic resolution of neutral SLs. The final step carries Chol and contaminants to the solvent front (Farwanah et al., 2009). Finally, replacing water with acetic acid in the solvent system decreases the mobility of Cer, separating it from Chol (van Echten-Deckert, 2000).

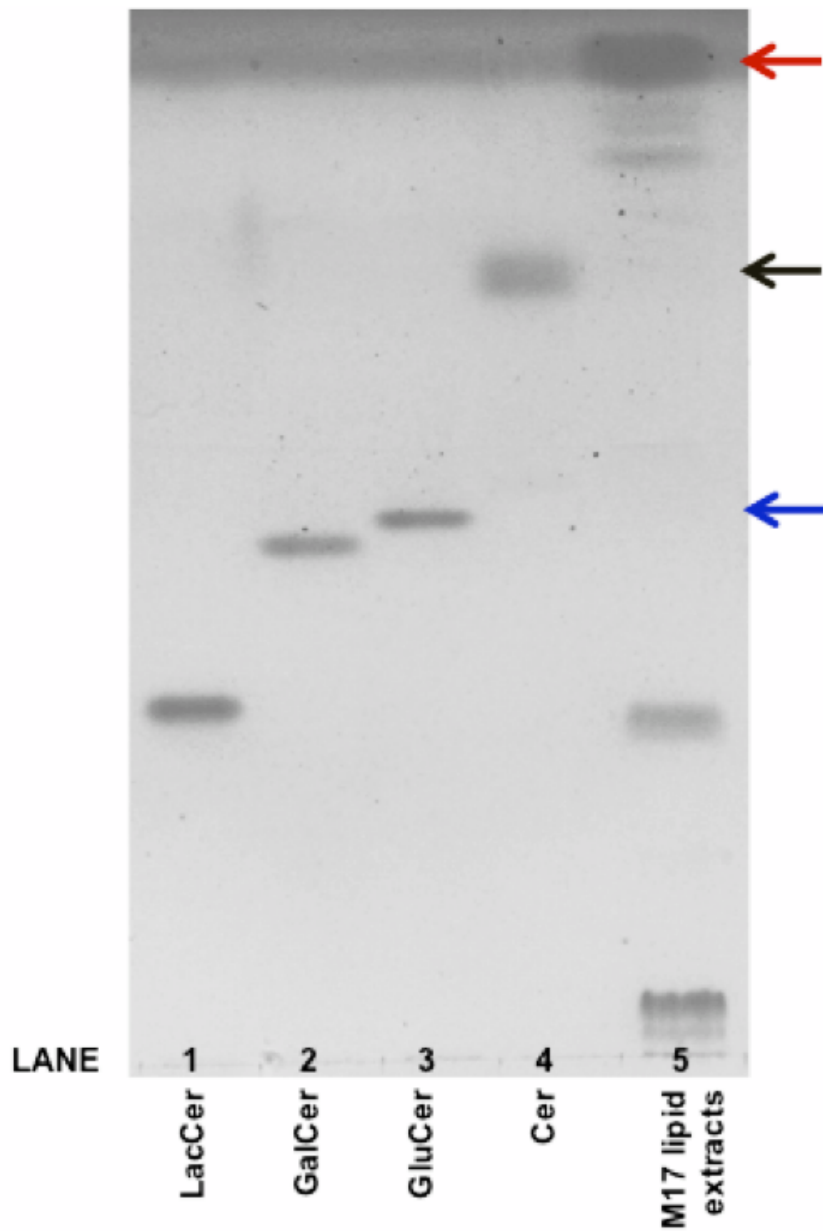
As shown in **Figure 6**, this protocol allows several neutral SL species to be resolved, including the lipid standards Cer, galactosylceramide (GalCer), glucosylceramide (GluCer), and lactosylceramide (LacCer). Additionally, Cer (black arrow) and Chol (red arrow) now acquire different  $R_f$  values, and can therefore be successfully separated.

To determine the limit of detection for this HPTLC protocol, a serial dilution and chromatographic separation of a mixture containing the lipid standards Cer (black arrow), GluCer (red arrow), and GluSph (blue arrow) was performed. As shown in **Figure 7**, the serial dilution establishes that approximately 15-30 ng of each lipid



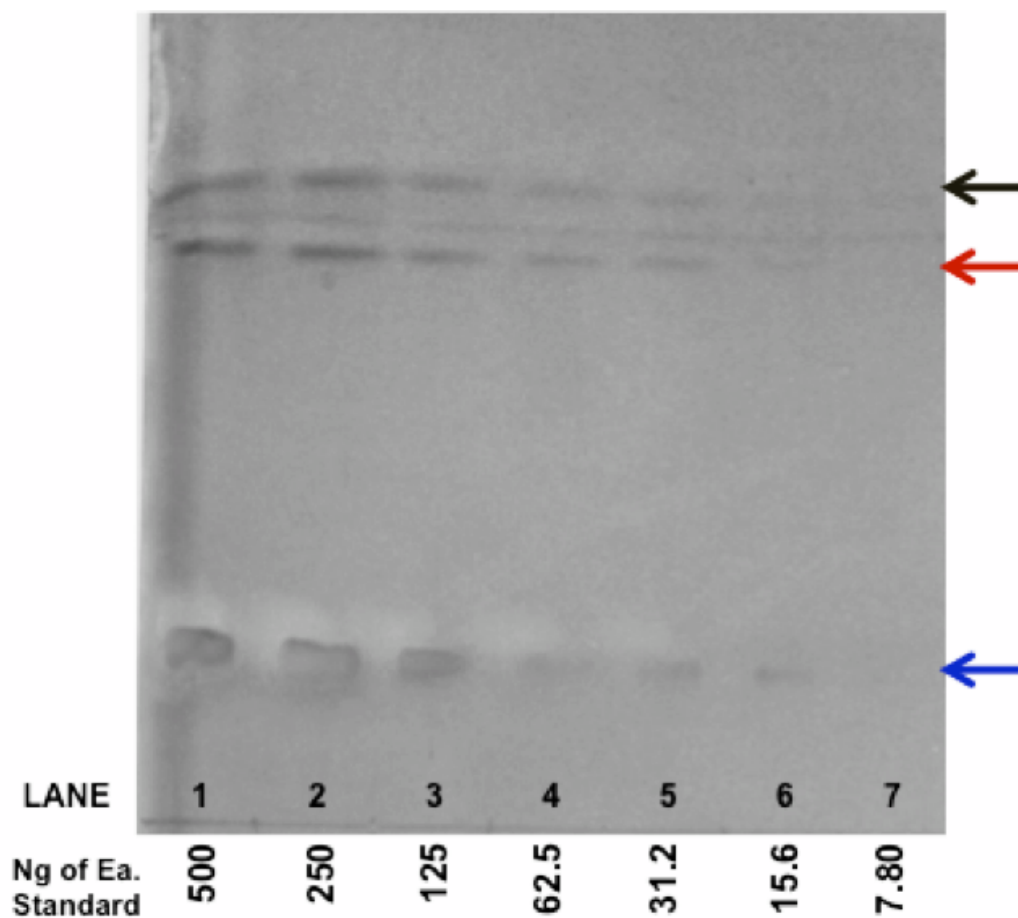
**Figure 5. HPTLC Separation of Neutral SLs.**

Lipid standards (3  $\mu$ l) were applied to the HPTLC plate and developed in a chloroform, methanol, and water (120:70:17; v/v/v) solvent system used for the separation of neutral SLs (Meisen et al., 2004). Cer, ceramide standard; Chol, cholesterol standard; PC, phosphatidylcholine standard; PE: phosphatidylethanolamine standard; SM, sphingomyelin standard.



**Figure 6. Lipid Extraction and HPTLC Separation of Neutral SLs and Chol.**

Neutral SLs were extracted from M17 cells, as described (Farwanah et al., 2009). Lipid extracts (25  $\mu$ l) and standards (3  $\mu$ l) were applied to the HPTLC plate and developed in a chloroform, methanol, and acetic acid (70:30:8; v/v/v) solvent system used for the separation of neutral SLs (Farwanah et al., 2009). Cer, ceramide standard; GalCer, galactosylceramide standard; GluCer: glucosylceramide standard; LacCer, lactosylceramide standard.



**Figure 7. Serial dilution and HPTLC Separation of Neutral SL Standards.**

A serial dilution was performed on a mixture of the lipid standards Cer (black arrow), GluCer (red arrow), and GluSph (blue arrow). Samples were applied to the HPTLC plate and developed in a chloroform, methanol, and acetic acid (70:30:8; v/v/v) solvent system (Farwanah et al., 2009). The weight (ng) per lane of each lipid standard is labeled along the bottom.

standard can be detected using this HPTLC protocol (**Figure 7, lanes 5-6**). Furthermore, there is no observable interference between these three SL standards during HPTLC separation.

### **3.2 Optimization of a Lipid Extraction Protocol for the High Yield**

#### **Extraction of Neutral SLs**

Farwanah and colleagues (2009) have developed a protocol for the extraction of neutral SLs from human fibroblasts. The procedure features the extraction of lipids in a 40°C water bath overnight. This heating step is required for the phase transition of SLs to occur (Farwanah et al., 2009; Sullards and Merrill, 2001; van Echten-Deckert, 2000). Of note, phospholipids are also extracted using this chloroform-methanol extraction mixture. Some of these phospholipid species co-migrate with particular SLs, and it is therefore necessary to remove these major lipid contaminants via alkaline hydrolysis (Farwanah et al., 2009; Sullards and Merrill, 2001; van Echten-Deckert, 2000).

Non-transfected, non-treated M17 cells were subjected to lipid extraction and HPTLC separation of neutral SLs using the protocol described by Farwanah and colleagues (2009). Surprisingly, neither Cer (black arrow) nor GluCer (blue arrow) could be detected using this extraction procedure (**Figure 6, lane 5**) (Farwanah et al., 2009). A possible explanation for this finding is that the protocol must be further optimized for the specific cell or tissue type being processed.

