Exploring a Role for the Parkinson Disease-Linked *GBA1* Gene in Host Responses to Infections

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Abstract

Typical Parkinson's Disease (PD) is a complex disease that arises from a combination of factors including genetics, environment, gene-environment interactions, sex and age. How these factors interact has yet to be elucidated. We have previously published roles for PD-linked genes in response to microbial infections in an effort to model gene-environment interactions in PD. Mutations in the *GBA1* gene, encoding a protein that confers glucocerebrosidase (GCase) activity, represent the commonest risk factor for PD development. In the present study, we sought to understand the role of murine *Gba1* in microbial infection. GCase activity was found to be sex- and organ-dependent in young adult mice carrying the p.D409V mutation. Mice carrying *Gba1* p.D409V knock-in mutations did not show altered immune outcomes in response to Influenza virus H1N1, bacterial *Salmonella typhimurium*, or serial infections of the two. In response to Vesicular Stomatitis Virus (VSV), p.D409V mice survived at a higher rate overall compared to their wild type littermates, while homozygous males survived at a higher rate compared to wild type males in a sex-dependent manner. Heterozygous females had a lower viral load in the lung after VSV infection. GCase activity was found to be altered in VSV-infected p.D409V mice in a sex-and organ-dependent manner. Taken together, this study identifies a possible role for *Gba1* in host response to an acute neurotropic infection and highlights the importance of exploring sex-dependent outcomes in *Gba1*-focused studies.

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Abbreviations Used

BBB	Blood Brain Barrier
CBE	Condurital B Epoxide
CFU	Colony Forming Units
CNS	Central Nervous System
DLB	Dementia with Lewy Bodies
DPI	Days Post-Infection
ER	Endoplasmic Reticulum
ERAD	Endoplasmic Reticulum associated protein degradation
ERT	Enzyme Replacement Therapy
GCase	Glucocerebrosidase
GCS	glucosylceramide synthase
GD	Gaucher Disease
GlcCer	Glucosylceramide
GlcSph	Glucosylsphingosine
GSC	Glucosylceramide Synthase
iPSC	induced Pluripotent Stem Cells
LB	Lewy Body
L-DOPA, Levodopa	l-3,4-dihydroxyphenylalanine
LN	Lewy Neurite
MPTP	1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine
nGD	Neuronal Gaucher Disease
OB	Olfactory Bulb
PBS	Phosphate Buffered Saline
PD	Parkinson's Disease
PFU	Plaque Forming Units
SNpc	Substantia Nigra Pars Compacta
SRT	Substrate Reduction Therapy

UPR	Unfolded Protein Response
VSV	Vesicular Stomatitis Virus
WT	Wild Type

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Introduction

1.1 Parkinson's Disease

Parkinson's disease (PD) is a progressive neurodegenerative disease that affects 1% of the population over 60 and ~4% over the age of 80 worldwide (Deng et al., 2018; Tysnes et al., 2017). The disease was first identified in 1817 by Dr. James Parkinson in "An Essay on the Shaking Palsy", in which he accurately described manifestations of PD in six individuals, some through causal observation in the street (Parkinson, 1817; reviewed in Obese et al., 2017). Two-hundred years later, the etiology of typical PD is still unknown (Tysnes et al., 2017). Improved diagnostics and an aging population are resulting in increased prevalence of the disease, and the need for preventative therapeutic interventions is paramount (Armstrong et al., 2020; Dorsey 2018).

1.2 Pathology and Symptoms

Pathologically, PD is typically defined by the progressive degeneration of dopaminergic neurons in the *substantia nigra pars compacta* (SNpc) and in most, but not all cases, by intraneuronal protein inclusions known as Lewy bodies (LB) and Lewy neurites (LN) (Simon et al., 2020; Tysnes et al., 2017). Loss of dopaminergic neurons are responsible for the motoric deficits of PD (Zeng et al, 2018; Tysnes et al., 2017). Neuronal loss is not exclusive to the SNpc, and affects the *Locus coeruleus*, nucleus basalis of Meynert, pedunculopontine nucleus, raphe nucleus, dorsal motor nucleus of the vagus, amygdala and hypothalamus (Kalia and Lang, 2015).

Lewy bodies and Lewy neurites are intraneuronal inclusions, primarily comprised of aggregated α-synuclein in cell bodies and processes, respectively (Kalia and Lang, 2015; Simon et al., 2020). Lewy pathology can be found outside of the brain, in both the central and peripheral

nervous system (Kalia and Lang, 2015). A staged brain pathology in idiopathic PD was proposed by Braak, Del Tredici and colleagues, where they identified six stages associated with increasing severity of LB pathology (Braak et al., 2003a). Stages 1 and 2 are limited to the medulla oblongata and pons, with pathology appearing in the dorsal IX/X motor nucleus and/or intermediate reticular zone initially, followed by the first LB-lesions in caudal raphe nuclei, gigantocellular reticular nucleus and coeruleus-sub coeruleus complex. Also observed in the first stage is pathology in the olfactory bulb (Braak et al., 2003b). Stages 3 and 4 see the beginning of lesions in the midbrain, in particular, the SNpc. Mesocortex lesions follow with absence of neocortical involvement (Braak et al., 2003a). Lastly stages 5 and 6 present with lesions in several cortical regions (Braak et al., 2003a). While not the intent of the study, Braak staging can be correlated to PD symptoms, with stages 1 and 2 representing pre-motor symptoms, 3 and 4 representing motoric symptoms and 5 and 6 representing later cognitive deficits, as an increase in cortical LB pathology has been associated with more severe cognitive deficits (Kalia and Lang, 2015). The Braak-Del Tredici staging scheme has been critiqued by some, as not all idiopathic PD cases follow the proposed staging (Kalia and Lang, 2015).

Clinically, PD is represented by several motor impairments, including resting tremor, bradykinesia, rigidity, and impaired gait (Simon et al., 2020; Tysnes et al., 2017; Kalia and Lang, 2015). Non-motor symptoms are observed later in disease, such as autonomic dysfunction, psychiatric disturbances, and dementia (Obeso et al., 2017). Typically, signs of clinically diagnosed PD follow several non-motor symptoms, such as olfactory dysfunction (hyposmia), constipation, REM sleep disorder and depression and anxiety, which can pre-date diagnosis by decades (Obeso et al., 2017). This period has been termed the “prodromal phase” of disease (Kalia and Lang, 2015).

1.3. Treatment

The identification of dopaminergic loss in SNpc neurons led to the development of therapeutics using the dopamine precursor, L-3,4-dihydroxypheylalanine (L-DOPA, levodopa) (Muthuraman et al., 2018). Levodopa is still the most effective therapy at relieving PD symptoms and helps to partially restore depleted dopamine levels due to neuronal loss (Muthuraman et al., 2018). Low bioavailability and poor blood brain barrier (BBB) permeability of dopamine is the reason for its precursor's use in therapy (Muthuraman et al., 2018). Dopa carboxylase inhibitors are typically administered in conjunction with the drug, reducing peripheral degradation and the side effects with peripheral dopamine such as nausea, vomiting, hypotension (Muthuraman et al., 2018). Prolonged administration of levodopa can cause unwanted motor symptoms, such as dyskinesia. And what is known as "on-off" states, where patients experience periods of relief and periods without (Zahoor et al., 2018). A balance between relief of PD symptoms and reduction in unwanted side effects is important (Zahoor et al., 2018).

Surgical treatment of PD symptoms exists in the form of Deep Brain Stimulation (DBS). This technique, which has shown efficacy in other neurological disorders, involves applying electrical stimulation to brain regions associated with disease (Kalia et al., 2013). In PD, electrodes are typically placed in the subthalamic nucleus or globus pallidus internus (Kalia et al., 2013). DBS is effective in treating patients early in PD, but it is not known if progression is slowed and the mechanism of how DBS ameliorates motor symptoms is not fully understood (Kalia et al., 2013). Ultimately, while these two treatments, among others, have found use in alleviating symptoms of PD patients, the need for therapeutics aimed at preventing disease is of great need (Zahoor et al., 2018).

1.4 Genetics in Parkinson's Disease

Roughly 15% of PD cases can be traced through family history, with 3-5% of cases being monogenic with Mendelian inheritance. The remainder of cases are deemed "sporadic" (or idiopathic) and are without a known cause (Deng et al., 2018; Klein and Westenberger, 2012). Several disease-causing genes for PD have been identified, three of which will be summarized here. Among them are genes associated with autosomal dominant inheritance, autosomal recessive inheritance, x-linked inheritance and inheritance patterns that are not yet known (Deng et al., 2018). The *SNCA* gene was the first linked to heritable PD in an Italian family and three Greek families in 1996 and 1997 (Deng et al., 2018; Klein and Westenberger, 2012; Polymeropoulos et al., 1996; Polymeropoulos et al., 1997). Since, six point mutations and various allelic multiplications (duplication, triplication, quadruplication) have been identified (Deng et al., 2018). Mutations in the *LRRK2* gene, encoding the leucine-rich repeat kinase 2 (LRRK2) protein, identified in 2004 as pathogenic, are the most common in autosomal dominant PD and are also implicated in sporadic PD (Deng et al., 2018). Seven *LRRK2* pathogenic mutations have been identified, with the most common being the p.G2019S mutation, accounting for 1-2% of all sporadic cases and 5% in familial cases (Deng et al., 2018). *PRKN* was the second gene associated with PD and the first to show an autosomal recessive inheritance pattern (Klein and Westenberger, 2012). Mutations in both alleles of the *PRKN* gene are most frequently associated with early-onset PD (Klein and Westenberger, 2012). In addition to monogenic forms of PD, over 90 loci have now been implicated as risk factors for PD (Bressan et al, 2021), and the appreciation that typical PD is a complex disease with a genetic component as well as environmental contributions has grown in recent years (Schlossmacher et al., 2017).

1.5. Environmental Factors in Parkinson's Disease

Before genetic involvement was known, PD was thought to arise from environmental exposures (Kalia and Lang, 2015). While epidemiological studies have identified environmental risk modifiers, the precise mechanism or degree to which environmental exposures contribute to PD is complicated (Chen and Ritz, 2018). Late-onset PD takes decades to develop and identifying and defining the entirety of one's exposome is challenging (Chen and Ritz, 2018). Discerning between environmental exposures that initiate PD versus those that modify progression adds an additional layer of complexity. An early example of secondary PD, *i.e.*, arising from an external toxin, comes from several individuals in the United States that developed parkinsonian symptoms overnight (Langston. 1983; Langston 2017). It was determined that all of these individuals had taken a synthetic form of heroin containing the compound 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine (MPTP) and that this compound led to the onset of their symptoms (Langston 1983; Langston 2017). Pesticides were subsequently suspected as PD risk modifiers, paraquat in particular, due to their structural similarity to MPTP (Vaccari et al., 2017). Many studies have associated pesticides, rural living, well water drinking, farm life, increased milk consumption and brain injury with increased risk of PD (Vaccari et al., 2017; Chen and Ritz 2018; Zhang et al., 2021). Several other environmental factors have been identified as potential modifiers. Smoking, coffee drinking, ibuprofen use, vigorous exercise and plasma urate are among modifiers associated with decreased risk (Chen and Ritz, 2018). In 2016, Bellou and colleagues performed an umbrella review of 75 meta-analyses identifying environmental modifiers (Bellou et al., 2016). Across meta-analyses, many cases of heterogeneity among studies or small-study effects were observed (Bellou et al., 2016). Of all risk factors studied, only four met convincing (type I) or suggestive (type II) evidence:

constipation (I), physical exercise (I), anxiety/depression (II) and smoking (II) (Bellou et al., 2016). Constipation and anxiety/depression were associated with increased risk, and physical exercise and smoking associated with lower risk (Bellou et al., 2016). Overall, it is important to note that epidemiologic studies have the potential for biases and that the contribution of environmental exposures to PD is complex and dynamic (Bellou et al., 2016; Vaccari et al., 2017; Chen and Ritz, 2018).

Evidence exists for the involvement of viral infection in PD development (Jang et al., 2009). A popular example is an apparent rise in individuals exhibiting parkinsonian features around the 1918 Spanish flu pandemic. Post encephalitic parkinsonism has been reported to be 2-3-fold higher in people born outside of the years 1888-1924 (Martyn 1997). The link between the two remains controversial, with lack of viral RNA in post-mortem PD brains one reasoning (Jang et al., 2009). However, the late onset nature of disease could result in a viral infection that is transient in nature being no longer detectable, *i.e.,* that viral infection leads to inflammatory processes that persist long after the virus is cleared (Jang et al., 2009). Other viruses have been implicated in development of late onset PD or acute secondary PD based on cluster events including, but not limited to; West Nile virus, Herpes Simplex virus, Japanese Encephalitis virus B, Measles, Coxsackie virus and Polio (Reviewed in Jang et al., 2009).

2.1. GBA1 Structure and Function

The *GBA1* gene encodes the lysosomal acid hydrolase glucocerebrosidase (Gcase) (Migdalska-Richards and Shapira et al., 2016). Synthesis of GCase occurs in the endoplasmic reticulum and is shuttled to lysosomes by way of lysosomal integral membrane protein 2 (LIMP-2) where it exerts its catalytic function under acidic lysosomal conditions (Migdalska-Richards

and Shapira, 2016; Boer et al., 2020). The protein contains 497 amino acids and has a molecular weight of 56-65kDa, depending on the degree of a complex glycosylation pattern (Do et al., 2019). The protein consists of three domains: (1) a three-stranded anti-parallel beta sheet; (2) an immunoglobulin-like domain; and (3) an eight-stranded b/a triose phosphate isomerase (TIM) barrel, containing the protein's active site (Dvir et al., 2003). Gcase requires a co-factor, Saposin C, which is generated in the lysosomes by cleavage of prosaposin (Boer et al., 2020). Condurital B epoxide is an irreversible inhibitor that binds the catalytic site of Gcase and is used as a pharmacological model of GCase deficiency in mice (Boer et al., 2020). GCase is responsible for the degradation of the sphingolipid glucosylceramide (GlcCer) into glucose and ceramide and is active against many GlcCer molecules with varying fatty acyl chains (Boer et al., 2020). Increases in glucosylsphingosine (GlcSph) can occur as a consequence of GlcCer accumulation in GCase deficiency, a transformation mediated by lysosomal acid ceramidase (Boer et al., 2020). GCase has also been known to have direct catalytic activity against GlcSph molecules (Yang et al, 2013; Do et al., 2019). Both GlcCer and GlcSph accumulation is important in Gaucher disease (GD) pathology.

2.2. GBA1 in Gaucher Disease

Mutations in the *GBA1* gene have been heavily studied prior to their identification in PD due to its association with GD, a lipid storage disorder (Do et al., 2019). The disease, first identified by Philippe Gaucher in 1882, is a heterogeneous disease that results in lipid-laden macrophages known as "Gaucher cells" (Sidransky and Lopez, 2012). GD is diagnosed by severely reduced enzymatic activity of GCase (<15%) in peripheral blood leukocytes or the presence of biallelic mutations (Pastores and Hugues, 2018). Mutations in *GBA1* are particularly

prevalent in those of Ashkenazi Jewish ancestry (Pastores and Hughes, 2018). Sequencing of *GBA1* is difficult due to the proximal homologous pseudogene *GBAP* (Sidransky and Lopez, 2012; Pastores and Hughes, 2018). The most common mutations in *GBA1* are 84dupG (a duplication), IVS2+1G>A (insertion) and two-point mutations p.N370S and p.L444P which account for 50-60% of pathogenic variants in non-Jewish populations and approximately 90% in Ashkenazi Jews (Pastores and Hughes, 2018). Manifestations of the disease are varied, and relative activity levels do not always correlate to disease severity (Westbroek et al., 2012; Pastores and Hughes, 2018). Despite complications in genotype-phenotype correlations, certain observations are well maintained. For example, patients carrying at least one p.N370S allele do not develop primary neurological disease (although may still be associated with PD risk) and homozygous p.N370S carriers (or p.R496H variant) tend to have milder disease. Patients homozygous for the p.L444P mutations are likely to develop severe disease with primary neurological manifestations (Pastores and Hughes, 2018). Typical manifestations of GD include anemia, thrombocytopenia, hepatosplenomegaly, bone involvement with osteoporosis, pain crisis or pathological fractures (Sidransky and Lopez, 2012). GD can be classified under three different subtypes. Type 1 is the most common and is deemed non-neuronopathic (Sidransky and Lopez, 2012). Type 1 has a wide range of symptom severity and can produce several visceral symptoms or individuals with very few symptoms (Westbroek et al., 2012). While termed "non-neuronopathic", patients with type 1 GD can develop neurological symptoms later in life, including PD (Westbroek et al., 2012). Type 2 GD is the acute neuronopathic form, leading to rapid neurodegeneration and death in late infancy (Sidransky and Lopez, 2012). Type 3 is the, "chronic" neuronopathic form, wherein patients with variable neurological involvement who survived infancy are classified. (Sidransky and Lopez, 2012). Treatment for GD includes

substrate reduction therapy (SRT) and enzyme replacement therapy (ERT), both of which are able to ameliorate the visceral effects of those with type 1 GD but are not effective in treating the neuronopathic forms of the disease due to lack of penetration across the BBB (Pastores and Hughes, 2018).

2.3. GBA1 in Parkinson's Disease

The link between *GBA1* mutations and PD first arose in clinical observation of Gaucher patients who had developed parkinsonian symptoms (Neudorfer et al., 2001; Tayebi et al., 2001; Tayebi et al., 2003). It was then shown that family members of Gaucher patients who were obligate carriers of *GBA1* mutations were more likely to develop PD (Goker-Alpan 2004; Lwin et al., 2004; Aharon-Peretz et al., 2004). Today, mutations in *GBA1* are known as the commonest risk factor for PD, where carriers of p.N370S and p.L444P mutations are over five times more likely to develop PD compared to non-carriers (Sidransky et al., 2009). It is estimated that between 7 and 12% of PD patients carry a mutation in *GBA1* and 11-31% in Ashkenazi Jews (Aflaki et al., 2017; Do et al., 2019). Two mutations that are not associated with GD, p.E236K and p.T369M, have been identified solely as PD risk factors (Duran et al., 2013; Davis et al., 2016; Mallet et al., 2016). Mutations in specific regional cohorts have been demonstrated to confer PD risk, such as the p.K198E (Columbian and Peruvian), p.W378R (Australian) and p.L216I (Chinese) variants (Velez-Pardo et al., 2019; Lubomski et al., 2018; Jin et al., 2018). Despite the increased risk associated with *GBA1* mutations, penetrance for the mutation is relatively low, with estimations ranging from 10-30% (Alcalay et al., 2014; Rana et al., 2013; Anheim et al., 2012). Similarly, very few GD patients, who are at an estimated 20-fold elevated risk compared to non-carriers, only develop PD at a rate estimated around 9-12% by age 80

(Bultron et al., 2010; Rosenbloom et al., 2011). Some reports suggest increased risk for PD in patients with severe or null *GBA1* mutations (Gan-Or et al., 2015; Cilia et al., 2016; Liu et al., 2016). Heterozygote carriers are more commonly diagnosed with PD, but there is evidence that the overall risk of heterozygous carriers and homozygous carriers is similar for PD development, with the latter experiencing earlier onset (Alcalay et al., 2014). Some studies posited that *GBA1* mutation carriers exhibited features of dementia with Lewy bodies (DLB), a related synucleinopathy (Goker-Alpan et al., 2008; Goker-Alpan et al., 2012). DLB patients develop parkinsonian features, but course of disease is much more rapid with significant cognitive impairment early in disease (Aflaki et al., 2017). A multi-centre analysis determined *GBA1* carriers were over eight times more likely to develop DLB than non-carriers (Nalls et al., 2013).

PD patients with *GBA1* mutations do not differ from PD patients without *GBA1* mutations clinically, in that they display the same cardinal motor symptoms (Migdalska-Richards and Shapira, 2016; Ryan et al., 2019). However, more rapid progression, an earlier age of onset, increased prevalence of cognitive decline and rate of decline are observed (Mullin et al., 2019; Thaler et al., 2017; Gan-Or et al., 2010; Lesage et al., 2011; Thaler et al., 2018; Adler et al., 2017; Mata et al., 2016; Scherzer et al., 2016). Disease length in *GBA1*-linked PD was shown to be the same as other idiopathic forms of PD, but with earlier onset and death (Adler et al., 2017). PD patients carrying *GBA1* mutations have been associated with an increased prevalence of several non-motor features, which include olfactory dysfunction, REM-sleep behaviour disorder, depression, anxiety and hallucinations (Stoker et al., 2018). Response to dopaminergic therapy in PD patients carrying *GBA1* mutations appears to be the same as non-*GBA1* mutation carrying PD patients (Ziegler et al., 2007; Neumann et al., 2009). Pathologically, *GBA1*-linked PD patients

are indistinguishable from other PD patients with both dopaminergic loss, LB and LN pathology (Migdalska-Richards and Shapira, 2016).

2.4. GBA1-Linked PD: Proposed Mechanisms

The precise mechanism by which *GBA1* mutations confer risk for PD is unknown (Migdalska-Richards and Shapira, 2016). Both loss-of-function and gain-of-function theories exist (Migdalska-Richards and Shapira, 2016; Sardi et al., 2012). Loss-of-function, associated with reductions in GCase activity, are often studied in the context of *GBA1*-linked PD (Migdalska-Richards and Shapira, 2016). Studies have shown mild reductions in GCase activity in the brains of idiopathic cases (Gegg et al., 2012; Murphy et al., 2014; Rocha et al., 2015). However, the low rate of PD development in Gaucher patients suggests that activity may not be the sole contributor, and heterozygous mutants are at a high risk for PD development, despite less severe reductions in activity. Similarly, it has been suggested that *GBA1* mutations, which can cause misfolded GCase and are present in the heterozygous state in PD, give rise to gain-of-function effects that can contribute to disease (Cullen et al., 2011; Sardi et al., 2012). This theory is hindered by the presence of deletion events, such as c.84dupG mutations in humans and inhibition of GCase using CBE leading to pathological outcomes in mice (Migdalska-Richards and Shapira, 2016). Overall, reductions in GCase activity and other consequences of *GBA1* mutation are, at most, contributing factors with other external factors at play and may act through both loss-of-function and gain-of-function mechanisms (Sardi et al., 2012).

The interaction of GCase and α-synuclein is of high interest in understanding *GBA1*-linked PD pathogenesis. A post-mortem study identified a correlation between lowered GCase activity in the SNpc and increases in α-synuclein (Günder et al., 2019). In neurons, Mazzulli et

al., demonstrated that GCase dysfunction could result in misprocessing of α-synuclein and accumulation of toxic oligomeric α-synuclein species (Mazzulli et al., 2011). They also demonstrate, in what they term a "bidirectional feedback loop", that those toxic α-synuclein species could inhibit GCase trafficking to the lysosome, leading to further propagation of disease (Mazzulli et al., 2011). Inhibition of GCase activity using CBE in both mice and neuronal cells have been shown to increase α-synuclein levels (Manning-Bog et al., 2009; Cleeter et al., 2013). This effect; however, was not recapitulated in a study by Dermentzaki et al. in the same cell type under different experimental conditions (Dermentzaki et al., 2013). Cullen et al. demonstrated that in cells overexpressing mutant *GBA1*, increases in α-synuclein were associated with the amount of mutant protein expressed and not with activity (Cullen et al., 2011). Another study demonstrated a direct interaction between GCase and α-synuclein and that this interaction can be altered in the presence of *GBA1* mutations or inhibition with CBE (Yap et al., 2011). The interaction between these two PD-linked proteins is still not fully understood but is an important part in understanding *GBA1*'s role in development of PD synucleinopathy.

Endoplasmic reticulum (ER) stress is another proposed mechanism of *GBA1*-linked PD. The ER is responsible for re-folding misfolded proteins. Proteins that are unable to be refolded by ER chaperones are degraded via the ER-associated protein degradation (ERAD) system. Failure of the ERAD system to correct the issue can lead to ER stress and trigger the unfolded protein response (UPR). It has been hypothesised that misfolded GCase can cause ER stress by the aforementioned mechanisms (Migdalaska-Richards and Shapira, 2016). The UPR in turn can increase *GBA1* expression causing increased ER stress (Ryan et al., 2019). Two publications cite misfolded GCase in GD fibroblasts accumulating in the ER and undergoing ERAD (Ron and Horowitz 2005; Bendikov-Bar et al. 2011).

Mitochondrial dysfunction in *GBA1*-linked PD has also been postulated. Dysfunctional mitochondria accumulation in neurons can lead to the production of toxic oxygen radical species that are detrimental to neuronal health (Migdalska-Richards and Shapira, 2016). Mitochondrial defects have been reported in mouse and neuronal cell models of GD (Cleeter et al, 2013; Osellame et al., 2013; Xu et al., 2014). Heterozygous p.L444P mice have also demonstrated to carry mitochondrial defects (Yun et al., 2018; Li et al, 2019) and similar defects have been identified in post-mortem brain tissue of *GBA1* heterozygous carriers with PD (Li et al., 2019). In induced pluripotent stem cell (iPSC) neurons from PD patients and a Drosophila fly model of *GBA1*-linked PD, it was demonstrated that increasing a precursor for NAD+ is able to improve health of neurons in both models and age dependent neuronal loss in flies (Shöndorf et al., 2018).

Autophagy is a lysosome-mediated process involved in cell clearance and is thought to play a role in PD pathogenesis (Migdalska-Richards and Shapira, 2016). Sphingolipid accumulation has been associated with autophagy impairment (in particular GlcSph) (Migdalska-Richards and Shapira, 2016). This accumulation is at the center of GD, and autophagy impairment in GD models has been observed (Migdalska-Richards and Shapira, 2016). Autophagy impairment has also been linked to α-synuclein, and *GBA1*'s involvement could come from its interactions with α-synuclein (Migdalska-Richards and Shapira, 2016).

Lipids are of therapeutic interest in PD with α-synuclein in particular having been shown to interact with lipids, and that these interactions can result in disruption of cellular processes and the aggregation of α-synuclein (Alecu and Bennett, 2019). In the context of *GBA1*, it is tempting to consider alterations in GCase substrates, GlcCer and GlcSph, in potential pathogenic mechanisms due to the loss of enzymatic activity in both typical PD and *GBA1*-linked PD. The accumulations of GCase substrates in PD are not well defined; however, despite some evidence

in experimental models, in part by lack of substrate accumulation in post-mortem tissues (Alecu and Bennett, 2019; Gegg et al., 2012). Ceramide, which is a central figure in sphingolipid metabolism, is of particular interest in understanding the role of lipid metabolism in PD (Alecu and Bennett, 2019). Ultimately, *GBA1* and GCase play a role in a large network of lipids, and their interactions with or consequential effects on other lipid enzymes and their substrates could contribute to PD pathogenesis (Alecu and Bennett, 2019).

Taken together, there are several proposed mechanisms by which mutations in *GBA1* mutations contribute to development of PD. All are not mutually exclusive and disease progression likely stems from, and is contributed to, by many factors.

2.5. Current Therapeutic Approaches in GBA1-PD

As previously mentioned, SRT and ERT have been successful in treating visceral symptoms of GD. However, the lack of BBB penetration restricts their ability to be effective in treating the brain (Do et la., 2019). Currently, SRT therapies that can enter the brain are in clinical trials for *GBA1* heterozygous carriers with PD (Do et al., 2019).

One avenue for therapeutic intervention in *GBA1*-PD is the use of gene therapy through infection with adeno-associated viruses (AAV) (Do et al., 2019). AAV allows for the reliable insertion of corrected DNA into the cell (Do et al., 2019). *GBA1* gene delivery in mouse models has been shown to increase GCase activity, decrease lipid and α-synuclein levels and reduce neurodegeneration and neuroinflammation (Marshall et al., 2002; Sardi et al., 2011; Sardi et al., 2013; Massaro et al., 2018).