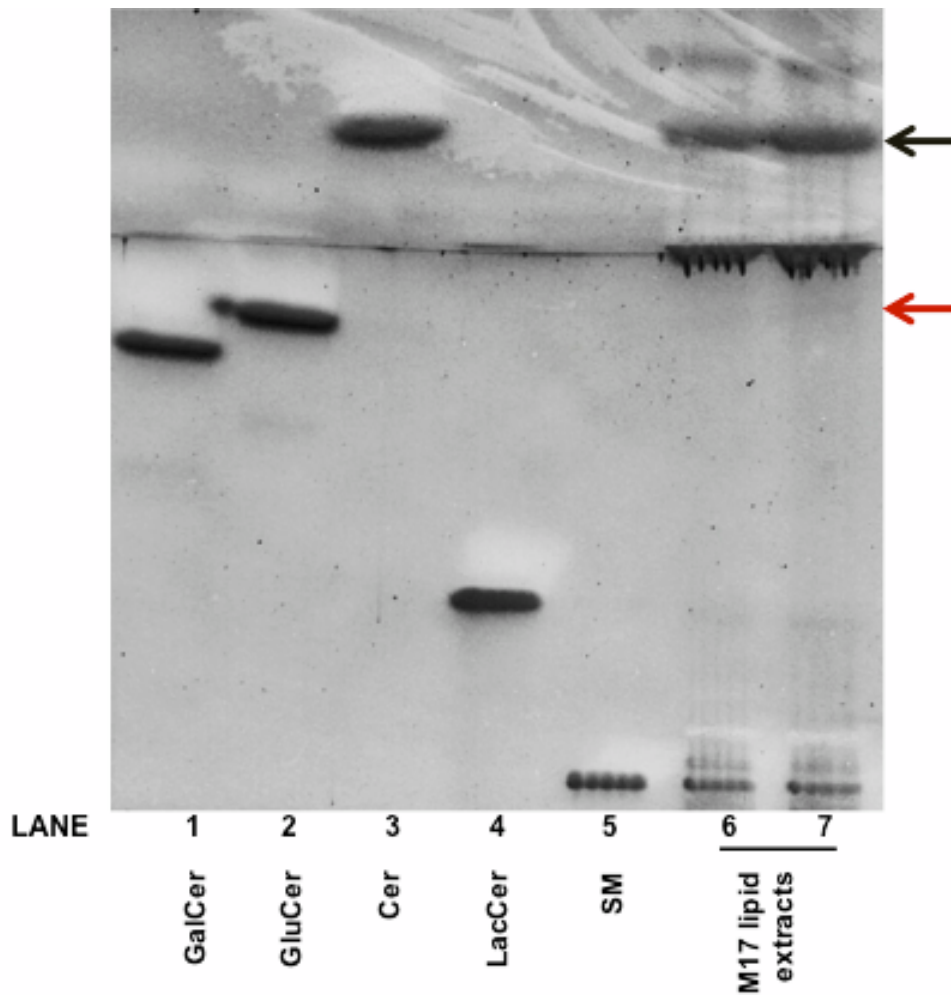
To increase the yield of neutral SLs extracted from M17 cells, a series of back-extractions with chloroform were performed (see **Materials and Methods**). Further, lipid extracts were concentrated so larger amounts ( $\mu\text{g}$ ) of lipids

could be loaded per lane. This was carried out by drying the chloroform layer containing the SL extracts under a stream of nitrogen gas, and then re-dissolving the extracts in consecutively lower volumes of solvent. As shown in **Figure 8**, these modifications successfully increased the ability to detect SLs extracted from M17 cells. The ability to detect Cer (black arrow) increased dramatically, whereas the ability to detect GluCer (red arrow) also increased, albeit to a barely detectable lipid band.

### **3.3 The Effect of WT and Mutant GBA1 Proteins on Neutral SL Levels in Neural Cells**

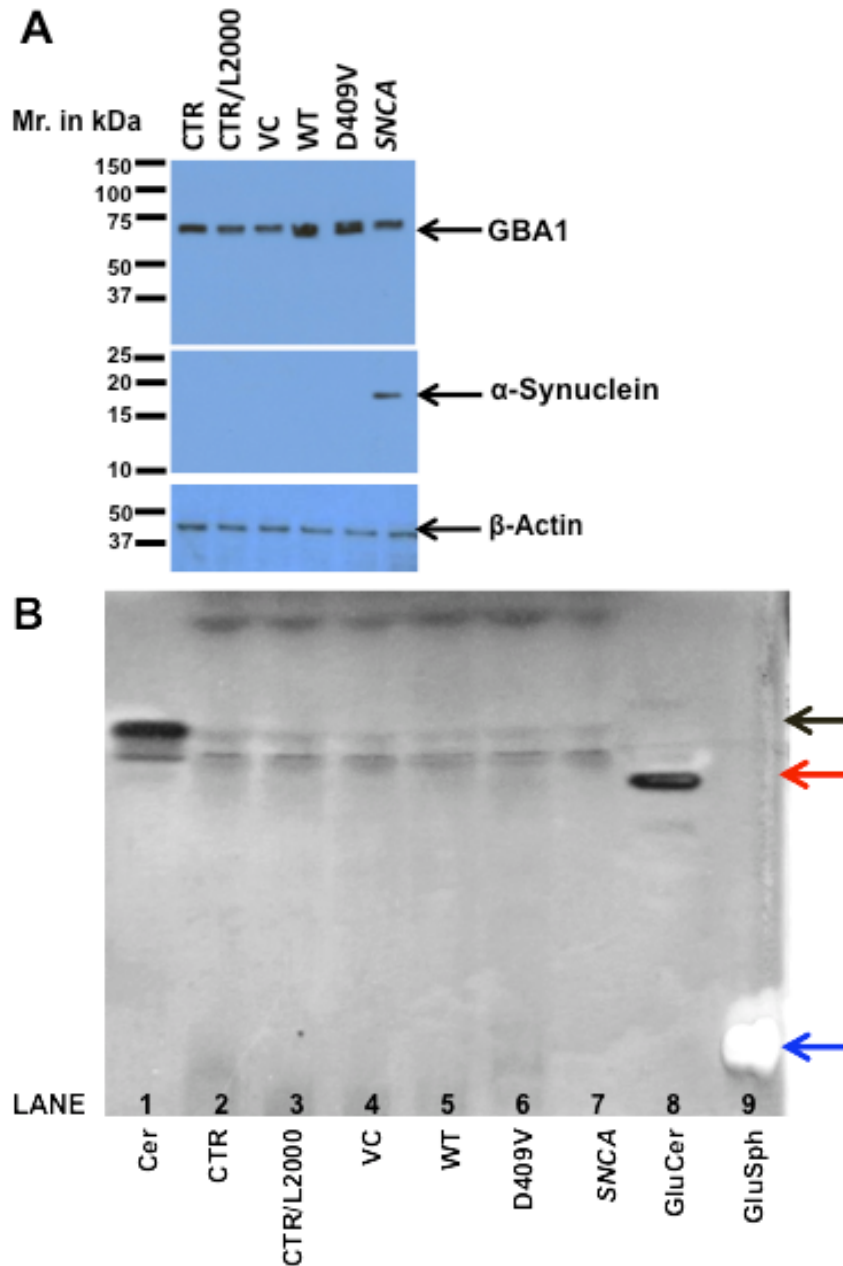
To analyze the effects of human GBA1 proteins on SL levels, *GBA1* cDNA-containing plasmids were transiently expressed in human dopaminergic M17 neuroblastoma cells. M17 cells were subsequently analyzed using the modified lipid extraction and HPTLC protocol as described above. Initially, three control arms were included: non-transfected cells (CTR), non-transfected cells treated with the transfection reagent Lipofectamine 2000 (CTR/L2000), and cells transfected with the empty pCMV-XL5 vector (VC) (**Figure 9**). These conditions were included to ensure that the liposomes in the transfection reagent would not affect the lipid readout of transfected cells.

As expected, no significant difference was observed in the levels of endogenous GBA1 protein (**Figure 9A**) or neutral SLs (**Figure 9B**) between the three control arms. Western blotting demonstrated that similar levels of exogenous WT and mutant D409V GBA1 proteins were expressed (**Figure 9A**). Under these conditions, there was no detectable difference in levels of GluCer (red



**Figure 8. Lipid Extraction and HPTLC Separation of Neutral SLs Using the Modified Protocol.**

Neutral SLs were extracted from M17 cells using the modified lipid extraction protocol. Lipid standards (3  $\mu$ l) and lipid extracts from M17 cells (25  $\mu$ l) were applied to the HPTLC plate and developed in a chloroform, methanol, and acetic acid (70:30:8; v/v/v) solvent system (Farwanah et al., 2009). Cer, ceramide standard; GalCer, galactosylceramide standard; GluCer: glucosylceramide standard; LacCer, lactosylceramide standard; SM, sphingomyelin standard.



**Figure 9. Effects of Exogenous GBA1 and  $\alpha$ -Synuclein Proteins on Neutral SL Levels in M17 Cells.**

(A) Western blot analysis using denaturing SDS/PAGE under reducing conditions of M17 cell lysates. Anti- $\beta$ -actin was used as a loading control. (B) Neutral SLs were extracted from M17 cells using the modified lipid extraction protocol. Lipid standards (3  $\mu$ l) and lipid extracts from M17 cells (25  $\mu$ l) were applied to the HPTLC plate and developed in a chloroform, methanol, and acetic acid (70:30:8; v/v/v) solvent system (Farwanah et al., 2009). Cer, ceramide; GluCer: glucosylceramide standard; GluSph, glucosylsphingosine standard.

arrow), GluSph (blue arrow), or Cer (black arrow) between the VC condition and cells overexpressing WT or mutant D409V GBA1 (**Figure 9B**).

Schlossmacher et al (2005) demonstrated that  $\alpha$ -synuclein binds with high affinity to brain-derived GSLs that contain GluCer at their core *in vitro*. We therefore sought to determine whether  $\alpha$ -synuclein overexpression had an observable effect on neutral SL levels by transiently expressing human *SNCA* cDNA-containing plasmids in M17 cells (**Figure 9A, SNCA**). There was no demonstrable effect of exogenous  $\alpha$ -synuclein on the levels of neutral SLs relative to the VC condition (**Figure 9B**).

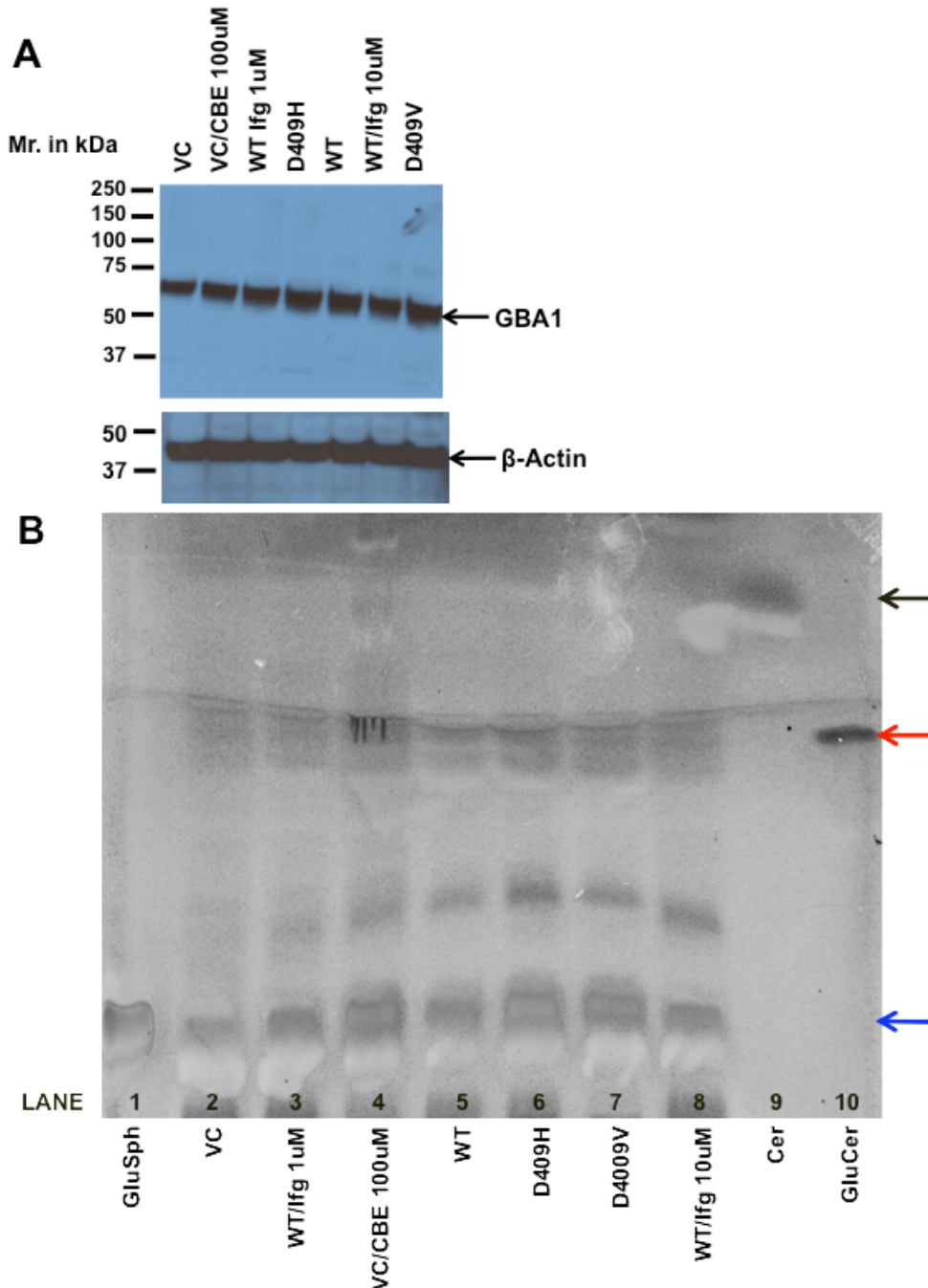
To date, differences in cellular GluCer, GluSph, or Cer have not been observed between the experimental conditions. A possible explanation could be that a larger amount of starting material is required in order to detect subtle changes in SL levels by HPTLC. To address this, the number of cells was increased from  $1 \times 10^6$  to  $10 \times 10^6$  cells per condition for all subsequent experiments.

Next, the protein levels, GCase activity, and SL levels were analyzed in the following experimental arms: (1) VC, vector-transfected control cells; (2) VC/CBE 100 $\mu$ M, vector control cells treated with 100  $\mu$ M of the GCase inhibitor conduritol  $\beta$ -epoxide (CBE); (3) WT, cells expressing exogenous WT GBA1 protein; (4) WT/Ifg 1 $\mu$ M, cells expressing exogenous WT GBA1 protein co-treated with 1  $\mu$ M of the GBA1 chaperone / partial antagonist isofagomine (Ifg) (Lieberman et al., 2007); (5) WT/Ifg 10 $\mu$ M, cells expressing exogenous WT GBA1 protein treated with 10  $\mu$ M Ifg; (6) D409H, cells expressing exogenous mutant D409H GBA1 protein; and (7) D409V, cells expressing exogenous mutant D409V GBA1 protein.

Treatment of M17 cells with CBE will inhibit endogenous (and exogenous) GCCase activity, and thus provide a cellular model of the homozygous genotype. In contrast, M17 cells expressing both endogenous WT GBA1 and exogenous GBA1 variants will be used as a cellular model of the heterozygous genotype. As shown in **Figure 10A**, overexpression of all exogenous GBA1 proteins was achieved.

To ensure that transfection resulted in proper ER/Golgi trafficking of GBA1 and led to the expression of functional lysosomal GBA1 proteins, cellular GCCase activity was quantified, as summarized in **Table 1**. M17 cells expressing exogenous WT GBA1 (WT) demonstrated a significant rise in GCCase activity (to 145.1%,  $p < 0.0001$ ) compared to the vector control cells (VC, set at 100% activity), as expected. Cells expressing exogenous WT GBA1 that were treated with Ifg (WT/Ifg 10 $\mu$ M) revealed a similar increase in GCCase activity (to 140.8%;  $p < 0.0003$ ). Of note, there was no difference in the enzymatic activity between M17 cells co-treated with Ifg (WT/Ifg 10 $\mu$ M) versus those without any chaperone added (WT). This indicates that most of the overexpressed WT GBA1 protein was successfully processed through the ER and Golgi apparatus, and delivered into the lumen of the lysosomes (Lieberman et al., 2007).

As expected, the enzymatic activity of vector control cells treated with CBE (VC/CBE 100 $\mu$ M) was markedly and significantly reduced by greater than 98% (to 1.2%,  $p < 0.0001$ ) relative to vector control cells without treatment (VC). Finally, there was no significant difference in the enzymatic activity of cells overexpressing the D409H (to 95.5%,  $p = 0.9216$ ) or D409V (to 100.5%,  $p = 0.3121$ ) variants compared to vector control cells (**Table 1**). This indicates that these GBA1 variants



**Figure 10. Effect of Exogenous GBA1 Proteins on Neutral SL Levels in M17 Cells.**

(A) Western blot analysis of M17 cells using denaturing SDS/PAGE under reducing conditions. (B) Neutral SLs were extracted from M17 cells using the modified lipid extraction protocol. Lipid standards (3  $\mu$ l) and lipid extracts from M17 cells (25  $\mu$ l) were applied to the HPTLC plate and developed in a chloroform, methanol, and acetic acid (70:30:8; v/v/v) solvent system (Farwanah et al., 2009). Cer, ceramide standard; GluCer: glucosylceramide standard; GluSph, glucosylsphingosine standard.

<b>TABLE 1: Quantification of GCase Activity in M17 Cells Expressing Human GBA1 Proteins</b>				
<b>Condition (Repeats<sup>a</sup>)</b>	<b>Mean GCase Activity ± SD (ng/h/mg prot)</b>	<b>Mean GCase Activity (%VC)</b>	<b><i>p</i> Value<sup>b</sup></b>	<b>Statistical Difference<sup>c</sup></b>
<b>VC (n=3)</b>	35.0 ± 6.1	100.0	N/A	N/A
<b>VC/CBE 100uM (n=3)</b>	0.4 ± 0.1	1.2	< 0.0001	Yes
<b>WT (n=3)</b>	50.8 ± 7.4	145.1	< 0.0001	Yes
<b>WT/lfg 10uM (n=3)</b>	49.3 ± 4.6	140.8	0.0003	Yes
<b>D409V GBA1 (n=3)</b>	33.5 ± 2.6	95.5	0.3121	No
<b>D409H GBA1 (n=3)</b>	35.2 ± 2.8	100.5	0.9216	No

a. Each number represents an independent experiment run in triplicates.  
b. Determined using the student *t* test to compare mean GCase Activity (ng/h/mg prot) between Vector Control cells (VC) and all other conditions.  
c. Determined using the student *t* test as mentioned above. In addition, a one-way analysis of variance (ANOVA) confirmed an interaction (*p* < 0.05) between the conditional arm and mean GCase activity level (ng/h/mg prot).  
CBE = conduritol β-epoxide; lfg = Isofagomine; N/A = not applicable; VC = vector control cells; SD = standard deviation; WT = wild-type.  
Quantification of GCase activity was performed by Dr. Pablo Sardi from the Genzyme Corporation.

do not act in a dominant-negative manner towards the endogenous WT GBA1 expressed by M17 cells. Furthermore, the mutant proteins do not seem to have residual enzymatic activities, under the conditions tested.

To determine whether changes in SL levels could be detected between the seven experimental arms, samples were analyzed using the modified lipid extraction and HPTLC protocol. It should be noted that samples were additionally concentrated prior to application onto the HPTLC plate. This was performed by re-dissolving lipid extracts in only 50ul of chloroform and methanol (2:1; v/v) and applying 25ul of the final preparation onto the plate. As shown in **Figure 10B** this modification further enhanced the detection of GluCer.

Although cells overexpressing WT GBA1 (treated with or without Irg) demonstrated a robust increase in GCase activity compared to vector control cells (VC), as measured by a pseudo-substrate *in vitro* (**Table 1**), HPTLC analysis did not show any detectable difference in SL levels between these conditions (**Figure 10B**). M17 cells treated with CBE (VC/CBE 100µM) had a significant reduction in GCase activity relative to vector control cells. In parallel, HPTLC analysis demonstrates a small but noticeable accumulation in the substrate GluSph in cells treated with CBE relative to cells without treatment (**Figure 10B**). The streaking observed in **Lane 4** likely represents an overload of certain lipids (Variyar, 2011). Since the streaking occurs where GluCer is resolved, it may indicate an accumulation in this lipid. However, since this streaking can be observed higher on the plate, it may indicate a non-specific overload of SLs instead (**Figure 10B**).

Finally, GCase activity levels in cells expressing the D409H or D409V variants were not significantly different than vector control cells, likely owing to the endogenously expressed WT GBA1 protein (**Table 1**). Under these conditions, there appears to be a subtle elevation in the levels of both GluSph and GluCer in cells expressing the D409H and D409V GBA1 variants (**Figure 10, lanes 6-7**). Contrary to my hypothesis, this finding may indicate that the expression of mutant GBA1 protein is sufficient to result in the accumulation of GluCer and GluSph lipid substrates, even without a corresponding loss of GCase function. However, since the HPTLC technique only allows for the partial characterization and limited quantification of changes in SL levels, these results must be confirmed using a more reliable quantitative technique. To address this, sister samples were prepared for analysis by high-profile liquid chromatography coupled to tandem mass spectrometry (HPLC-MS/MS). At the time of thesis submission, samples are still being analyzed for changes in cellular GluCer/GluSph/Cer levels between the different conditions mentioned above.