Chemical chaperons are small molecules that are able to cross the BBB and correct misfolded proteins (Do et al., 2019). GCase chaperones are of therapeutic interest in *GBA1*-PD

for the correction of GCase misfolding and consequences stemming from misfolded GCase, such as ER stress, trafficking to the lysosome, mitochondrial impairment and GCase activity reduction (Do et al., 2019). Some chemical chaperones aim at increasing effectivity of endogenous chaperones to aid in GCase re-folding (Do et al., 2019). Arimoclomol induces heat shock protein response and has shown to increase GCase activity in homozygous p.L444P patient-derived fibroblasts (Fog et al., 2018). The chemical chaperone celastrol acts by stabilizing the BAG protein family regulator 3 to refold mutant GCase (Yang et al., 2014). Another mechanism of action of chemical chaperones for mutant GCase is to stabilize the misfolded protein in the ER and allow for proper trafficking to the lysosome (Do et al., 2019). These chaperones typically bind at cytosolic pH and dissociate in the acidic pH of the lysosome (Do et al., 2019). Ambroxol has been shown to increase GCase activity levels in patient derived cell lines and mouse models (Parenti et al., 2015; Migdalska-Richards et al., 2016), although PD pathology was unaffected (Migdalska-Richards et al., 2016). Chemical chaperones NCGC607 administered to iPSC-derived dopaminergic neurons from GD and PD patients showed increased GCase levels and reduced substrate accumulation and has shown an effect in decreasing α-synuclein in PD patient-derived neurons (Aflaki et al., 2016). NCGC758 has been shown to increase GCase activity and α-synuclein clearance (Mazzulli et al., 2011). Isofagomine (IFG) can bind to wild type and mutant GCase and has demonstrated increased GCase levels in cells derived from PD patients (Do et al., 2019). Administration *in vivo* has resulted in GCase activity, but not improved PD pathology (Sun et al., 2012; Khanna et al., 2010). Chaperones provide several benefits including cost and oral administration (Do et al., 2019). While drawbacks in *GBA1* chaperone therapy are present, such as lack of effect in deletion mutants or difficulty in re-folding p.L444P mutant GCase, they represent a promising avenue for disease amelioration (Do et al., 2019).

3.1 Inflammation in PD

Inflammation is a mechanism against pathogenic stimuli or tissue injury used to protect the host and promote tissue repair (Pajares et al., 2018). Inflammation and immune responses are present in various forms in PD. Whether these inflammatory processes are causative in neurodegeneration or a consequence thereof is yet to be determined (Deleidi and Gasser, 2013). Microglia are the resident macrophages in the CNS and make up roughly 5-15% of brain cells (Deleidi and Gasser, 2013). Upon neuronal injury, macrophages become activated, which can have negative or positive effects on surrounding neuronal cells (Deleidi and Gasser, 2013; Pajares et al., 2018). Microglial activation leads to their polarization towards pro-inflammatory (M1) and regulatory/anti-inflammatory (M2) phenotypes (Wang et al., 2015; Pajares et al., 2018). The M1 phenotype is characterized by upregulation of pro-inflammatory mediators which help facilitate BBB permeability and involvement from circulating blood leukocytes (Pajares et a., 2018). Chronic microglial activation can lead to increased neuronal death (Wang et al., 2015). Dying neurons can secrete molecules such as ATP, α-synuclein and metalloproteinase-3 which further activate microglia leading to an amplification effect with negative impacts on neuronal health (Wang et al., 2015). Pronounced activation of microglia in PD brain in the SNpc and other regions, along with evidence of microglial activation in PD mouse models highlight the importance of microglia in PD pathogenesis.

Astrocytes are glial cells found in the CNS responsible for metabolic support of neurons, tissue repair and maintenance, permeability of the BBB and cerebral blood flow (Pajares et a., 2018). Evidence of astrocyte dysfunction can be found in the brains of PD patients (Pajares et a., 2018; Wang et al., 2015). Astrocytes can take on similar phenotypes to microglia (A1= pro-inflammatory, A2= anti-inflammatory) (Pajares et a., 2018). A1 astrocytes are associated with a

lack of typical astrocytic function and secretion of neurotoxic factors that result in death of neurons and oligodendrocytes (Pajares et a., 2018). In several neurodegenerative disorders, including PD, microglial activation can cause chronic astrogliosis (Deleidi and Gasser, 2013). Uncontrolled inflammation as a result of simultaneous activation of microglia and astrocyte contributes to dopaminergic cell death and neurodegeneration, highlighting the importance of both in neuroinflammation in PD (Wang et al., 2015).

The role of inflammation in PD is not limited to the CNS. Under normal conditions, peripheral immune cells have limited detection in the CNS (Wang et al., 2015). The CNS is considered to be an immune privileged site due to the impermeability of the BBB; however, in the event of tissue injury or pathogenic insult, peripheral immune cells can enter the CNS through disrupted BBB permeability, a mechanism that is identified as a contributor to neurodegeneration, such as in PD (Wang et al., 2015). Endothelial cells in the SNpc are morphologically different in PD brains, further linking BBB damage, peripheral immune involvement, and PD (Wang et al., 2015). Systemic infections, including respiratory infections, gastrointestinal infections and *helicobacter pyrlori* infection are all linked to PD, highlighting the importance of peripheral immune cells and inflammation in PD pathogenesis. With increasing needs for preventative measures and the proposed role of inflammation in PD, understanding these inflammatory mechanisms and development of immunotherapies are of great interest (Pajares et al., 2018; Wang et al., 2015).

3.2. Gene-Environment Interaction in PD

Typical PD is considered to be a complex disease involving several factors, including genetic predisposition(s) and environmental exposure(s) (Schlossmacher et al., 2017). Our team

hypothesizes that gene-environment interactions are present in PD pathogenesis, *i.e.*, PD-associated genes mediate responses to environmental insult, which in turn trigger downstream pathological outcomes (Schlossmacher et al., 2017). This is informed by our studies focused on *Lrrk2*. Mutations in *LRRK2*, in addition to having been linked to PD, are associated with two immune-related disorders: Crohn's disease (Barrett et al., 2008) and leprosy (Fava et al., 2016). We have demonstrated that in response to microbial insults, mice carrying *Lrrk2* mutations mediate inflammation-rich responses to infection that have direct consequences on brain health (Hakimi et al., 2011; Shutinoski et al., 2019). Accepting the presence of both genetic and environmental risk factors is likely not sufficient, and the interaction between these two is important in understanding PD pathogenesis (Schlossmacher et al., 2017).

3.3. Braak and Del Tredici's Dual-hit Hypothesis

As reviewed above, Braak, Del Tredici and colleagues proposed a staging mechanism in PD Lewy pathology that begins in the lower brain regions, olfactory bulb and dorsal nucleus of the vagus nerve (Braak et al., 2003a). In a separate publication the same year, Braak et al. suggested the possibility of onset of PD pathology by exposure to a pathogen (Braak et al., 2003b). They suggest that because LB pathology begins in the dorsal motor nucleus of the vagus nerve and advances rostrally within the brain, that a pathogen capable of passing the mucosal barrier in the gastrointestinal tract, may initiate damage by travelling across postganglionic enteric neurons of the peripheral nervous system and entering the CNS by way of unmyelinated pre-ganglionic fibers from the vagus nerve (Braak et al., 2003b). This hypothesis was refined in a later publication (Hawkes et al., 2007). Termed the "dual-hit hypothesis", it was proposed that damage to the enteric nervous system and olfactory bulb in the initial stage of disease was

unlikely to be via independent mechanisms, and that introduction of a pathogen by simultaneous inhalation and ingestion of a pathogen could allow access to both olfactory and enteric neurons (Hawkes et al., 2007). This pathogen could initiate disease pathology, and travel via retrograde (vagus nerve) and anterograde (olfactory nerve) transport in the previously proposed, staged manner (Hawkes et al., 2007). The focus of this thesis hereafter is to understand how one or more microbial insults may affect *Gba1* function in mice and vice versa.

3.4. A Role for GBA1 in Immune Function

GBA1 is ubiquitously expressed in human tissues (Human Protein Atlas). Importantly, *GBA1* is highly expressed in cells of the mononuclear phagocyte lineage (Deleidi and Gasser, 2013). *GBA1*'s involvement in GD has provided insight into several possible aspects of *GBA1* PD, among them is the gene's role in immune system. Increased inflammation is seen in patients of GD (Vitner et al., 2012; Lugowska et al., 2019; Pandey et al., 2017). Mutations in *GBA1* causes lipid substrate accumulation in macrophages, the pathological hallmark of GD, key players in innate immunity (Sidransky and Lopez 2012). However, the aberrations in the immune system are not limited to the macrophage. Experimental models have identified roles for *GBA1* in increased cytokine production, activation of microglia and astrocytes, modulation of T and B cells and increased type 1 interferon response (Deleidi and Gasser, 2013; de la Mata et al., 2017; Liu et al., 2012; Vitner et al., 2016).

One example of *GBA1* in host response to infection comes from Maródi et al. who, citing increased susceptibility to bacterial infection in GD patients, explored the abilities of granulocytes and mononuclear phagocytes from GD patients and healthy controls to kill *S. aureus* bacteria. They found that cells from afflicted individuals, in particular mononuclear

macrophages, were less able to kill viable *S. aureus* bacteria and produce oxygen radicals. This phenotype was reversed via treatment with a macrophage-targeted GCase treatment (Maródi et al., 1995). More recently, a study implicating *GBA1* and GD in infection employed a neuronal *Gba1* knock-out mouse model and pharmacological inhibition of GCase with CBE as models of neuronopathic GD (nGD) (Melamed et al., 2020). They were able to demonstrate that these models of nGD survived better and had reduced viral load in response to neurotropic infection with Sindbis virus (Melamed et al., 2020). They posit that this is specific to neuronal cells (as demonstrated by their neuronal knock-out) and mediated by downstream changes of CBE treatment rather than CBE itself, as effects were seen with 8-day pre-treatment with CBE but not 2-days pre-treatment (Melamed et al., 2020).

Lipid substrates of *GBA1* and the disruption of lipid homeostasis in related knock out models have been studied in the context of viral infection (Drews et al., 2019; Soudani et al., 2019; Drews et al., 2020; Vitner et al., 2021). In a 2019 publication, Drews et al. demonstrated that A549 and HEK293 cells with *GBA1 k*nockout were able to inhibit infection of Influenza A H1N1 (PR8 strain). This effect was mediated by a disruption in endosomal trafficking of the virus (Drews et al., 2019). Soudani and colleagues identified an upregulation of ceramide in A549 cells in response to Influenza A infection, and that ceramide is able to suppress infectivity (Soudani et al., 2019). More recently, Drews et al. and Vitner et al. demonstrated a role for glucosylceramide synthase (GCS) in influenza infection, an enzyme responsible for the reverse reaction to that of GCase (Drews et al., 2020; Vitner et al., 2021). Both demonstrate an antiviral effect for inhibition or knock out of this enzyme in influenza infection in cell lines, seemingly contradicting the report that Drews et al., published in 2019. In their most recent manuscript, Drews and colleagues suggest that the inhibition of infection by knockout of both *GBA1* and

GCS can be explained by disruptions in lipid homeostasis, wherein changes in either direction can have consequences on viral infectivity (Drews et al., 2020). A mechanism involving GCase-relevant lipids and *GBA1*'s role in response to infection is not hard to imagine. Lipids are highly important in viral infection (Mazzon and Mercer, 2014; Heaton and Randall, 2011; Martín-Acebes, 2013) and their dysregulation has been shown to alter GCase-α-synuclein interactions (Mazzulli et al., 2011).

GBA1's interactions with other PD-associated genes provide additional insight into a possible role in immune function. GCase has been shown to interact with both α-synuclein and mutant LRRK2 (Mazzulli et al. 2011; Cullen et al., 2011; Yap et al., 2011; Ferrazza et al. 2016; Ysselstein et al., 2019). Our group and others have identified roles for these PD-associated genes in immune function and host response (Hakimi et al., 2011; Beatman et al., 2015; Fava et al., 2016; Tomlinson et al., 2017; Shutinoski et al., 2019). Theoretically, these genes could interact in response to infection or in innate immunity. It will be important to consider readouts involving these two genes in *GBA1* studies of infection.

GBA1 is expressed in the olfactory bulb, lung, and gut (Human Protein Atlas). These systems lie at the interface of environment and host and make *GBA1* an intriguing candidate in the study of host-pathogen interactions in the context of PD, in concordance with Braak and Del Tredici's dual-hit hypothesis. This, along with the immune abnormalities and implications in infections previously identified, lead us to believe that *GBA1* is involved in immune function and may be involved in host response to microbial infection, and that this may have implications in *GBA1* PD pathogenesis.

4.1. Gba1 p.D409V Mouse Model

Despite increased understanding of *GBA1* in the context of PD and DLB, modeling these diseases in mice remains a challenge as mutations in *GBA1* contribute to disease in a way that likely includes unknown additional genetic or environmental factors (Farfel-Becker et al., 2019). Several *Gba1* mouse models are still useful in determining biological changes associated with mutant GCase, whether through its loss of activity or other unknown gained functions (Farfel-Becker et al., 2019). The p.D409V model, originally created and characterized by Dr. Gregory Grabowski and colleagues as a model for GD was chosen for this thesis (Xu et al., 2003). Mice carrying p.D409V knock-in mutations exhibit loss of GCase activity in both heterozygous and homozygous states (Xu et al., 2003; Clarke et al., 2019; Polinski et al., 2021) while exhibiting very mild visceral effects typically seen in GD models (Xu et al., 2003; Weber et al., 2021). Mice carrying two p.D409V mutations have been shown to exhibit sphingolipid accumulation (Xu et al., 2003; Sardi et al., 2011; Polinski et al., 2021). Cognitive deficits have been identified in both heterozygous and homozygous p.D409V mice (Clarke et al., 2019; Sardi et al., 2011). An age-associated change in α-synuclein in p.D409V mice has been observed (Cullen et al., 2011; Sardi et al., 2011; Clarke et al., 2019). Although residue D409 has been linked to PD, the p.D409V mutation has not been identified in patients with PD or DLB, with one known heteroallelic case in GD (Farfel-Becker et al., 2019). Nevertheless, use of this well-characterized mouse model allowed us to explore how mutant mutations in *Gba1* biology affect microbial infection outcomes in mice and vice versa.