### **3.4 The Effect of WT and Mutant GBA1 Proteins on SL Levels in Animal Tissue**

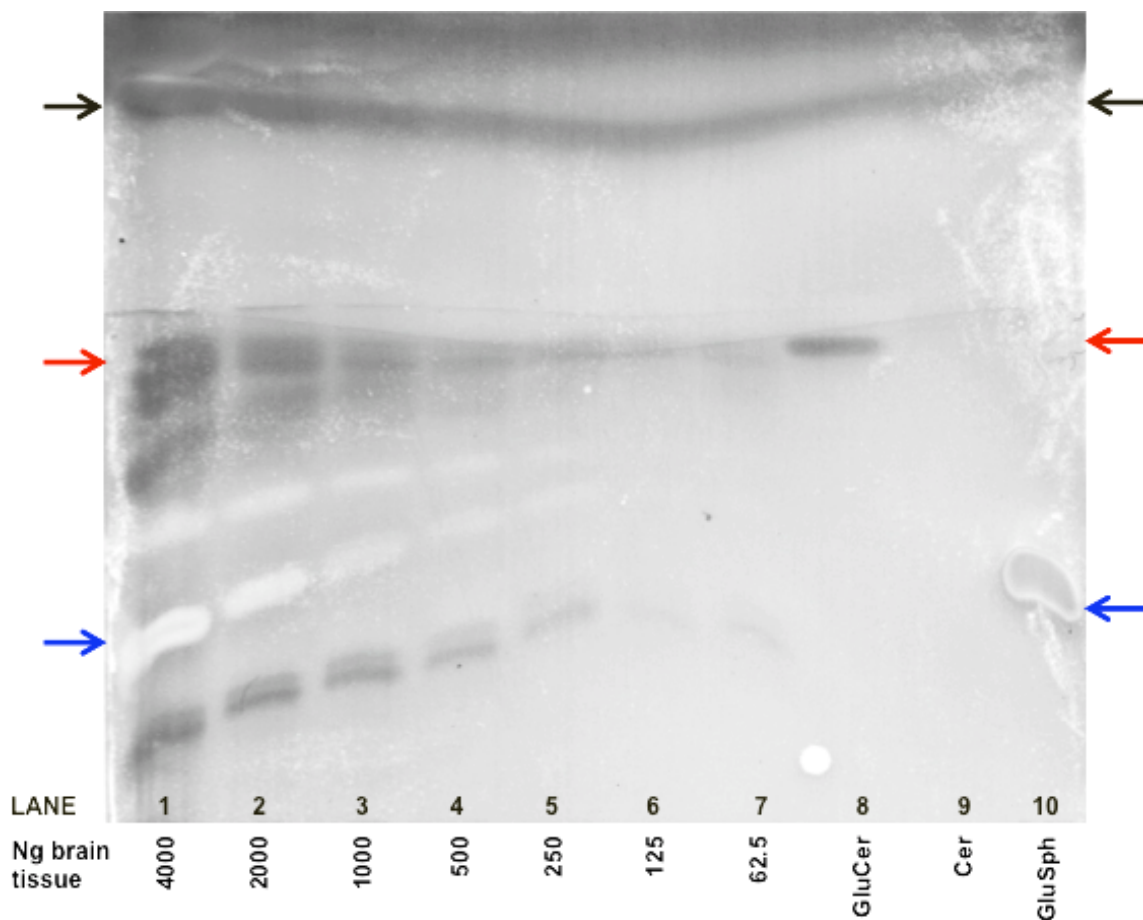
To determine whether the modified lipid extraction and HPTLC protocol mentioned here could be used to detect a more robust change in SL levels from animal tissue, we decided to next analyze a well-characterized mouse model expressing mutant D409V - encoding knock-in *gba1* alleles (Xu et al., 2003).

### 3.4.1 Serial Dilution of Lipid Extracts from a Mouse Brain

We first sought to determine the optimal loading level for lipids extracted from mouse brains. Lipid extraction was performed using 10 mg (wet weight) of brain tissue from a control mouse. Once neutral SL's were extracted, the dry extracts were re-suspended in a chloroform and methanol (2:1; v/v) solution. A serial dilution was performed and samples were loaded onto a plate, as shown in **Figure 11**. The optimal loading level seems to be the equivalent of 1 to 2  $\mu\text{g}$  of brain tissue, which represents a 1:10 or a 1:5 dilution of the extracts, respectively. At a higher lipid concentration (**lane 1**), both GluCer (red arrow) and GluSph (blue arrow) appear oversaturated and the solvent front does not ascend in a linear manner. At lower concentrations (**lanes 4-7**), the lipid bands are too faint.

### 3.4.2 SL Analysis in Mutant D409V *Gba1* Mice

To determine the relationship between *GBA1* point mutations, GCCase activity, and SL levels, we next analyzed a well-characterized mouse model expressing two mutant D409V knock-in *gba1* alleles (Xu et al., 2003) and their WT littermates. Heterozygous (WT/D409V) *gba1* mice were also used and all animals were 3.5-months-old. **Table 2** summarizes the GCCase activity measured in the brain tissue of these D409V *gba1* mice. There was a greater than 50% reduction in brain GCCase activity (to 46.8%,  $p < 0.05$ ) in homozygous D409V/D409V *gba1* mice compared to their WT littermates (set to 100% GCCase activity). Although heterozygous WT/D409V *gba1* mice had a close to 15% reduction in brain GCCase activity (to 85.5%) compared to WT mice, this difference was not significant ( $p > 0.05$ ).



**Figure 11. Serial Dilution and HPTLC Separation of Neutral SLs from Mouse Brain.**

Neutral SLs were extracted from 10 mg (wet weight) of mouse brain tissue using the modified lipid extraction protocol. A serial dilution was performed on lipid extracts and samples were applied to the HPTLC plate and developed in a chloroform, methanol, and acetic acid (70:30:8; v/v/v) solvent system (Farwanah et al., 2009). The 'Ng brain tissue' represents the estimated weight (ng) of tissue from which the specific dilution would have been extracted. Cer, ceramide standard; GluCer, glucosylceramide standard; GluSph, glucosylsphingosine standard.

<b>TABLE 2: Quantification of GCCase Activity in the Brain of D409V <i>Gba1</i> Mice</b>				
<b>GENOTYPE</b> (Repeats <sup>a</sup> )	<b>Mean GCCase Activity ± SD</b> (ng/h/mg prot)	<b>Mean GCCase Activity</b> (%WT)	<b>p Value<sup>b</sup></b>	<b>Statistical Difference<sup>c</sup></b>
<b>WT</b> (n=7)	34.7 ± 5.4	100	N/A	N/A
<b>WT/D409V</b> (n=7)	29.7 ± 3.3	85.5	0.0805	No
<b>D409V/D409V</b> (n=4)	16.3 ± 0.6	46.8	<0.0002	Yes

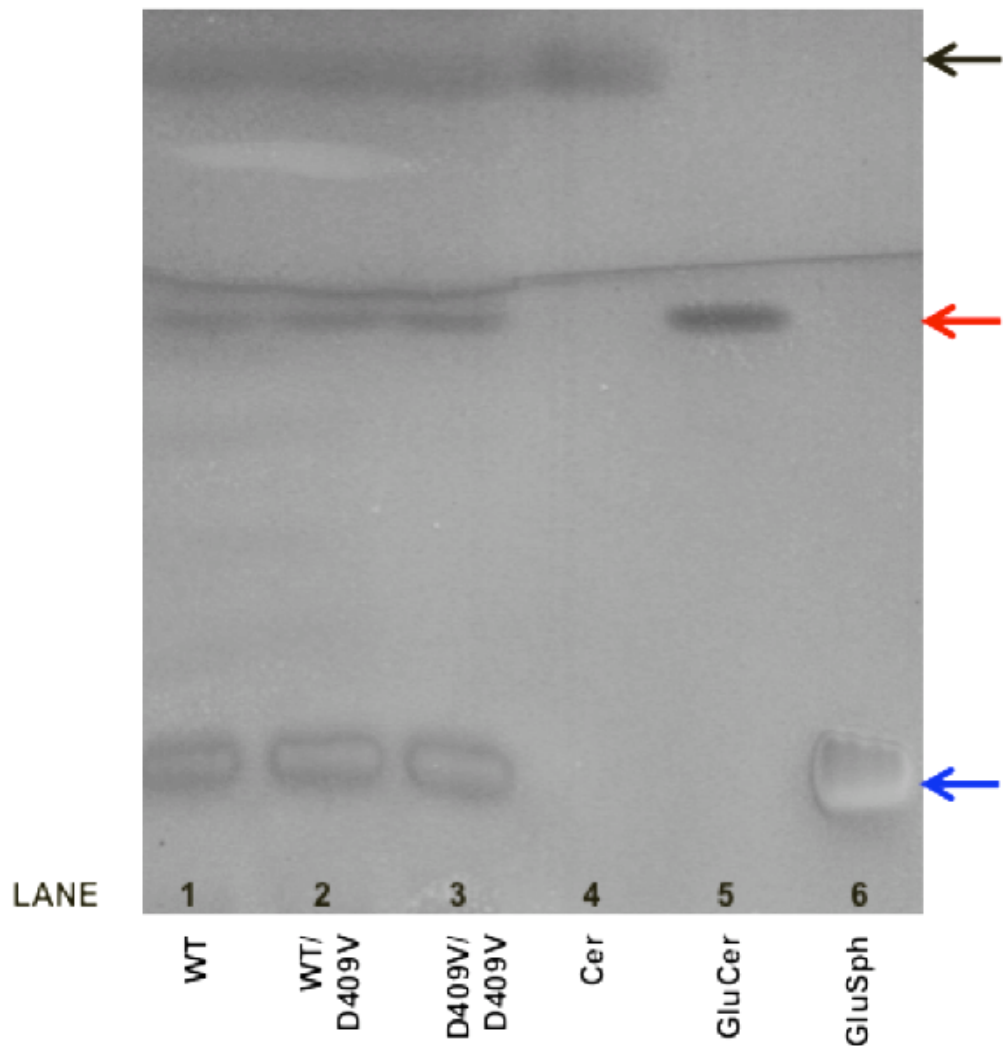
a. Each number represents an individual mouse

b. Determined using the student *t* test to compare mean GCCase Activity (ng/h/mg prot) between WT *gba1* mice (WT) and all other genotypes.

c. Determined using the student *t* test as mentioned above. In addition, a one-way analysis of variance (ANOVA) confirmed an interaction ( $p < 0.05$ ) between the genotype and mean GCCase activity level (ng/h/mg prot).  
 N/A = not applicable; SD = standard deviation; WT = wild-type.  
 Quantification of GCCase activity was performed by Dr. Pablo Sardi from the Genzyme Corporation.