4.2. Pathogen Selection

With the goal of identifying a role for *Gba1* in response to infection, we employed a selection of infection paradigms that cause illness in adult mice that differ in structure and target organs. *Salmonella typhimurium* is a virulent bacterial pathogen that induces death in C57Bl/6J mice within seven days (Robinson et al., 2012). Macrophages control *S. typhimurium* infection and death in macrophages is employed by *S. typhimurium* to increase virulence (Robinson et al., 2012). Strain SL1344 is able to induce apoptosis in macrophages of the spleen early in infection (Wei et al., 2019). Type 1 interferon has also been demonstrated in increasing lethality from *S. typhimurium* infection (Robinson et al., 2012). Spleens become enlarged and have signs of severe necrosis (Wei et al., 2019). Using a *S. typhimurium* SL1344 infection paradigm allows us to test the role of *Gba1* in response to bacterial infection and in an organ in which it is expressed and involved in GD pathogenesis (Merra et al., 2008).

To assess the role of *Gba1* in viral infection targeting the lung, a mouse-adapted Influenza A H1N1 (1947 Fort Monmouth strain) (hereafter referred to as MA-FM H1N1) was employed. MA-FM H1N1 causes lethal infection in C57Bl/6 mice with severe lung pathology (Mahmoud et al., 2016; McCormick et al., 2011). *Gba1* is expressed in the lung and results in high levels of glucosylceramide accumulation in the lungs of Gaucher mice (Sun et al., 2013). Several studies, reviewed above, highlight a role for ceramide metabolism in Influenza A H1N1 infectivity (Drews et al., 2019; Drews et al., 2020; Soudani et al., 2019; Vitner et al., 2021). Using MA-FM H1N1 allows us the opportunity to explore organ- and pathogen-specific effects of *Gba1*.

Vesicular stomatitis virus (VSV) was chosen to measure *Gba1*-mediated effects in the context of a neurotropic virus. VSV causes lethal encephalitis in C57/Bl6 mice (Nair et al., 2014;

Fensterl et al., 2012) and is able to infect the olfactory epithelium (Huneycutt et al., 1994). The type 1 interferon response is critical in host defense in response to VSV infection (Detje et al, 2009; Detje et al., 2015; Nair et al., 2014; Fensterl et al., 2012). The virus' s tropism for the olfactory system is of great interest in study centered around Braak and Del Tredici's dual-hit hypothesis (Hawkes et al., 2007). Studying *Gba1*'s role in the context of a virus infecting the brain is of great interest in the context of PD as neurotropic viruses have been identified, as mentioned above, to be affected in nGD models (Melamed et al., 2020) and as risk factors for idiopathic or secondary PD development (Jang et al., 2009).

4.3. Rationale and Hypothesis

The overarching goal of this thesis is to identify a role for *Gba1* in response to microbial infection. Heterogeneity, low penetrance, and the apparent complexity of loss-of-function vs. gain-of-function in *GBA1*-linked PD suggests that there are additional factors at play in pathogenesis. We believe that *GBA1*-linked PD, like other forms of 'idiopathic' PD, is the cumulative result of several factors including genetic susceptibility, lifetime environmental exposures, sex, and age. In line with Braak and Del Tredici's dual-hit hypothesis, we believe the environmental component of this equation could include pathogen exposure (one or more; microbe or non-microbe-based; transient or chronic). Our group and others have demonstrated roles for two PD-associated genes, *Snca* and *Lrrk2*, in response to virulent microbial infections.

I **hypothesized** that mutations in the *Gba1* gene, modeled through mice carrying one or two p.D409V mutant alleles would alter immune outcomes in response to a microbial infection. Both heterozygous and homozygous animals were employed to examine the relevance of a single allele mutation carrier status vs. a two altered alleles carrier status, as is seen in dementia with Lewy bodies and Parkinson's disease.

Study Objectives

- To determine if p.D409V mutations alter immune outcomes in *S. typhimurium* infection, *i.e.,* an acute bacterial infection with splenic tropism.
- To determine if p.D409V mutations alter immune outcomes in Influenza A infection, *i.e.*, an acute viral infection with lung tropism.
- To determine if p.D409V mutations alter immune outcomes in Vesicular Stomatitis Virus infection, *i.e.,* an acute viral infection with neurotropism.
- To understand how GCase activity is involved in microbial insult.

Materials and Methods

Animal model

All animals were kept in single cage housing, containing up to 5 mice with access to water and food *ad libitum*. Mice were kept on a 12-hour light/dark cycle. Mice with a knock-in p.D409V mutation were initially obtained by Dr. Gregory Grabowski and are described in Xu et al., 2003. Mice were backcrossed on the C57bl/6J background (Jackson Laboratories) by Dr. Q. Jiang for 10 generations. Experiments were performed under the guidelines of the Canadian Councils on Animal Care and approved by the Ethics Board of the Animal Care Committee at the University of Ottawa. Infection protocols *in vivo* were performed in a containment level 2 biohazard facility. Both males and females were used in this study.

Tissue Processing for GCase Activity in 7-Week-Old Uninfected Mice

Tissues were obtained from wild type, heterozygous and homozygous mice from heterozygous breeding pairs. Mice were euthanized at 7 weeks of age by injection of Euthanyl followed by cardiac puncture. Blood was collected by cardiac puncture and cardiac perfusion with phosphate buffered saline (PBS) was performed to remove circulating blood. Brain, lung, and spleen were snap frozen upon dissection over dry ice. Snap frozen tissue was lysed in citric acid buffer containing 176mM K_2HPO_4, 50 mM citric acid, 10mM sodium taurocholate and 0.01% Tween-20 at pH 5.9 as previously published (Fishbein et al., 2014). Tissues were homogenized using metal beads and Magna Lyser (Roche) set to 7000 for 10 seconds. Homogenized tissue was centrifuged at 14000 rpm for 10 minutes at 4^{o}C and supernatant was collected and frozen. Tissue lysates were measured for concentration using Pierce bicinchoninic

acid (BCA) assay (Thermo Fisher Scientific) and all samples were diluted to a final concentration of 0.625mg/ml.

Measuring Lysosomal GCase Activity in Mouse Tissue

Samples were assayed in black flat-bottom 96-well plates. 40µL of tissue lysate was measured in duplicate with or without the presence of 5µl of 20mM CBE (Millipore Sigma), a selective GCase inhibitor for 30minutes at 37^{o}C. Samples were then incubated with 40µL of 10mM 4-Methylumbelliferyl β-D-glucopyranoside substrate (Millipore Sigma) for one hour at 37^{o}C. Standard curve using 4-methylumbelliferone (Millipore Sigma) was run in parallel. After one hour, 40µL of glycine stop solution (1M glycine, 1M NaOH, pH 10.6) was added. Samples were subsequently measured for fluorescence at excitation and emission wavelengths of 365nm and 450nm, respectively. Relative fluorescent units were converted to pmol of substrate using the standard curve.

Intraperitoneal Infection with Salmonella typhimurium and Measurement of CFUs

Salmonella typhimurium strain SL1344 was used in the bacterial infection paradigm. Stock aliquots of *S. typhimurium* were prepared, and the concentration determined by Dr. B. Shutinoski. Wild type, heterozygous and homozygous p.D409V littermates were intraperitoneally inoculated with 200 colony forming units (CFUs) of *S. typhimurium* in 100µL of PBS. Mice were euthanized 5 days post-infection (DPI) and spleens were collected in 10mL of RPMI serum-free media (Gibco). Spleens were homogenized and serially diluted (10-fold, v:v). 100µL of homogenate was spread on sterile agar culture plates. Plates were incubated at

37ºC for 24 hours. Colonies were counted and bacterial load was measured as colonies per spleen.

Intranasal infections with Mouse-Adapted Influenza A H1N1

Mouse-adapted Influenza A H1N1 (1947 Fort Monmouth) was obtained from Dr. E. Brown (University of Ottawa). Titre was determined by Dr. Brown and was aliquoted for single use in intranasal paradigm. Wild type, heterozygous and homozygous p.D409V litter mates were anesthetized with 3% isoflurane at 1L/m for 1 minute. $2x10^3$ plaque forming units (PFU) of MA-FM Influenza A H1N1 diluted in 50µL PBS were administered to the nostrils of anesthetized mice. Mice were monitored for weight loss, sickness, and survival from 4 to 14 DPI. Mice were observed twice daily during peak infection, between 6- and 11- DPI. Mice were considered endpoint upon respiratory distress, with mice losing 30% body weight euthanized as humane endpoint. Sickness scores were developed based on those reported in Celestino et al., 2018 and are summarized below in Table 1. In addition to monitoring from days 4-14 post infection, weights were recorded 1- and 2-weeks post infection.

Table 1. Sickness Score Assessment of H1N1-Infected Mice.

Score	Presentation
0	no sickness
1	ruffled fur
2	piloerection, rapid shallow breathing, slight hunch
3	piloerection, deeper rapid breathing, hunched, 20% weight loss
4	respiratory distress, severely hunched, slow movement, moribund

Serial Infection with Salmonella typhimurium in H1N1 Survivors

Survivors of H1N1 that had regained 100% of starting weight were infected with *S. typhimurium* 4 weeks after initial H1N1 infection, (two weeks after the end of the 14-day H1N1 infection course) (see Fig. 5a). 200 CFU of *S. typhimurium* diluted in 100µL of PBS were administered intraperitoneally. Mice were euthanized and spleens were harvested 5 days after *S. typhimurium* infection. Spleens were processed and assessed for bacterial load as described above.

Intranasal infection with Vesicular Stomatitis Virus

Vesicular stomatitis virus was obtained from Dr. E. Brown (University of Ottawa). Adult mice aged 6-10 weeks were used in survival studies (11-13-weeks for PFU, GCase and α-synuclein analyses). Mice were anesthetized with 3% isoflurane at 1 L/m for 1 minute. Subsequently, mice were inoculated with $3x10^3$ PFU VSV (Indiana strain), unless otherwise stated (e.g., dose study), diluted in 50µL PBS. Mice were observed for 14 DPI in survival studies. Mice were weighed from 4-14 DPI and monitored twice daily during peak infection (5-10 DPI). Mice were considered endpoint as a result of encephalitis, characterized by moribund state, hind limb paralysis and inability to walk. Mice that lost 30% body weight without signs of encephalitis or that displayed hind limb paralysis were sacrificed as a humane endpoint. Mice were sacrificed at 14 DPI at the end of disease course, as indicated by amelioration in disease presentation and steady weight gain.

Tissue Processing of VSV-Infected Mice

Adult mice aged 11-13 weeks for PFU, GCase and α-synuclein analyses were sacrificed 2 DPI, corresponding to peak viral titre in brain and lung (Nair et al., 2014; Fensterl et al., 2012). Blood was collected via cardiac puncture and cardiac perfusion with PBS was performed to remove blood from organs of interest. Brain, olfactory bulbs, and lung were excised and snap frozen. Frozen tissue was lysed in PBS and homogenized using metal beads and Magna Lyser machine (Roche) at a speed of 7000 for 10 seconds. Tissues were aliquoted for PFU and GCase measurement, and a third aliquot was prepared for male olfactory bulbs for α-synuclein quantification. Samples for PFU were flash frozen in liquid nitrogen and allowed to thaw on ice. Thawed lysate was centrifuged at 5000 rpm for 5 minutes. Supernatants were collected and frozen at -80^{o}C for titre analysis. GCase samples were incubated with citric acid buffer (contents listed above) in a 1:4 ratio, as previously described (Sanyal et al., 2020). Samples were vortexed and placed on ice for 10 minutes. Samples were then centrifuged at 14000 rpm for 10 minutes, and supernatants were collected. Protein concentration was measured using Pierce bicinchoninic acid assay (Thermo Fisher Scientific) and samples were diluted to a final concentration of 0.625mg/ml. Samples were frozen at -80^{o}C for GCase activity measurement as described above. In samples for α-synuclein measurement, Triton X-100 was added to a final concentration of 0.5% and samples were vortexed and incubated on ice for 10 minutes. Samples were then centrifuged at 14000 rpm for 10 minutes 4^{o}C. Supernatant protein concentration was quantified by Bradford protein assay (Bio-Rad) and standardized at a concentration of 5mg/mL. Samples were frozen at -80^{o}C.

Cell culture

Vero (African green monkey kidney, ATCC CCL-81) cells were grown in high glucose Dulbecco's modified Eagle's medium (Millipore Sigma) supplemented with 10% fetal bovine serum (Thermo Fisher Scientific, Gibco), 4mM L-glutamine (Millipore Sigma), 1mM sodium pyruvate (Millipore Sigma) and 1x penicillin/streptomycin (Millipore Sigma). Cells were incubated at 37ºC at 5% CO_2.

Measuring Viral Load in VSV-Infected Mice

Vero cells were seeded in 12-well culture plates the day before plaque assay, so as to reach ~90% confluency the next day. Plaque assay samples were serial diluted (10-fold, v:v) in serum-free DMEM media (Millipore Sigma) and 250µL was overlayed per well. Cells overlayed with viral suspensions were incubated at 37ºC at 5% CO_2 for 1 hour, with rocking every 15 minutes to ensure even distribution among cells. After one hour, viral suspensions were removed and cells were overlayed with 1ml of a 1:1 mixture of 1% agarose and 2xDMEM (Gibco, Thermo Fisher Scientific) supplemented with 10% FBS. Cells were incubated at 37ºC at 5% CO_2 for 24 hours. The next day, cells were stained with 1ml fixative Carboy's reagent (methanol:acetic acid 3:1) and incubated for 1 hour at room temperature. Cell overlay was removed by gentle lifting and cells were stained with Coomassie blue solution (0.1% Coomassie Brilliant Blue, 20% methanol, and 10% acetic acid) and incubated for 30 minutes at room temperature. Coomassie stain was removed, and plaques were counted by hand. Titres were converted to PFU/g tissue, according to organ dilution in PBS upon homogenization (brain 2x, lung 2x, olfactory bulb 5x).

Measurement of a-Synuclein in VSV-infected tissues

Measurement of α-synuclein in the olfactory bulbs of VSV-infected males was performed by N. Lengacher in the Schlossmacher lab. Total α-synuclein concentration was determined by sandwich ELISA using Syn1/HSA4 (MJFR1, Abcam) antibodies, as previously described (Tomlinson et al., 2017).

Statistical Analyses

Significance was measured by unpaired t-test, One-Way Anova or Two-Way Anova with post comparisons, as indicated in the figure legends. Significance in survival curves was measured by Mantel-Cox log rank test. Differences were considered statistically significant at p values <0.05. Statistical analysis was performed using GraphPad Prism, version 9 (GraphPad Software Inc.)

Results

GCase Activity in 7-Week-Old p.D409V Mice is Organ- and Sex-Dependent

This thesis set out to employ a selection of pathogens targeting different organs to explore a possible role for *Gba1* in host response to infection. With this in mind, we first sought to characterize the activity profile in multiple organs across all three genotypes and both sexes in *Gba1* mice carrying a knock-in p.D409V mutation. Multiple-organ activity profiles have been demonstrated in p.D409V homozygous mice on C57Bl/6-129/SvEvBrd backgrounds (Xu et al., 2003; Liou et al., 2019). Heterozygous mice have been less completely explored (Clarke et al., 2019; Polinski et al., 2021) and sex comparisons have not been, to our knowledge, demonstrated. GCase activity was measured in brain, lung and spleen in wild type, heterozygous and homozygous p.D409V littermates of both sexes (Fig. 1).

Mice were sacrificed at 7 weeks of age to collect a representative profile of young adult mice, which are the focus of our selected infection paradigms. Heterozygous male brains did not have a significant reduction in GCase activity compared to wild type ($p=0.3450$) despite a reduction of nearly 20% (Fig. 1a). Activity reductions in the brains of homozygous p.D409V males were more severe, with ~18% residual activity In females, heterozygous p.D409V mutants had a significant reduction in activity compared to wild type ($p=0.0034$), with nearly 30% less activity (Fig. 1a). Activity in homozygous females, as seen in males, was more severely reduced, with roughly 11% residual activity (Fig. 1a). In male lungs, heterozygous animals had significantly reduced activity compared to wild type ($p=0.0049$), having a residual activity level around 56% (Fig. 1a). Homozygous p.D409V lungs, had a severe reduction in activity, more pronounced than in the brain, with <1% residual activity compared to wild type (Fig. 1a). Female heterozygotes had significantly less GCase activity in the lung compared to wild type ($p=0.0307$)

with just under 50% residual activity (Fig. 1a). Homozygous female lung samples were more severely reduced, with residual activity around 1.5% (Fig. 1a). In male spleens, heterozygous activity was significantly reduced ($p<0.0001$) with a reduction of just over 50% (Fig. 1a). Activity in homozygous spleens was more severely reduced with residual activity of roughly 1.5% compared to wild type animals (Fig. 1a). In females, heterozygous spleens had significantly less activity compared to wild type ($p<0.0001$) with 55% residual activity (Fig. 1a). Spleens of homozygous, female p.D409V mice showed a more severe reduction with less than 3% residual activity (Fig. 1a).

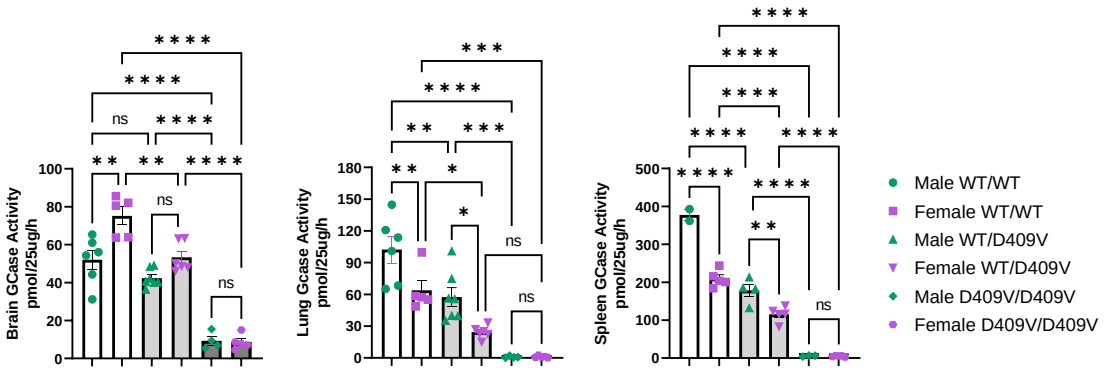
When sex was compared, significant differences between males in females were detected to some degree in all three organs (Fig. 1a). In brains, wild type females had significantly higher GCase activity compared to males ($p=0.0019$) (Fig. 1a). Heterozygous female p.D409V mice showed a similar trend but did not reach significance ($p=0.2260$). Activity in the brains of homozygous animals was not different between the two sexes (Fig. 1a). Contrary to what was seen in the brain, wild type males had significantly higher activity in the lung ($p=0.0062$) compared to females (Fig. 1a). This effect was also seen in the lungs of heterozygous animals ($p=0.0133$) (Fig. 1a). As seen in the brains, differences in lung activity between sexes were not observed in homozygous animals (Fig. 1a). Sex differences in the spleen mirrored those seen in lung, with wild type males having significantly higher activity ($p<0.0001$) compared to females (Fig. 1a). In heterozygotes, activity was also significantly higher ($p=0.008$) in males compared to females (Fig. 1a). Consistent across all organs tested, spleens of homozygous animals showed no differences between sexes (Fig. 1a).

GCase activity between organs was taken into account in both males and females to assess priority of infectious paradigm, with priority given to the paradigm targeting the organ

with the most activity with the intention of best assessing *Gba1*'s role in infection and eliciting a response. In males, activity was highest in the spleen, with roughly five times the activity in the brain and four times that in lung (Fig. 1a). Activity in the lung was higher than in brain (Fig. 1a). In females, GCase activity was highest in the spleen, with roughly two-and-a-half times higher activity compared to brain and lung (Fig. 1a). Comparable activity in the brain and lung of females was observed (Fig. 1a).

Taken together, GCase activity was confirmed to be genotypically dose-dependent in all organs tested (although not significantly lowered in heterozygous male brain). It was also shown that GCase activity differs between males and females and between organs, highlighting the importance of including both sexes and multiple organs in *Gba1* studies.

a



b

Male	Brain	Lung	Spleen
WT/WT	100	100	100
WT/D409V	82.18	56.15	47.21
D409V/D409V	17.85	0.82	1.51
Female			
WT/WT	100	100	100
WT/D409V	70.82	47.21	55.06
D409V/D409V	11.48	1.51	2.36

Figure 1. Lysosomal GCase Activity is Sex-, Genotype- and Organ-Dependent in 7-week-old p.D409V *Gba1* Mice. (a) GCase activity in brain (left panel), lung (middle panel), spleen (right panel) of 7-week-old wild type (WT/WT), heterozygous (WT/D409V) and homozygous (D409V/D409V) *Gba1* p.D409V littermates. Mean GCase activity with SEM is shown in pmol/25ug/h. Each point represents a biological replicate (n= 2-7 per group). (b) Percent mean GCase activity compared to wild type in each organ and sex are summarized. Test used was Two-Way Anova with Tukey post-comparison. Significance is demonstrated by; *=p<0.05, **p=<0.01, ***=p<0.001, ****=p<0.0001, ns = not significant (p>0.05).

p.D409V Mutations in Gba1 do Not Alter Bacterial Load in Salmonella typhimurium Infection in 6-Month-Old Mice

The abundance of GCase activity in the spleen in wild type mice, coupled with a significant impairment in enzymatic activity due to heterozygous and homozygous mutations, led to the prioritization of infection with *Salmonella typhimurium* strain Sl344, a bacterial sepsis model with splenic involvement (reviewed above). Typically, 8-10-week-old adult mice would be employed for this model; however, mice available due to facility shutdowns associated with the COVID-19 pandemic were 6 months of age. Mice were infected intraperitoneally with 200 colony forming units (CFUs) of *S. typhimurium*. Colony forming units in the spleen were measured after 5 days, as previously published (Tomlinson et al., 2017; Shutinoski et al., 2019) (Fig. 2). Independent of genotype, males and females did not display significant differences in bacterial load (Fig. 2a). Separating for genotype alone, no significant differences were seen between wild type, heterozygous and homozygous animals. Separating for sex and genotype, no significant differences were detected (Fig. 2c, d).

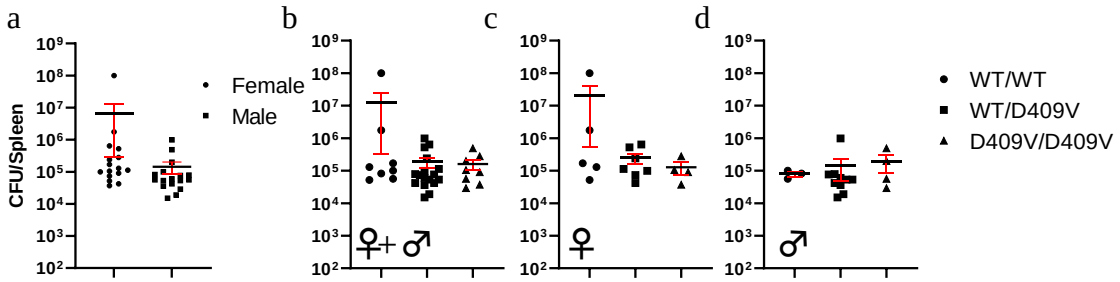


Figure 2. ***S. typhimurium*** **Bacterial Load is Not Altered in 6-Month-Old p.D409V *Gba1* Mutant Mice.** Wild type, heterozygous and homozygous littermates were infected intraperitoneally with 200 colony forming units (CFUs) of *Salmonella typhimurium* SL1344 at 6 months of age. Animals were sacrificed 5 days post-infection and spleens were harvested and homogenized. Bacterial load is compared between sexes independent of genotype (a), separated for genotype alone (b) and separated for sex and genotype (c,d). Mean bacterial burden is presented as CFU/spleen with SEM (n= 3-10 per group). Tests used were unpaired t-test (a) and One-Way Anova with Tukey post comparison (b-d). No significant differences were measured ($p>0.05$).

Infection with Influenza A H1N1 Does Not Result in Altered Infection Outcomes in Mice Carrying p.D409V Mutations

To elucidate mechanisms of *Gba1* in host response, we sought an infection paradigm that would elicit the largest *in vivo* effect. No differences in bacterial load in our *S. typhimurium* paradigm, along the increased activity compared to the brain in males (not seen in females) and the marked reduction in GCase activity in the lungs of p.D409V homozygous mice, led us to explore the effect of an infection paradigm which targets the lung: Influenza A H1N1 (reviewed above). Mouse-adapted Influenza A H1N1 (Fort Monmouth 1947 strain) was intranasally administered to littermate mice aged 6-9 weeks at a dose of $2x10^3$ PFU diluted in a volume of 50µL of PBS. Overall, lethality was low with ~94% of infected mice surviving. Mice typically presented with illness between 6 and 12 DPI, which included symptoms of piloerection, hunched posture, breathing difficulties and weight loss. Disease severity typically peaked at 8 DPI and death occurring between 8 and 11 DPI, which was determined by the presence of respiratory distress. While females appeared to be more severely affected, an observation that did not translate to a worsened survival outcome (Fig. 3a, left panel). When separated for sex and genotype, or genotype alone, no significant differences were found in percent survival (Fig. 3b-d, left panels). Interestingly, no homozygous mice succumbed to infection (n=30), while wild type (n=30) and heterozygous (n=49) had survival rates of ~93% and ~90%, respectively (Fig. 3b, left panel).

In addition to survival, weight and sickness of mice infected with H1N1 were recorded from 4 DPI until the end of the infection course (14 DPI), with weights being additionally measured at 1- and 2-weeks post-infection (Fig. 3). Body weight loss is shown as a percentage of starting weight (Fig. 3, middle panels), while sickness was graded from 0-4 (Fig. 3, right panels).

Sickness score is described in both Figure 3's legend and Table 1 and was developed based on a previously published scoring system (Celestino et al., 2018). Despite a high survival rate, mice did present with apparent weight loss and signs of illness. Sickness scores closely reflected recorded weight losses at peak infection. Overall, females lost more weight throughout early and peak infection and scored higher during peak infection (Fig. 3a, middle and right panel). When separated for genotype alone, no significant differences seen between genotypes in weight loss or sickness score (Fig. 3b, middle and right panels). All three genotypes were able to recover weight equally both 1- and 2-weeks after the 14-day infection course (Fig. 3b, middle panel). When separated for sex and genotype, no statistical differences were observed between genotypes in weight loss or sickness scores (Fig. 3c, d, middle and right panels).