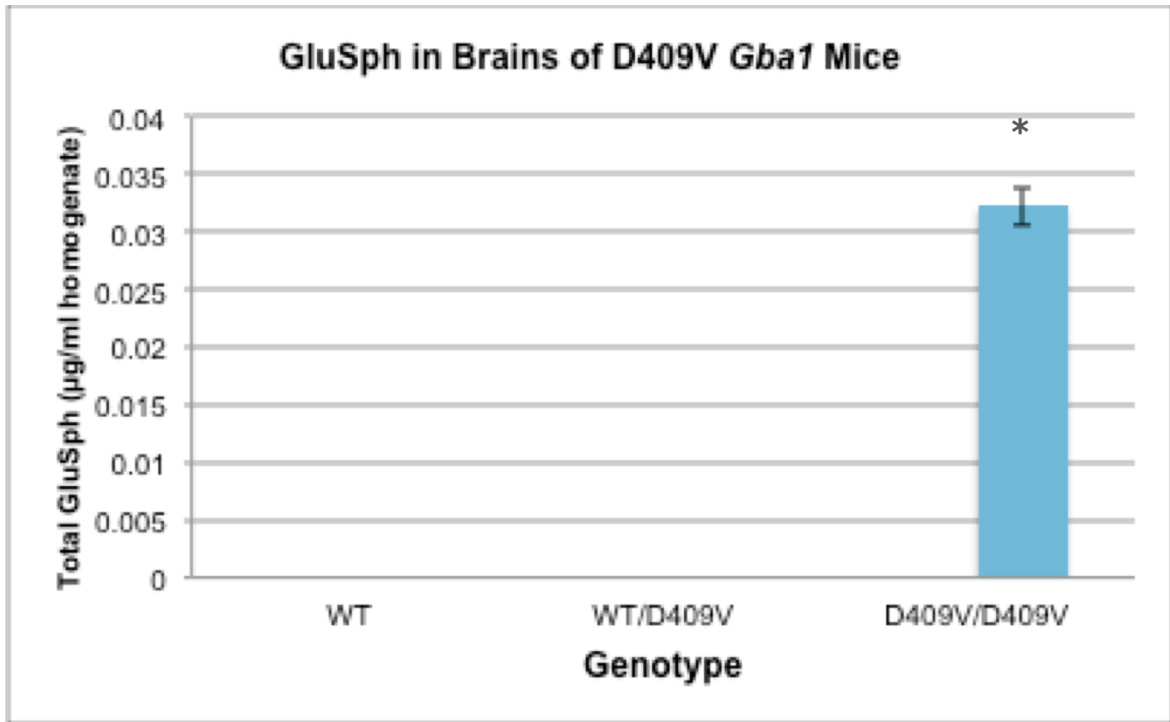
Brain tissue from D409V *gba1* mice was subsequently analyzed by the modified lipid extraction and HPTLC protocol for changes in SL levels. For lipid extraction, 10 mg (wet weight) of brain tissue from each mouse was used. Dry lipids were re-suspended in a chloroform and methanol (2:1; v/v) solution, and 2  $\mu$ l were loaded onto each lane. As shown in **Figure 12**, a subtle rise in GluCer (red arrow) can be detected in the homozygous D409V/D409V *gba1* mouse brain compared to the WT littermate. It is difficult to discern whether there is an even subtler rise in GluCer in the heterozygous WT/D409V *gba1* mouse brain compared to the WT littermate. Finally, at this lipid concentration, there is saturation of GluSph, and thus changes in this SL cannot be detected (blue arrow).

Since the limit of detection has been reached using this modified lipid extraction and HPTLC methodology, these findings must be confirmed using a more quantitative analysis. Accordingly, D409V *gba1* mouse brains were processed for analysis by HPLC-MS/MS. **Figure 13** shows GluSph levels ( $\mu$ g/ml homogenate) in brain tissue from these mice. There is a significant rise in GluSph ( $p < 0.05$ ) in homozygous D409V/D409V *gba1* mice compared to their WT littermates. In contrast, there was no accumulation in GluSph in the heterozygous WT/D409V *gba1* mice, consistent with other studies (see **Discussion**). At the time of thesis submission, brain tissues are still being analyzed for changes in brain GluCer and Cer levels between the genotypes.



**Figure 12. Neutral SL Levels in the Brain of D409V *Gba1* Mice**

Neutral SLs were extracted from the brain of 3.5-month-old wild-type (WT), heterozygous (WT/D409V), and homozygous (D409V/D409V) *gba1* mice (n=1 of each) using the modified lipid extraction protocol. Lipid standards (3  $\mu$ l) and extracts (2  $\mu$ l) were applied to the HPTLC plate and developed in a chloroform, methanol, and acetic acid (70:30:8; v/v/v) solvent system (Farwanah et al., 2009). Cer, ceramide standard (black arrow); GluCer: glucosylceramide standard; GluSph, glucosylsphingosine standard.



**Figure 13. HPLC-MS/MS Analysis of GluSph in the Brain of D409V *Gba1* Mice.**

The levels of the lipid substrate glucosylsphingosine (GluSph) in mouse brain lysates of 3.5-month-old wild-type (WT, n=7), heterozygous (WT/D409V, n=7), and homozygous (D409V/D409V, n=4) *gba1* mice were quantified by mass spectrometry. Data shown represents mean  $\pm$  SEM. Bar with symbol (\*) is significantly different from all other genotypes ( $p < 0.05$ ) as determined by the student *t* test and a one-way analysis of variance. Mass spectrometry experiments and data analysis was performed by our collaborators at Genzyme Corporation.

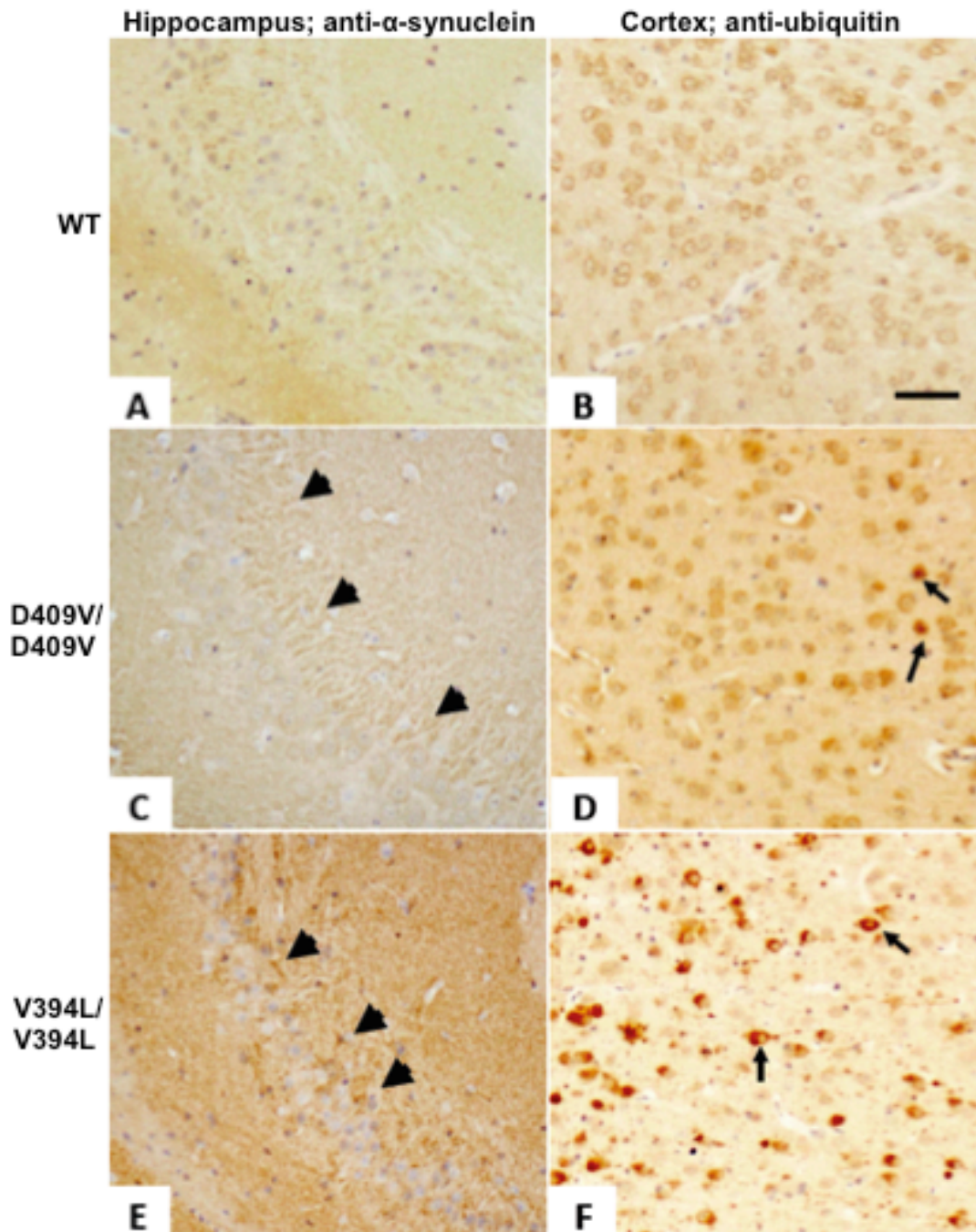
### 3.5 Immunohistochemistry of Brain Sections from *Gba1* Mice

To determine if  $\alpha$ -synuclein / ubiquitin accumulation and aggregation could be detected in brain tissue, slides containing brain sections from 12-month-old homozygous D409V/D409V *gba1* and V394L/V394L *gba1* mice were probed by routine IHC. As shown in **Figure 14**, ubiquitin-positive neurons (arrows) can be observed in the cortex of D409V/D409V *gba1* mice (**D**), which are more abundant in V394L/V394L *gba1* mice (**E**). Furthermore, endogenous  $\alpha$ -synuclein (arrowheads) can be detected in the hippocampus of D409V/D409V *gba1* (**C**) and in corresponding sections of V394L/V394L *gba1* (**E**) mice.