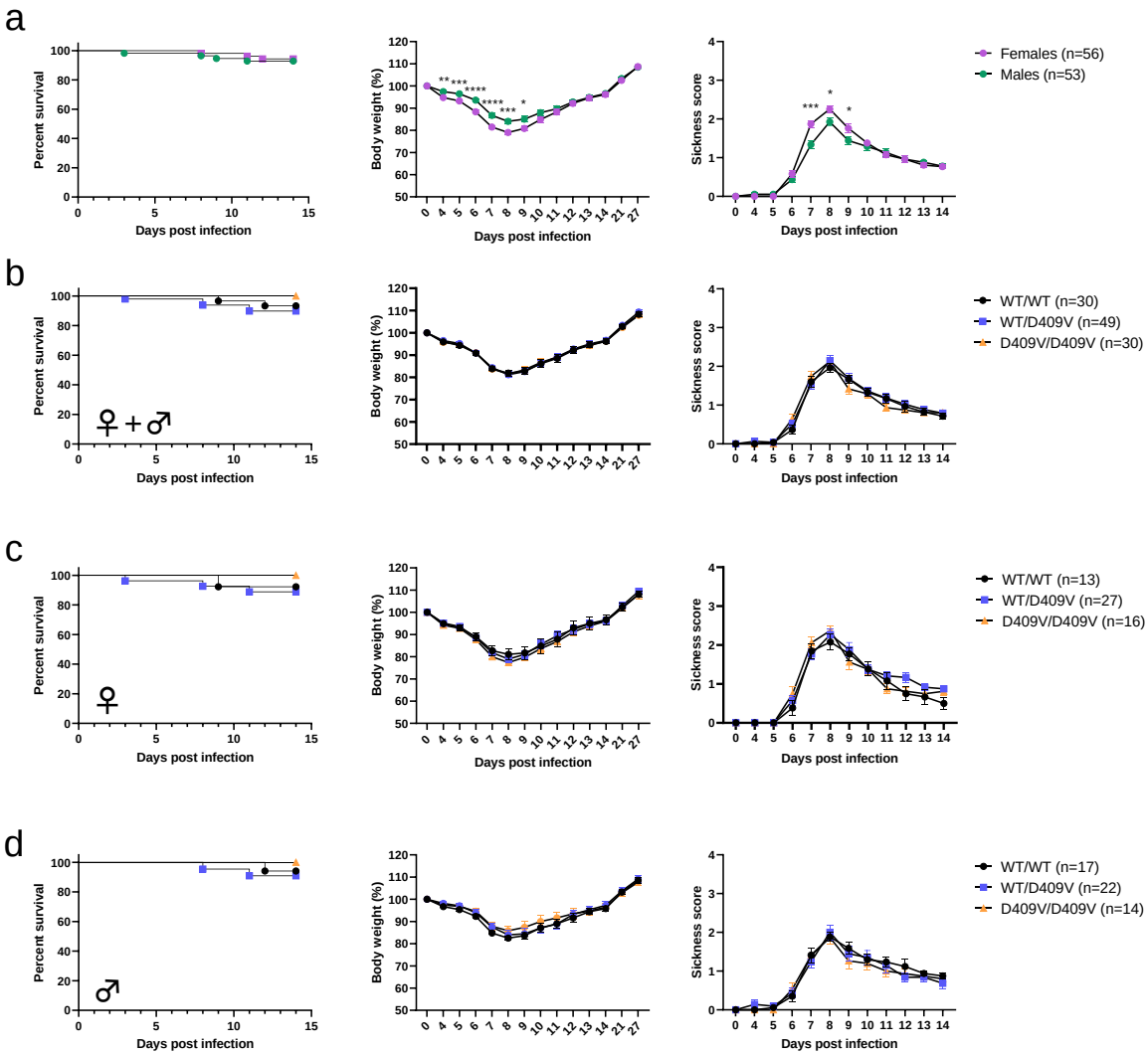


Figure 3. Intranasal Infection with H1N1 Does Not Lead to Altered Survival or Sickness Outcomes in Mutant p.D409V *Gba1* Mice. Wild type, heterozygous and homozygous p.D409V *Gba1* mutant littermates were infected at 6-9 weeks of age with Influenza A H1N1 (1947 Fort Monmouth strain). Mice were intranasally inoculated with $2x10^3$ PFU of virus diluted in 50µL PBS. Survival, weight and sickness were measured from 4 to 14 DPI, with weight being recorded 1- and 2- weeks post-infection. Data is shown comparing males and females independent of genotype (a), males and females combined, separated by genotype (b) females separated by genotype (c) and males separated by genotype (d). Survival is shown as percent surviving (left panels). Mean body weight loss is shown as a percentage of starting weight (middle panels). Sickness score is measured visually, with a score of 0-4 given based on presentation (0= no sickness; 1= ruffled fur, 2=piloerection, rapid shallow breathing, slight hunch; 3= piloerection, deeper rapid breathing, hunched, 20% weight loss; 4= respiratory distress, severe hunch, slow movement, moribund) and mean values are plotted (right panels). Results are pooled from three independent experiments. Mantel-Cox test was used to measure significance in survival, with no significant differences found. Significance in weight and sickness was measured using unpaired t-test (a) or One-Way Anova with Tukey post comparison (b-d) between genotypes on each individual day. Mean

body weight and sickness scores are plotted with SEM. No significant values were measured between genotypes. Significant values between sexes are denoted as: *=$p<0.05$, **=$p<0.01$.

Serial Infection with H1N1 and S. typhimurium Does Not Lead to Differences in Bacterial Load

The low lethality seen in our H1N1 paradigm allowed us to take advantage of a large group of survivors to inoculate them with a second pathogen in a serial infection model to test whether a pre-existing infection could alter the response to a second infection in p.D409V *Gba1* mice (Fig. 4). Mice surviving H1N1 infection which regained 100% of their starting weight were therefore infected 2 weeks post-H1N1 infection course with 200 CFU (intraperitoneal) of *S. typhimurium,* and bacterial load was measured by CFUs in the spleen 5 DPI (Fig. 4a). Independent of genotype, no significant differences in bacterial load were seen between males and females (Fig. 4b). When separated for genotype alone, no significant differences in bacterial load were observed (Fig. 4c). In both females (Fig. 4d) and males (Fig. 4e), no significant differences in bacterial load were observed.

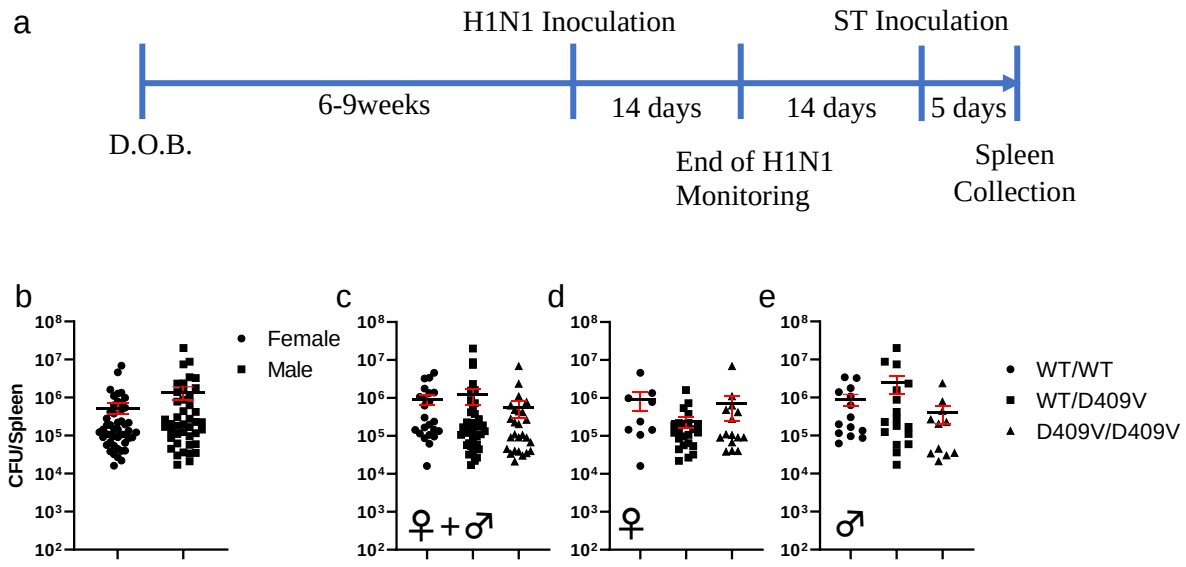


Figure 4. Outcome of *Salmonella typhimurium* Infection in H1N1-Surviving *Gba1* p.D409V Mice as Measured by Bacterial Load. (a) Surviving mice that regained a minimum of 100% of original body weight 4 weeks after initial H1N1 infection were inoculated intraperitoneally with 200 CFUs of *S. typhimurium* SL1344. Bacterial load at 5DPI is shown in the form of colony forming units per spleen between sexes, independent of genotype (b), separated by genotype alone (c), and separated by sex and genotype (d,e). Female and male mice used for this experiment were infected on separate days. Each data point represents one biological replicate (n= 9-23 per genotype, per sex). Mean bacterial load with SEM is shown in red. Results are pooled from three independent infections. Tests used were unpaired t-test (a) and One-Way Anova with Tukey post-comparison (b-d). No significant differences were measured ($p>0.05$).

Intranasal Infection with VSV Causes Lethality in Wild Type and Heterozygous p.D409V Mice.

Little signal observed in our infection paradigms targeting the lung and spleen led us to test a VSV paradigm, given its neurotropism and effect on the olfactory system (of interest due to Braak and Del Tredici's dual-hit hypothesis; reviewed above). To ensure that lethality observed would be sensitive enough to identify genotypic differences, several doses were tested in wild-type and heterozygous littermates with a goal of determining the lethal dose 50 (LD_{50}) for this genetic background (Fig. 5). Wild type mice inoculated with $3x10^5$ or $3x10^7$ PFU exhibited 100% mortality (Fig. 5a). Male mice appeared to be more severely affected than females (Fig. 5a). Doses of $3x10^2$ and $3x10^3$ PFU led to similar survival rates in females of both genotypes, where a $3x10^3$ PFU appeared to more severely affect males compared to $3x10^2$ PFU, which was significant in heterozygous animals (Fig. 5b, right panel). With a survival rate of 50% in wild types overall (i.e., LD_{50}), a dose $3x10^3$ PFU was chosen for subsequent experiments. The possibility of insufficient lethality in $3x10^2$ PFU in females (as seen in H1N1 paradigm) further supported this decision.

In a pilot study, cohorts of wild type, and heterozygous littermates were inoculated intranasally with established LD_{50} ($3x10^3$ PFU) of VSV diluted in 50µL of PBS and monitored for survival in three independent experiments (including the dosing experiment group). Here, there were no differences observed between the genotypes in either sex, or independent of sex (Fig. 5c).

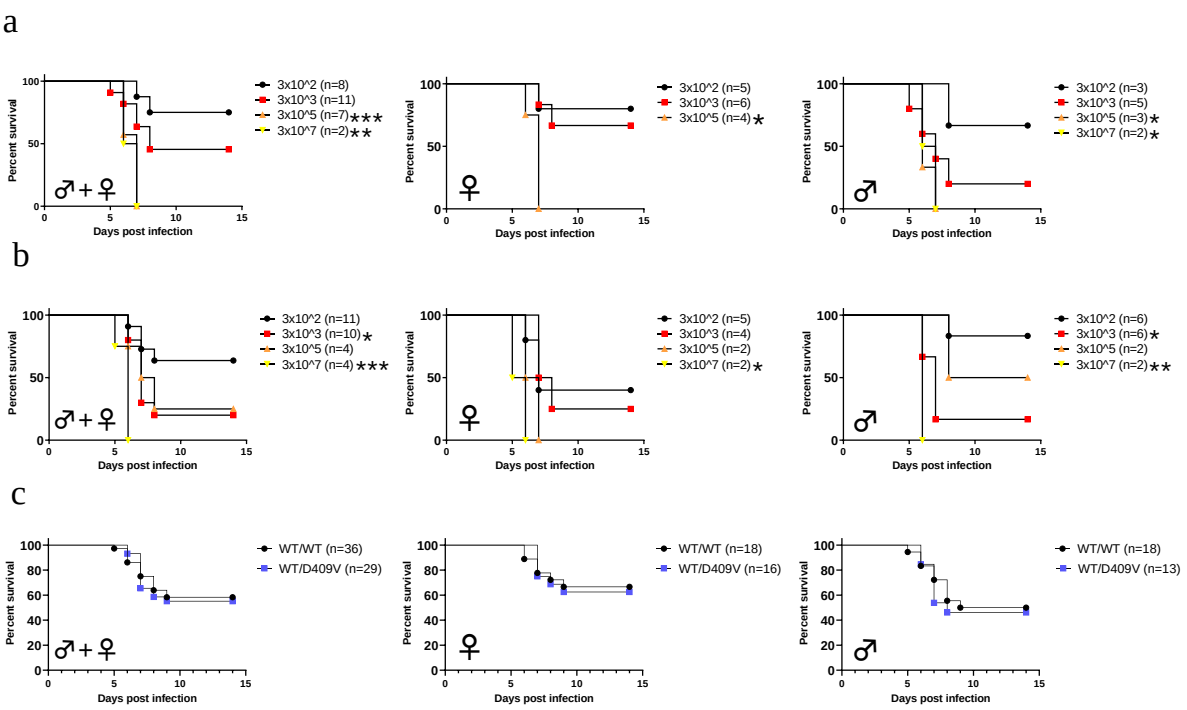


Figure 5. Intranasal VSV Dose-Finding and Pilot Studies in p.D409V *Gba1* Mice. Wild type and heterozygous *Gba1* p.D409V littermates were intranasally inoculated with escalating doses of VSV and monitored 2 weeks for survival (a,b). Doses of $3x10^2$, $3x10^3$, $3x10^5$ and $3x10^7$ PFUs were administered diluted in 50µL of PBS. Wild type (a) and heterozygous (b) mice were tested. Results are shown for both sexes combined (left panels), females (middle panels) and males (right panels). (c) Pilot study tested the response of wild type and heterozygous animals in three independent rounds of infection. Results are shown as percent of surviving mice. Test used was Mantel-Cox log rank test, where significant differences compared to a dose of $3x10^2$ PFU are shown as: *=p<0.05, **=p<0.01, ***=p<0.001.

Heterozygous p.D409V Gba1 Mice and Homozygous Males Show Increased Survival Rates in Response to VSV Infection

To assess the effect of homozygous mutation in VSV infection, littermates aged 6-9 weeks of all three p.D409V genotypes were intranasally infected with $3x10^3$ PFU of VSV diluted in 50µL of PBS. Survival and weight were monitored for 2 weeks (Fig. 6). Infected mice exhibited sickness as early as 5 DPI, with disease and death peaking between 7 and 9 DPI. First signs of disease included piloerection and weight loss, which was followed by slowness of movement, hunched posture, hind limb paralysis and inability to walk at endpoint. Overall, males survived significantly less than females (p=0.001) (Fig. 6a, top panel). When separated for genotype alone, ~67% of heterozygous mice survived, which was significantly more than wild types, who survived at a rate of ~42% (p=0.0249), inconsistent with the pilot study. Homozygous mice also survived at a significantly higher rate (~67%), compared to wild type, (p=0.0479) (Fig. 6b, top panel). In females, wild type mice survived a rate of ~64%, compared to ~91% in heterozygous mice and ~71% in homozygous mice (Fig. 6c, top panel). Despite a 27% difference in survival, heterozygous mice did not survive significantly better than wild type animals (p=0.0526). In males, wild type mice survived at a rate of ~27%, compared to ~46% in heterozygous and ~62% in homozygous animals (Fig. 6d, top panel). Homozygous animals survived significantly more than wild type (p=0.0289), while heterozygous did not (p=0.1359).

Weights were recorded in all mice from days 4 to 14 DPI. Overall, males lost significantly more weight compared to females from 7 DPI onward (Fig. 6a, bottom panel). When separated for genotype regardless of sex, no significant differences were seen in weight loss (Fig. 6b-d, bottom panels).

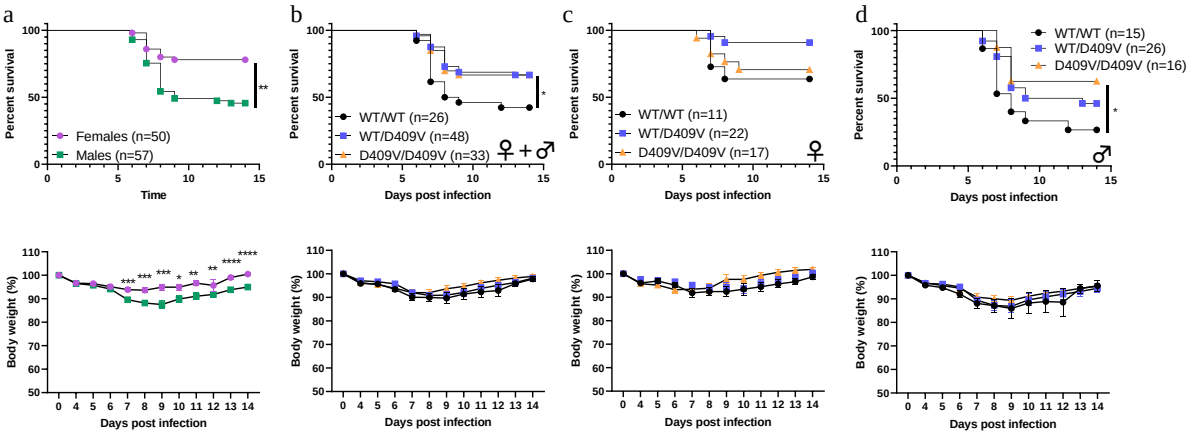


Figure 6. Intranasal Infection with VSV Leads to Genotype- and Sex-Dependent Survival Differences in p.D409V Mice. Wild type, heterozygous and homozygous p.D409V *Gba1* mutant littermates were infected at 6-10 weeks of age with VSV (Indiana strain). Mice were intranasally inoculated with $3x10^3$ PFU of virus in a volume of 50µL of PBS. Survival and weight were recorded from 4 to 14 DPI. Data is shown comparing males and females independent of genotype (a), males and females combined, separated by genotype (b) and females (c) and males (d) separated by genotype. Survival is shown as percent surviving (top panels). Mean body weight loss with SEM is shown as a percentage of starting weight (bottoms panels). Mantel-Cox test was used to analyze survival. Unpaired t-test (a) or One-Way Anova with Tukey post comparison (b-d) was used between genotypes on each individual day for weight loss. Significant values are denoted as: *=p<0.05, **=p<0.01, ***=p<0.001, ****=p<0.0001.

Heterozygous Females Exhibit Lower Viral Load in the Lung after VSV Infection

To understand differences seen in survival, 11-13-week-old p.D409V mice were infected with VSV and sacrificed 2 days post-infection to identify genotypic differences in viral load. Day 2 has been previously identified as a period of peak viral titre in VSV infection in brain and lung (Nair et al., 2014; Fensterl et al., 2012). Olfactory bulb (OB), brain and lung have all been demonstrated to carry viral load following intranasal infection (Nair et al., 2014; Fensterl et al., 2012). Titres are shown in the brains (-OB) and lungs of males and females (Fig. 7a,b). Unfortunately, olfactory bulbs could not be measured for titre because of a processing error in the initial infection group. Titres shown for olfactory bulbs represent a separate group of 7 female mice infected in which the brains and lungs were also titred and combined with the previous group (Fig. 7c).

In female brains, titres varied with several brains below the detection limit (Fig. 7a, left panel). Female brains with detectable titre had roughly 10-fold less titre than those seen in male brains and no significant differences in viral load were seen between genotypes (Fig. 7a, left panel). In male brains, titres were higher and more consistent than what was seen in female brains (Fig. 7a, middle panel). No significant differences in viral load were seen between genotypes in the brains of infected male mice (Fig. 7a, middle panel). In brains of males and females combined, no significant differences were observed between genotypes (Fig. 7a, right panel).

Titre in the lung was roughly 10-fold higher than in the brain in animals overall, and differences between male and female wild type animals seen in the brain were not seen in lung (Fig. 7b). All female lungs titred were well above the detectable limit and did not show the same variability as in infected brains (Fig. 7b, left panel). When comparing genotypes, heterozygous

females had the lowest titre, with an average of $1.77x10^4$ PFU/g compared to wild types and homozygous animals with average titres of $1.35x10^5$ PFU/g and $2.26x10^5$ PFU/g, respectively (Fig. 7b, left panel). Difference in titre between heterozygous and homozygous female infected lungs was significant (p=0.0395), while the difference between heterozygous and wild type animals did not reach significance (p=0.2240). In males, no significant differences were observed between genotypes (Fig. 7b, middle panel). When separated for genotype alone, no significant differences were observed (Fig. 7b, right panel). Wild type female olfactory bulbs (n=3) had roughly a 10-fold increase in viral titre compared to lungs of female mice (Fig. 7c). The variability seen in female brains was not seen in the olfactory bulbs of wild type mice tested. Homozygous mice (n=3) did not appear to have different viral load compared to wild types, although significance could not be calculated for this group due to insufficient sample size in heterozygous animals (n=1).

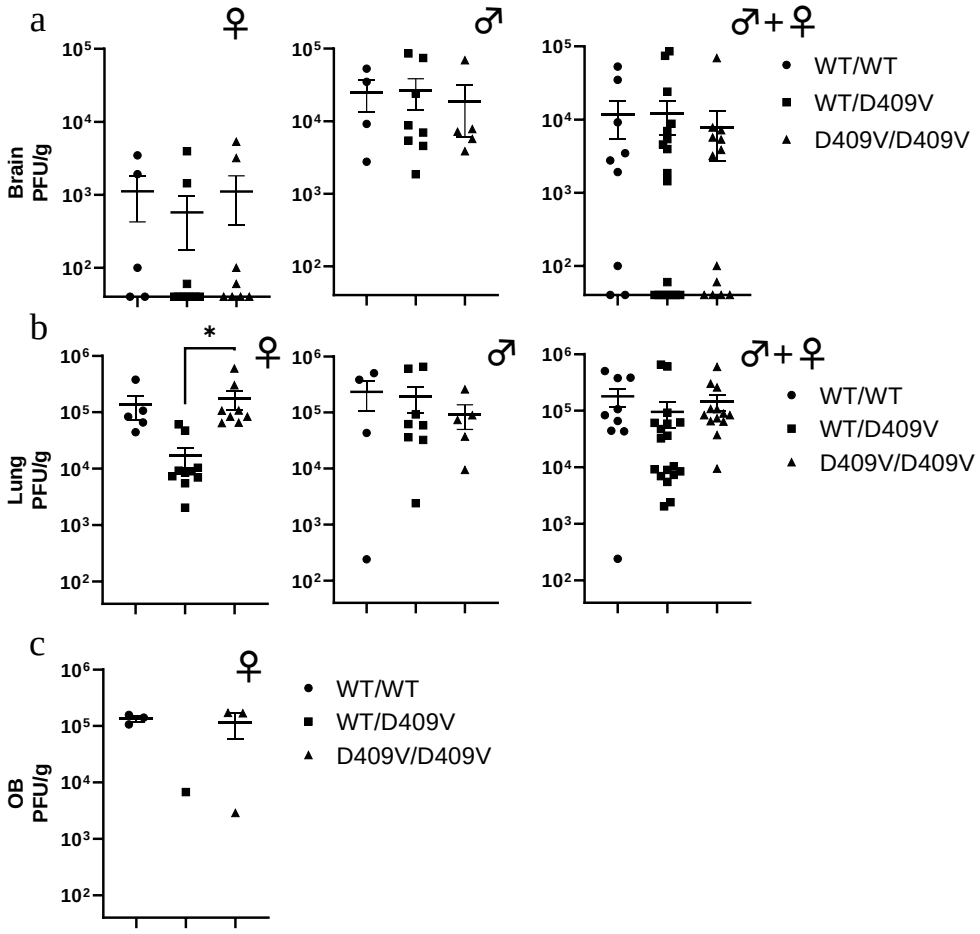


Figure 7. Measuring Viral Load in VSV-Infected *Gba1* p.D409V Mice. Wild-type, heterozygous and homozygous p.D409V littermates were intranasally infected with $3x10^3$ PFUs of VSV Indiana strain diluted in 50µL PBS and were sacrificed at 2 DPI. Titres are shown as PFU/g of tissue. Brains without olfactory bulbs (a), lungs (b) and olfactory bulbs (c) were measured. Genotypes are compared in females (left panels), males (middle panels) or in sexes combined (right panels). Mean titre with SEM is shown (n= 4-10 per genotype, per sex in brain and lung). Test used was One-Way Anova with Tukey post-comparison. Significance is shown as: *=$p<0.05$.

GCase Activity is Altered in a Sex- and Organ-Dependent Manner After VSV Infection

To understand how GCase activity may be involved in acute infection, activity was measured in the brains, olfactory bulbs and lungs of 11-13-week-old VSV-infected p.D409V mice at 2 DPI (Fig. 8). Activity was compared between mock and infected mice of each genotype (wild type= left panels, heterozygous= middle panels, homozygous= right panels), sex (for each panel left= female, right= male) and organ (brain [a], lung [b] and olfactory bulb [c]). VSV infection did not lead to significant changes in brain GCase activity at 2 DPI in across all genotypes and both sexes (Fig. 8a). In lungs, no significant differences between mock and infected animals were seen in GCase activity across all genotypes and both sexes (Fig. 8b). In female homozygous lungs, a 30% reduction in activity was seen after VSV infection, which did not reach statistical significance ($p=0.0712$). In the olfactory bulbs of VSV-infected mice, GCase activity was not altered compared to mock-infected animals in both sexes and all genotypes with the exception of female homozygous animals (Fig. 8c). Female homozygous mice infected with VSV showed a near-25% reduction in activity compared to mock in the olfactory bulbs, which was statistically significant ($p=0.0009$). Interestingly, GCase activity in mock-infected wild type olfactory bulbs was roughly doubled in both males and females compared to activity in the rest of the brain (Fig. 8a,c).

To identify genotypic effects in GCase activity changes in response to VSV infection, GCase activity was plotted as a ratio of activity in infected tissue relative to mock-infected tissue and subsequently normalized to wild type (Fig. 9). In female brains, no significant differences in GCase activity infected-to-mock ratios were observed in heterozygous or homozygous mice (Fig. 9a). Conversely, heterozygous mice showed significantly higher infected-to-mock ratios in GCase activity after infection compared to wild type animals in the brain ($p=0.0025$) while

homozygous mice showed a similar trend that did not reach statistical significance (p=0.0774) (Fig. 9a). In the lung, female homozygous mice had had a significantly lower infected-to-mock activity ratio compared to wild type animals (p=0.0179) (Fig. 9b). Heterozygous animals did not show the same effect (Fig. 9b). In male lung, no differences in infected-to-mock ratios were observed (Fig. 9b). Female olfactory bulbs mirrored results seen in the lung, with homozygous animals having a significantly reduced infected-to-mock ratio compared to wild type mice (p=0.002) (Fig. 9c). Heterozygous female mice did not show significant differences compared to wild type (Fig. 9c). In males, no significant differences were seen among genotypes in infected-to-mock activity ratios measured in the olfactory bulb (Fig. 9c).

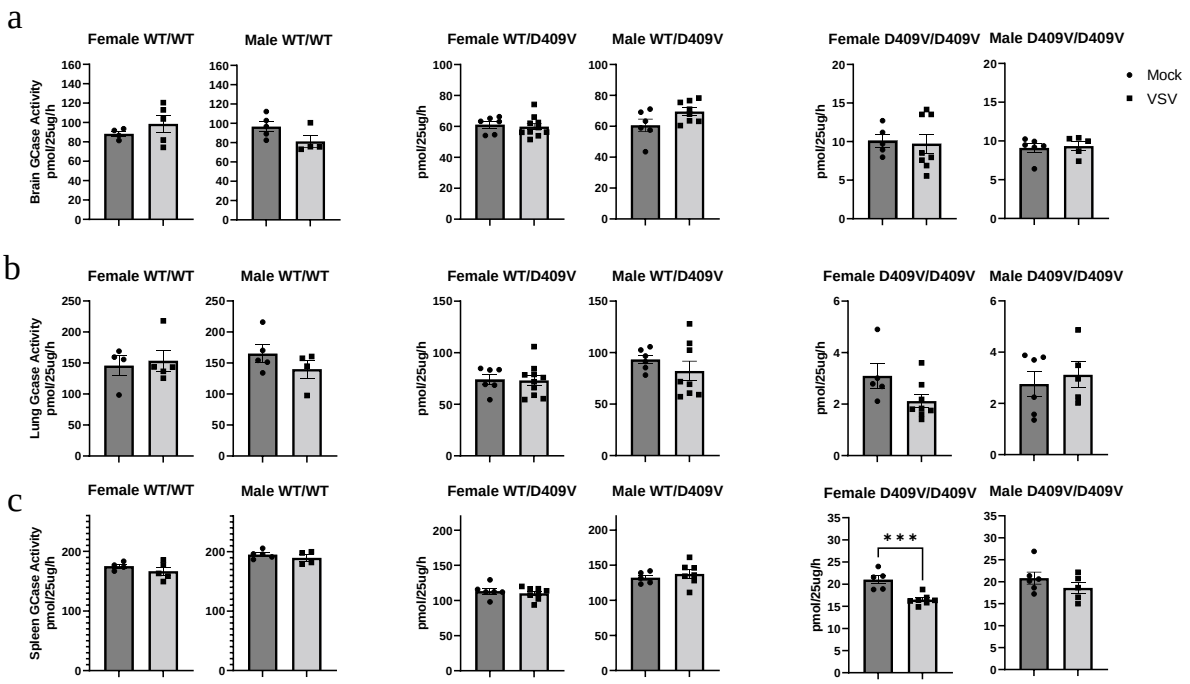


Figure 8. GCase Activity is Lowered in the Olfactory Bulb of Homozygous p.D409V Female Mice in Response to VSV Infection. Wild type, heterozygous and homozygous p.D409V littermates were intranasally inoculated with 50µL of PBS (mock) or $3x10^3$ PFU VSV Indiana strain (infected) diluted in 50µL of PBS. Activity is compared between mock and infected mice for each sex (left= female, right= male), genotype (wild type= left panels, heterozygous= middle panels, homozygous= right panels) and organ (brain [a], lung [b] and olfactory bulb [c]). Mice were sacrificed 2 DPI. Activity is shown as mean activity in pmol/25µg/h with SEM (n= 4-10 per sex, per genotype). Test used was unpaired test, where significance is demonstrated by ***=$p<0.001$.