### 3.6 Lipid Staining in Brain Sections of D409V *Gba1* Mice

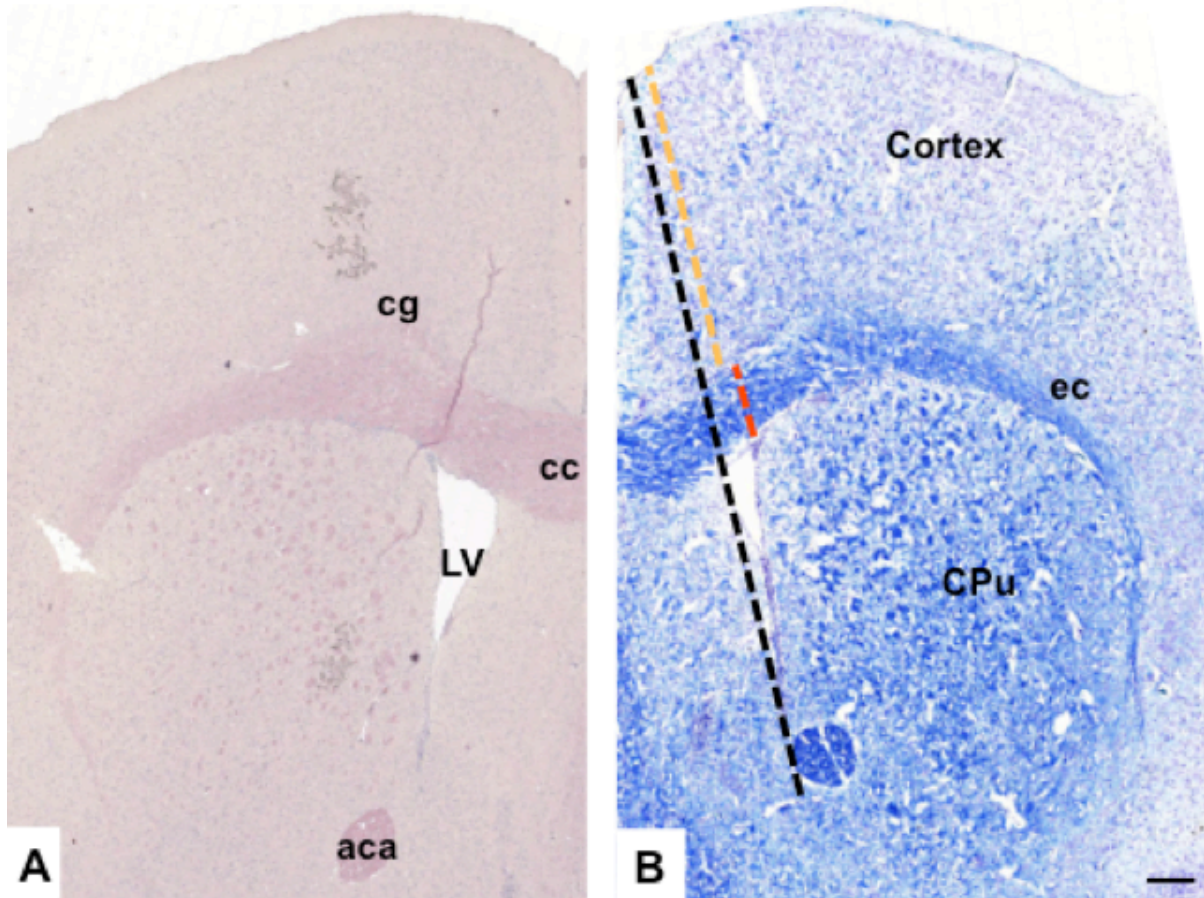
To determine whether mutant *gba1* mice display generalized lipid abnormality in brain tissue, brain sections from 3.5-month-old homozygous D409V/D409V *gba1* mice, heterozygous WT/D409V *gba1* mice, and their WT littermates were stained with Oil Red O (ORO) and Luxol Fast Blue (LFB) for neutral lipids and myelin/nerves, respectively.

Corpus callosum (cc) thickness as a percentage of cortex thickness (cc/Cort) was used as a quantitative indicator of any differences in the size of lipid-rich brain structures between the genotypes. As shown in **Figure 15**, thickness of the cc (red dashed line) and cortex (yellow dashed line) were measured on the line that extended from the medial side of the anterior part of the anterior commissure (aca) and lateral ventricle (LV) to the edge or border of the cortex (black dashed line). Measurements were made on mouse brain sections (at Bregma -0.98) stained with



**Figure 14. Immunohistochemistry of Brain Sections from *Gba1* Mice.**

Brain sections from 12-month-old, homozygous D409V/D409V *gba1* mice (C-D), homozygous V394L/V394L *gba1* mice (E-F), and age-matched, wild-type (WT) littermates (A-B) (n=3 each) were stained by IHC with monoclonal anti- $\alpha$ -synuclein antibody, Syn-1 (A, C, E) and anti-ubiquitin antibody (B, D, F). Arrows point to ubiquitin-positive neurons in the cortex. Arrowheads point to endogenous  $\alpha$ -synuclein signal in the hippocampus of these *gba1* mice. Bar length, 25  $\mu$ M. Original magnification, 20X. IHC experiments were performed by Adel Farah in the Schlossmacher laboratory.



**Figure 15. Corpus Callosum-to-Cortex Thickness in Brain Sections from D409V *Gba1* Mice.**

Serial brain sections from 3.5-month-old *gba1* mice were stained for neutral lipids with oil red o (A) and myelin and nerves with luxol fast blue (B). Regions labeled are the anterior part of anterior commissure (aca), corpus callosum (cc), cingulum (cg), striatum (CPu), external capsule (ec), and lateral ventricle (LV). Bar length, 500  $\mu$ M. Original magnification, 1X. Mouse brains were sectioned and stained by the Pathology Laboratory at the University of Ottawa.

ORO and LFB. As shown in **Table 3**, there was no difference in cc/Cort thickness between heterozygous WT/D409V *gba1* mice, homozygous D409V/D409V *gba1* mice, and age-matched, WT littermates.

Furthermore, brain sections stained with ORO demonstrate a diffuse lipid staining (**Figure 16, D-F**), indicating the absence of lipid deposits or the accumulation of neutral lipids, such as triglycerides and cholesterols (Kinkel et al., 2004; Kruth, 1984; Wang et al., 2011). In addition, there is no obvious difference in ORO staining between WT, heterozygous, and homozygous D409V *gba1* mice. Similarly, there is no difference in brain LFB staining between the three genotypes (**Figure 16, A-C**).

<b>TABLE 3: Quantification of Corpus Callosum-to-Cortex Thickness in the Brain of D409V <i>Gba1</i> Mice</b>			
<b>GENOTYPE</b> (Repeats <sup>a</sup> )	<b>Mean cc/Cort Thickness ± SD (%)</b>	<b>p Value<sup>b</sup></b>	<b>Statistical Difference<sup>c</sup></b>
<b>WT</b> (n=6)	24.5 ± 1.6	N/A	N/A
<b>WT/D409V</b> (n=6)	25.8 ± 1.9	0.2280	No
<b>D409V/D409V</b> (n=6)	24.8 ± 2.6	0.7940	No

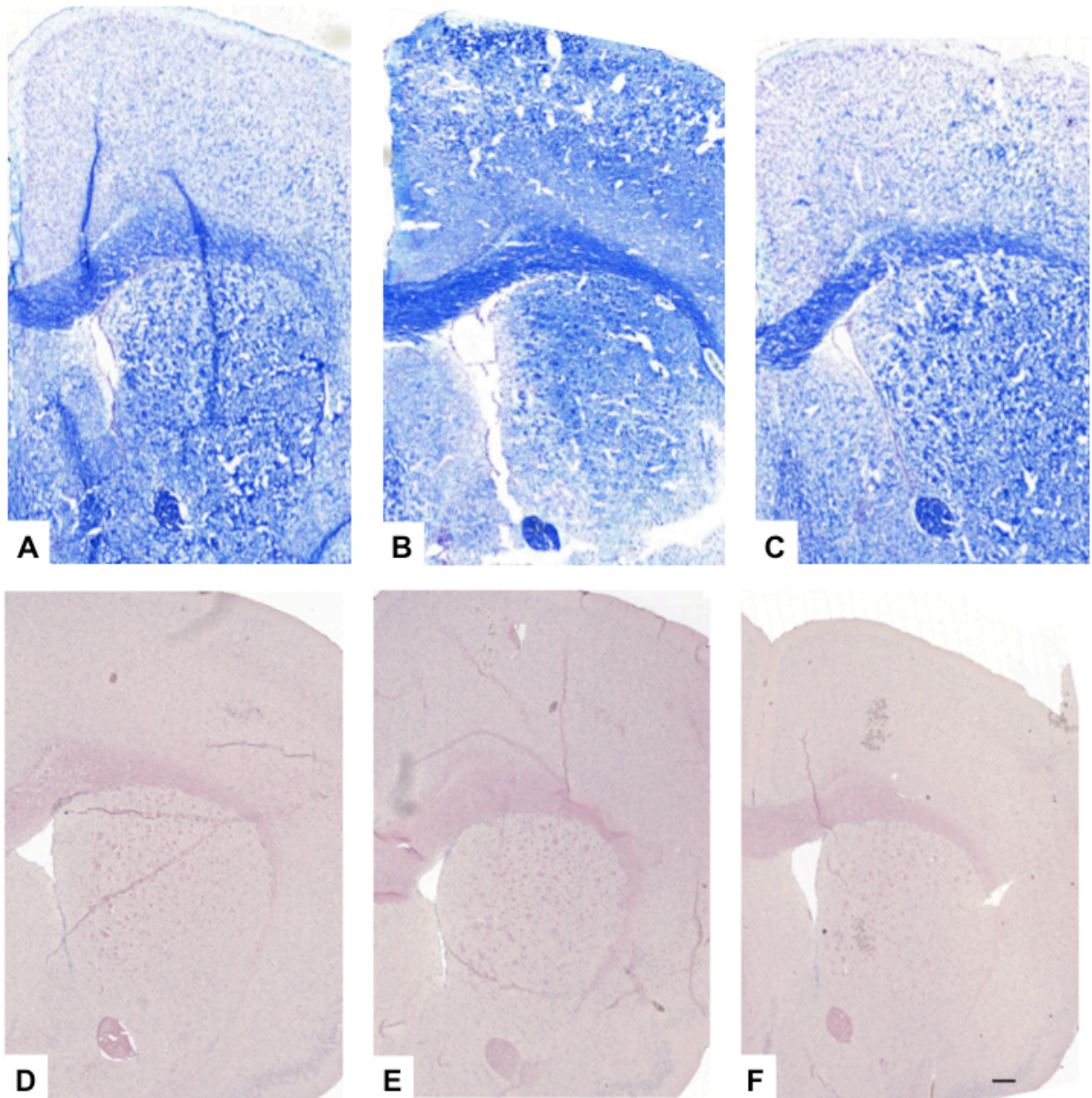
a. Each number represents an individual mouse

b. Determined using the student *t* test to compare mean cc/Cort thickness (%) between wild-type *gba1* mice (WT) and all other genotypes.

c. Determined using the student *t* test as mentioned above. In addition, a one-way analysis of variance (ANOVA) confirmed an interaction ( $p < 0.05$ ) between the genotype and mean cc/Cort thickness (%).

cc = corpus callosum; Cort = cortex; N/A = not applicable; SD = standard deviation; WT = wild-type.

Mouse brains were sectioned and stained with Oil Red O and Luxol Fast Blue by the Pathology Laboratory at the University of Ottawa.



**Figure 16. Lipid Staining in Brain Sections from D409V *Gba1* Mice.**

Serial brain sections from 3.5-month-old homozygous D409V/D409V *gba1* (C, F), heterozygous WT/D409V (B, E) *gba1* mice, and their wild-type (WT) littermates (A, D) were stained for myelin and neurites with luxol fast blue (A-C) and neutral lipids with oil red o (D-F). Bar length, 250  $\mu$ M. Original magnification, 2X. Mouse brains were sectioned and stained by the Pathology Laboratory at the University of Ottawa.

#### 4. DISCUSSION

A possible link between GD and the synucleinopathy disorders PD (and DLB) began to emerge with the clinical observation of parkinsonism in a subset of GD patients (Neudorfer et al., 1996). Since then, many studies have attempted to delineate the molecular mechanisms underlying this association. Several lines of evidence support the loss-of-enzymatic function (LOF) as well as the novel gain-of-toxic function (GOF) effect models; both mechanisms may conspire to fuel  $\alpha$ -synuclein accumulation and aggregation (DePaolo et al., 2009; Mazzulli et al., 2011; Sardi et al., 2011; reviewed in: Sardi et al., 2012; Velayati et al., 2010). According to the latter (GOF), the expression of a mutant GBA1 protein seems sufficient to raise  $\alpha$ -synuclein levels (Cullen et al., 2011; Sardi et al., 2011). According to the former (LOF), reduced levels of GCCase (which has not yet been unequivocally demonstrated in the brain of *GBA1* mutation carriers), lead to a rise in lipid substrates, thereby stabilizing  $\alpha$ -synuclein species within neuronal lysosomes. Growing evidence also supports the existence of a bidirectional pathogenic relationship between  $\alpha$ -synuclein aggregation and GBA1-mediated GCCase activity; there, accumulation in  $\alpha$ -synuclein (possibly oligomers) impairs the processing of GBA1 into a mature 63 kDa glycoprotein, thereby leading to a reduction in lysosomal GCCase (DePaolo et al., 2009; Mazzulli et al., 2011; Sardi et al., 2011; Sardi et al., 2012; Velayati et al., 2010).

Both of these proposed models have limitations. Importantly, the toxic GOF hypothesis does not explain why patients with 'null' mutations resulting in the absence of a mutant GBA1 protein being expressed (also known as

haploinsufficiency) are at a higher risk of developing PD than patients that have mutations resulting in the expression of a mutant protein with reduced GCase activity (Gan-Or et al., 2008; Sidransky et al., 2009). The LOF model does not provide an adequate explanation for why only few GD patients, who by definition have markedly reduced levels of GCase, develop PD (or DLB). Although the penetrance rate of *GBA1* mutations and the lifetime risk for PD and DLB in carriers are not yet known, only a small fraction of *GBA1* carriers develop PD. Moreover, it remains unclear whether the LOF and GOF effects are mediated through independent molecular mechanisms, or if they share changes in SL metabolism upstream of  $\alpha$ -synuclein misprocessing and accumulation. The objective of this thesis work was to optimize a lipid extraction and HPTLC protocol to assess changes in the GluCer/GluSph/Cer lipid profile of neural cells and tissue modeling the homozygous or heterozygous genotype.

Homozygous D409V/D409V *gba1* mice and vector control cells treated with CBE were both used as models of 'homozygosity'. M17 cells treated with CBE (VC/CBE 100 $\mu$ M) had a significant reduction in GCase activity (**Table 1**) and a noticeable accumulation in GluSph relative to vector control cells (**Figure 9B**). The streaking observed where GluCer is resolved on HPTLC may also have indicated an accumulation in this SL. Additionally, brain tissue from the homozygous D409V/D409V *gba1* mouse demonstrated a significant decrease in GCase activity (**Table 2**) and a corresponding rise in GluCer compared to the WT littermate (**Figure 12**). HPLC-MS/MS analysis also revealed a rise in GluSph in these homozygous D409V/D409V *gba1* mice (**Figure 13**), as expected from the published literature

(Sardi et al., 2011; Sun et al., 2012; Xu et al., 2003). Lastly, IHC experiments demonstrated  $\alpha$ -synuclein / ubiquitin signal abnormalities in brain sections from 12-month-old homozygous D409V/D409V and V394L/V394L *gba1* mice compared to their WT littermates (**Figure 14**), which was independently confirmed biochemically (Cullen et al., 2011) and by immunofluorescence microscopy (Sardi et al., 2011). Our results support previous cellular and *in vivo* studies demonstrating that a reduction in GCCase activity below a critical threshold (i.e., well below 50%; **Table 2**) leads to a rise in GluCer and GluSph substrates, and that these changes are associated with downstream  $\alpha$ -synuclein misprocessing either directly or indirectly.

For example, in primary neuronal cultures depletion of GCCase led to a rise in GluCer followed by  $\alpha$ -synuclein accumulation and aggregation (Mazzulli et al., 2011). Homozygous D409H/D409H *gba1* mice that have an ~80% reduction in GCCase activity and a 2-4-fold increase in GluCer in various visceral tissues (lung, liver, spleen), develop subsequent  $\alpha$ -synuclein aggregates in the cerebellum and in brainstem regions (Xu et al., 2003; Xu et al., 2011). Similarly, 12-month-old homozygous D409V/D409V *gba1* mice that show a reduction in brain GCCase activity to 21% of WT exhibit an age-dependent rise in GluSph and proteinase K-resistant  $\alpha$ -synuclein in the hippocampus (Sardi et al., 2011). However, we note with interest that under these conditions, an even more dramatic change occurs in neuronal ubiquitin signals (**Figure 14**) (Cullen et al., 2011; Sardi et al., 2011), thereby possibly indicating a broader change in lysosomal function that is not necessarily specific for  $\alpha$ -synuclein metabolism. For a complete review of the GCCase activity levels, associated lipid substrate accumulations, and related changes in  $\alpha$ -synuclein

metabolism, as identified in each cellular and mouse model published to date (to explore the GBA1-synucleinopathy link), I wish to refer the reader to a recent review article, which we recently published (Sardi et al., 2012).

Heterozygous WT/D409V *gba1* mice and neural cells expressing both endogenous WT GBA1 and exogenous mutant GBA1 were used as models of 'heterozygosity'. M17 cells over-expressing mutant D409V or D409H GBA1 proteins did not exhibit a reduction in GCCase activity compared to vector control cells (**Table 1**). Our HPTLC analysis seems to show a subtle increase in the lipid substrates GluSph and GluCer (**Figure 10**), although the low sensitivity of this technique (15-30 ng per lane) could be limiting to the accuracy of that observation. Similarly, heterozygous WT/D409V *gba1* did not demonstrate a significant reduction in GCCase activity when compared to WT littermates (**Table 2**), and it is difficult to discern whether there is a subtle rise in brain GluCer by HPTLC in these mice (**Figure 12**). If an accumulation of lipid substrates were confirmed using a more quantitative analysis, these findings would indicate, contrary to my hypothesis, that the presence of mutant GBA1 is sufficient to cause the accumulation of GluCer and GluSph substrates, even without a corresponding loss of GCCase function. However, since the HPTLC technique only allows for the partial characterization and relatively insensitive detection of changes in SL levels, these results must be confirmed using a more quantitative technique. In fact, our HPLC-MS/MS analysis of brain tissue demonstrated no elevation in GluSph in heterozygous D409V/D409V *gba1* mice, which is more consistent with other studies.

For example, heterozygous WT/D409V or WT/null *gba1* mice have

approximately 54% of WT GCase activity and do not show any accumulation of GluSph in the hippocampus (Sardi et al., 2011). Whereas heterozygous WT/D409V *gba1* mice show  $\alpha$ -synuclein and ubiquitin-positive aggregates at six months of age (Cullen et al., 2011; Sardi et al., 2011), heterozygous WT/null mice do not. These results seem to imply that one copy of the WT *gba1* allele is sufficient to degrade its cognate lipid substrates (GluCer and GluSph) and that the presence of a mutant GBA1 protein can lead to  $\alpha$ -synuclein accumulation through an – as of yet – undefined GOF mechanism that may be independent of altered lipid metabolism.

#### **4.1 Limitations and Future Directions**

There are several limitations to employing the HPTLC technique for the separation and detection of changes in the levels of neutral SLs. Firstly, overloading of samples usually results in the nonlinear advance of the solvent front (Variyar, 2011). This can not only impair the separation of different lipid species, but can also obscure detection of changes in lipid levels due to streaking (**Figure 10, lane 4**) and ‘tailing’ effects (**Figure 11, lanes 1-2**) (Variyar, 2011).  $R_f$  values depend on many factors, including humidity, ambient temperature, development distant, and sorbent quality and thickness (Variyar, 2011). Lastly, the limit of detection of neutral SLs using HPTLC was between 15-30 ng, which may be insufficient to detect subtle changes in SL levels from neural cells and brain tissue. All of these limitations decrease the precision and reproducibility of this technique, allowing for only the partial characterization and detection of changes in SL levels (Variyar, 2011). The modified lipid extraction and HPTLC protocol optimized here should therefore be coupled to a more quantitative method, such as HPLC-MS/MS,

to characterize and analyze changes in SL levels in neural cells and brain tissue.

#### **4.2 Potential Therapeutic Targets**

It is important to better understand the precise molecular cascade that connects *GBA1* mutations with PD and DLB risk in humans, as this promises to provide insights into new therapeutic targets – including repositioning of drugs approved for GD - for these incurable neurodegenerative diseases.

If *GBA1* mutations cause the accumulation of lipid substrates upstream of  $\alpha$ -synuclein misprocessing through a LOF effect, substrate reduction therapy using chemical inhibitors of GluCer Synthase could provide a possible therapeutic target (Grabowski, 2005; Sidransky et al., 2007). On the other hand, if the presence of mutant *GBA1* enhances  $\alpha$ -synuclein aggregation through a GOF mechanism that is independent of lipid substrate accumulation, administration of small molecule *GBA1* chaperones such as Ifg may provide a better therapeutic target (Lieberman et al., 2007; Sun et al., 2012). *In vivo* studies using fibroblasts from GD patients and mouse models of GD suggest that systemic administration of Ifg restores ER/Golgi trafficking and stabilizes *GBA1* in serum and certain tissues (Sun et al., 2012).

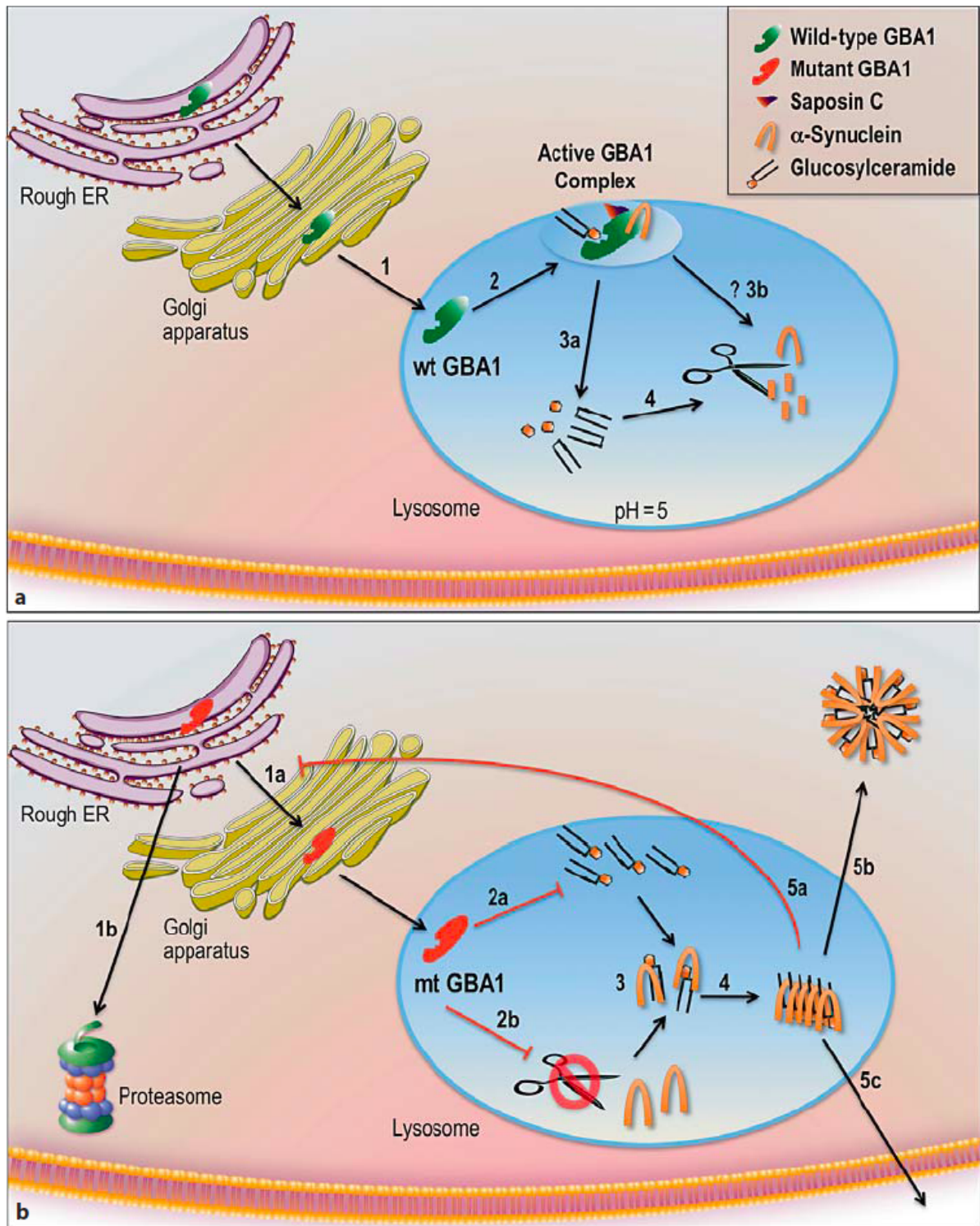
Therapies that work by augmenting *GBA1* function in the brain promise to address the pathogenic effects that have been associated to both the LOF and GOF models. In GD patients, enzyme replacement therapy with a glycan-modified recombinant enzyme (Cerezyme®) can reduce lipid substrate accumulation and improve disease manifestations in visceral tissue (Grabowski, 2005, 2008). However, systemic treatment with this recombinant enzyme does not improve CNS manifestations because of its inability to cross the blood brain

barrier (Grabowski, 2005, 2008). Exciting evidence shows that AAV – mediated expression of WT GBA1 in the hippocampus of homozygous D409V/D409V *gba1* mice lowers GluSph levels and ameliorates both  $\alpha$ -synuclein / ubiquitin neuropathology as well as memory impairment (Sardi et al., 2011). This study indicates that the expression of WT GBA1 (possibly through gene therapy in humans) and the resultant increase of GCCase activity in the CNS may provide a therapeutic approach to reversing  $\alpha$ -synuclein accumulation and slowing the progression of PD/DLB-type symptoms (Sardi et al., 2011).

#### **4.3 Our Proposed Model of the GBA1-Synucleinopathy Association**

Based on recent insights from biochemical, cellular, and *in vivo* studies, we have proposed a revised model for the interaction between WT GBA1, mutant GBA1, and  $\alpha$ -synuclein (Sardi et al., 2012). As shown in **Figure 17a**, WT GBA1 is trafficked to the lysosome as a mature protein (1) (Bergmann and Grabowski, 1989; Ron and Horowitz, 2005). Optimal GCCase activity depends upon the protein's association with anionic phospholipid-containing membranes, the activator protein saposin C (Sap C), and a lysosomal pH of 3-5 (2) (Atrian et al., 2008; Sinclair et al., 2006; Vaccaro et al., 1994; van Weely et al., 1993; Wilkening et al., 1998).

The 'Active GBA1 Complex' represents the physiological interaction between WT GBA1, Sap C, and possibly  $\alpha$ -synuclein itself, which may act by bringing the lipid substrate GluCer physically closer to GBA1 (Yap et al., 2011). As GCCase, GBA1 catalyzes the hydrolysis of its lipid substrate glucosylceramide (GluCer) into ceramide (Cer) and glucose (3a, GluSph metabolism is not shown for simplicity) (Atrian et al., 2008).



**Figure 17. Working Model of Interaction Between WT GBA1, Mutant GBA1, and  $\alpha$ -Synuclein.**

(a) Interaction under physiological conditions. (b) Interaction under pathological conditions. Figure is from Sardi, Singh et al. (2012).

WT GBA1 can also stimulate  $\alpha$ -synuclein degradation in cells and *in vivo* through undefined mechanism(s) (3b), thereby maintaining  $\alpha$ -synuclein homeostasis (Cullen et al., 2011; Sardi et al., 2011). The lipid product of GCase activity, i.e. Cer, is known to stimulate cathepsin D (Cath D), which is a candidate protease for degrading lysosomal  $\alpha$ -synuclein (4) (Heinrich et al., 1999).

As shown in **Figure 17b**, while some mutant GBA1 proteins are tagged as misfolded and transported to the proteasome for endoplasmic reticulum (ER)-associated degradation (1b), there is evidence that some *GBA1* mutations lead to the expression of unstable or misfolded proteins with residual GCase activity in the lysosome (1a) (Bendikov-Bar et al., 2011; Ron and Horowitz, 2005; Sawkar et al., 2006).

Through a loss-of-enzymatic function effect, mutations in *GBA1* cause the accumulation of lipid substrates such as GluCer (2a) (Manning-Bog et al., 2009; Mazzulli et al., 2011). Through a gain-of-toxic-function effect, mutant GBA1 inhibits  $\alpha$ -synuclein degradation via unknown mechanism(s) (2b) (Cullen et al., 2011; Sardi et al., 2011). Both the accumulation of GluCer and the inhibition of  $\alpha$ -synuclein degradation promote the buildup of  $\alpha$ -synuclein monomers that can associate with GluCer-containing lipids (3) (Mazzulli et al., 2011; Schlossmacher et al., 2005).

Accumulation of these  $\alpha$ -synuclein monomers eventually leads to the formation of neurotoxic soluble  $\alpha$ -synuclein oligomers (4), which can: inhibit ER maturation of GBA1 proteins (5a); form insoluble  $\alpha$ -synuclein - positive LBs with a lipid core (5b); or become secreted through exocytosis (5c) (Colla et al., 2012; Conway et al., 2000b; Karpinar et al., 2009; Marques and Outeiro, 2012; Mazzulli et al., 2011;

Mollenhauer et al., 2008; Winner et al., 2011).

#### **4.4 Conclusion**

The molecular mechanisms underlying the association between mutations in *GBA1* and risk of developing PD and DLB remain controversial and unclear. Several lines of evidence support the LOF and GOF models, and both mechanisms may conspire to fuel  $\alpha$ -synuclein misprocessing (Sardi et al., 2011). To better understand the precise molecular cascade that connects *GBA1* mutations with  $\alpha$ -synuclein dysregulation, a modified lipid extraction and HPTLC protocol were optimized to detect changes in neutral sphingolipids (SLs) from neural cells and tissue expressing wild-type (WT) *GBA1*, mutant *GBA1*, or both. We demonstrate that mutant *GBA1* does not confer a dominant-negative effect on WT *GBA1*-mediated activity; however, *bona fide* LOF mutation events led to the accumulation of lipid substrates in neural cells and tissue and enhance  $\alpha$ -synuclein/ubiquitin reactivity in brain tissue of homozygous mutant *gba1* mice. Our HPLC-MS/MS data are consistent with other studies demonstrating that heterozygous *GBA1* mutations do not lead to lipid accumulation, but may alter  $\alpha$ -synuclein degradation through a yet-to-be defined GOF event. Further studies are required to better understand the relationship between mutations in *GBA1* and the misprocessing of  $\alpha$ -synuclein, as well as to provide insights into possible therapeutic targets.

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