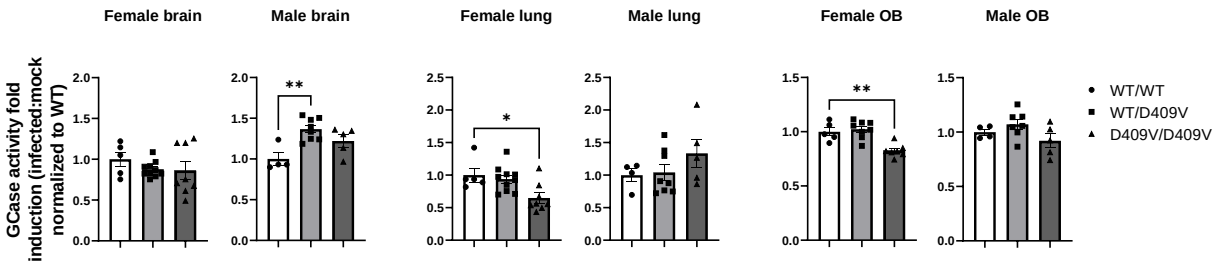


Figure 9. GCase Activity Changes in Infected p.D409V Mice Differs Between Genotypes in a Sex- and Organ-Specific Manner. Wild type, heterozygous and homozygous p.D409V littermates were intranasally inoculated with 50µL of PBS (mock) or $3x10^3$ PFU VSV Indiana strain (infected) diluted in 50µL PBS. Activity is shown as a ratio of infected activity compared to mock and normalized to wild type for brain (a), lung (b) and olfactory bulb (c) in both females (left of each panel) and males (right of each panel). Ratios are derived from activities shown in Figure 8. Mean ratios with SEM are shown. Significance was determined using One-Anova with Dunnett post-comparison, where significance is demonstrated by; *=$p<0.05$, **=$p<0.01$.

No Differences in α-Synuclein Levels are Seen in Male Olfactory Bulb 2 Days Post-VSV Infection in p.D409V Gba1 Mutant Mice.

Our group and others have identified a role for α-synuclein in infection, including its upregulation following infection of the brain in Lrrk2 p.G2019S mice (Beatman et al., 2015; Tomlinson et al., 2017; Shutinoski et al., 2019). Additionally, interactions between *Gba1* and *Snca* have been explored (Cullen et al., 2011; Sardi et al., 2011; Mazzulli et al., 2011). Taking this into account, we sought to measure α-synuclein in the olfactory bulbs of VSV-infected p.D409V mice. Sandwich ELISA was performed by N. Lengacher in the Schlossmacher lab, to measure concentration of total α-synuclein in the olfactory bulbs of male p.D409V mice infected with VSV at 2 DPI (Fig. 10). There, mock-infected animals showed no significant differences in α-synuclein concentrations (Fig. 10a). After infection, there were also no significant differences in α-synuclein levels of male olfactory bulbs (Fig. 10b). Similar to the analysis in GCase activity shown in Figure 9, differences in infected-to-mock changes in α-synuclein concentration were compared between genotypes (Fig. 10c). No significant differences were observed between genotypes in changes of total α-synuclein concentrations (Fig. 10c).

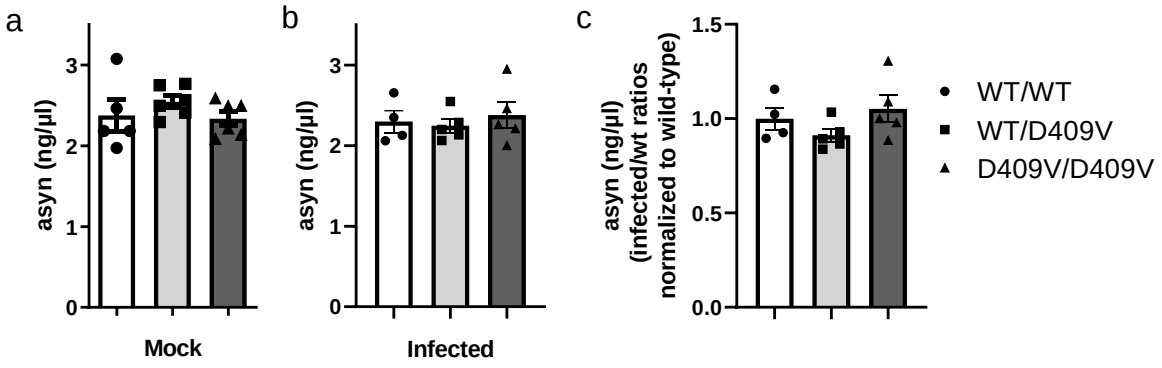


Figure 10. α-Synuclein Levels in the Olfactory Bulb of Male Mice 2 Days Post-VSV Infection. 11-13-week-old p.D409V littermates were intranasally infected with $3x10^3$ PFU of VSV Indiana strain diluted in 50µL of PBS. Mice were sacrificed 2-days post-infection. Samples were measured using sandwich ELISA by N. Lengacher. Mock (a) and infected (b) samples are shown as α-synuclein concentration in ng/µl. Ratio of infected:mock values, normalized to wild type are also shown (c). Mean values and SEM are shown (n= 4-6 per genotype). No significant differences were measured as determined by One-Way ANOVA with Tukey post-comparison (a,b) or Dunnett post-comparison (c) ($p>0.05$).

Discussion

Typical PD is believed to be multifactorial in nature and arise from a combination of factors such as environmental exposure, genetic predispositions, sex, and age (Schlossmacher et al., 2017). To model this, our work focuses on examining the impact of genetic susceptibility (mutations in PD-associated genes) and environmental exposure (microbial infection) on health outcomes in mice. Our results thus far have identified roles for *Snca* and *Lrrk2* in mice, important risk genes for PD in humans (*SNCA*, *LRRK2*), in response to microbial infection (Tomlinson et al., 2017; Shutinoski et al., 2019). The goal of this thesis was to understand whether murine *Gba1*, the commonest PD-risk gene (*GBA1*), is involved in response to microbial insults, and, if so, how pre-determined outcomes are affected by heterozygous or homozygous point mutations.

GCase Activity Profile in 7-Week-Old Mice Carrying p.D409V Mutations

Prior to administration of pathogens, it was important to confirm the distribution of activity in wild type and mutant mice for each sex and in several organs. GCase activity was measured in the brain, lung, and spleen of wild type, heterozygous and homozygous p.D409V mice in both sexes. Each of these measurements were included for the following reasons: (i) the importance of heterozygous mutations in PD and DLB risk stress the importance of understanding where single mutation carriers lie on the activity spectrum; (ii) having been backcrossed onto a pure C57Bl/6J background, we sought to confirm the activity profiles seen in homozygotes in studies performed by Grabowski and colleagues; (iii) brain, lung and spleen were chosen as previously identified areas of expression of *GBA1*, with all three being key organs in our selected infection paradigms; (iv) there is a known involvement of sex in both PD

expressivity and infection outcomes, and understanding how *Gba1* mutations affect each sex can help to determine *GBA1's* role in both PD and host response(s).

As expected, both heterozygous and homozygous p.D409V mutations resulted in a decrease in GCase activity, with deficits in homozygous animals being more severe (Fig. 1a). In the brain, activity was significantly reduced in heterozygous mice in females but not in males (Fig. 1a). A recent report of GCase activity in C56Bl/6 mice showed a reduction for heterozygous males of 45% in the brains of 5-month-old animals (Polinski et al., 2021). Both homozygous males and females exhibit significant reductions in activity, with approximately 18% and 12% residual activity, respectively. This value is in line with what has been previously published, with residual activity being around 10%-30% in the brain of p.D409V homozygotes on C57Bl/6-129; SvEvBrd and C56Bl/6 backgrounds (Xu et al., 2003; Liou et al., 2019; Polinski et al., 2021; Weber et al, 2021). Of note, these studies (when described) measured GCase activity in older mice than observed here, with ages ranging from 4-8 months. Genotypic dose-dependency was also seen in the lungs and spleens of both sexes by us (Fig. 1a). In both peripheral organs of either sex, homozygous mutants exhibited a reduction of activity that was more severe than that of the brain, with less than 3% residual activity, a pattern similar to what has previously been reported (Xu et al., 2003; Liou et al., 2019).

Interestingly, when sex was compared, all wild type organs exhibited a sex-dependent difference in activity (Fig. 1a). Female wild type brains had significantly higher activity compared to male wild types, while heterozygous females also had higher activity, but did not reach significance. In the lungs and spleen, the opposite effect was seen, as activity in the lungs and spleens in males was significantly higher compared to females in both wild type and heterozygous animals. Homozygous animals in the examined tissues did not exhibit any

differences, whether this is genotype-dependent or the residual activity is too low to allow for differences between sexes is unknown. To our knowledge, this is the first report of GCase activity differences between sexes in the p.D409V mouse model, with previous reports in this model failing to specify sex (Xu et al., 2003; Sardi et al., 2011; Sardi et al., 2013; Sun et al., 2013; Liou et al., 2019) or indicating male-only cohorts (Clarke et al., 2019; Weber et al., 2021; Polinski et al., 2021). The overall PD incidence rates display a bias towards males, with a 1.5-2-fold elevated risk **(**Miller and Cronin-Golomb, 2011; Moisan et al., 2016; Cerri et al., 2019). Some forms of PD do not follow this pattern, for example *LRRK2* PD patients show the opposite trend, apparently affecting more females than males that carry the p.G2019S mutation (Cilia et al., 2014). *GBA1*-linked PD has not been confirmed to have a sex-bias, although one cohort of DLB subjects observed an increased rate in males (Gámez-Valero et al., 2016). This result is intriguing, with GCase activity being a focal point in *GBA1*-linked PD and idiopathic PD (without *GBA1* mutations) that have shown a mild reduction in activity in post-mortem brains (Gegg et al., 2012; Murphy et al., 2014; Rocha et al, 2015). A recent study in C57Bl/6 mice showed evidence of different sphingolipid lipid species among both male and female mice and different tissues (Muralidharen et al., 2021). This could help to explain why GCase activity differs between sexes, and why these differences are not the same among different organs, with the ability of lipids to regulate GCase activity (Abdul-Hammed et al., 2017). Plasma of veinous blood has been collected from our mice, from which lipid profiles will be identified in collaboration with Dr. Steffany Bennett's team. It will be interesting to note whether changes in activity at age 7 weeks will correspond to differences in lipid species between sexes, even in wild type animals in the absence of mutation.

Enzymatic activity in organs of both males and females were compared. This organ profile was important in prioritizing which infection paradigm to employ first, with the prediction that organs displaying higher activities challenged with a pathogen may be more likely to elicit a response. This profile also gives a pseudo-measurement of expression, with difficulties by our group in probing for *Gba1* protein levels in mice via Western blotting. The true expression profile remains a high priority in our group. In males, activities in wild type organs differed in the order of spleen>lung> brain (Fig. 1a), consistent with previously published results (Liou et al., 2019). In females, spleens exhibited the highest activity; however, brains and lungs of female mice had comparable levels of activity (Fig. 1a). Overall, the activities in these 7-week-old mice highlight the importance of assessing *Gba1* in both sexes and in peripheral organs, as its expression is not limited to the brain.

Gba1's Role in Acute Bacterial Infection

The abundance in activity in the spleens of p.D409V mice, in addition to the severity in activity reduction in heterozygous and homozygous mutants led to the prioritization of infection with *S. typhimurium. S. typhimurium* SL 1344 is a highly virulent gram-negative bacterium that causes lethal sepsis in C57Bl/6J mice (Robinson et al., 2012; Shutinoski et al., 2019; Wei et al., 2019). Typically, in this paradigm, we administer *S. typhimurium* intravenously in 8-10-week-old mice (Tomlinson et al., 2017; Shutinoski et al., 2019). However, due to facility shutdowns associated with the COVID-19 pandemic, mice available for the study were 6 months of age; these were injected intraperitoneally, a route that has been established in C57Bl/6 mice (Chaudhuri et al., 2018; Cohen et al., 2021). An important consideration with these mice includes their age (6 months) and the possible presence of age-dependent splenic pathology in

homozygous mutants. Homozygous p.D409V mutant mice have been shown to show Gaucher-like storage cells in the spleen at age >7 months (Xu et al., 2003). This pathology was not confirmed in our mice but could have an effect on mutation-mediated response to infection and should therefore also be assessed in young adult mice.

Spleens of mice were collected at 5 DPI, as previously described (Shutinoski et al., 2019). When comparing males and females, independent of genotype, no differences in bacterial load were seen (Fig. 2a). When separated for genotype alone, or sex and genotype, no differences in bacterial load were seen (Fig. 2b-d). These results suggest that, under the conditions tested, p.D409V mutations in *Gba1* do not have an impact in *S. typhimurium* infection.

Gba1's Role in Viral Infection Targeting the Lung

As a first exploration of the involvement of mutations in *Gba1* in microbial infection, we sought a paradigm that provided the greatest signal in order to delve into mechanistic aspects of the possible relationship. As such, the negative result upon *S. typhimurium* infection led us to focus on an infection paradigm that involves the lung, due to its increased activity compared to the brain in male mice, the absence of this effect in female mice and the severe reduction in mutants compared to the brain (Fig. 1c,d). Mice were infected with mouse-adapted H1N1 Influenza A (Fort Monmouth 1947), a lethal viral infection paradigm targeting the lung (Brown and Bailley, 1999; McCormick et al., 2011; Mahmoud et al., 2016).

Studies involving C57Bl/6 mice infected with MA-FM H1N1 have reported varied survival rates, ranging from 10%-100% (Mahmoud et al., 2016; McCormick et al., 2011; Robbins et al., 2006). Upon consultation with our virologist colleague, Dr. Earl Brown, we

started with $2x10^2$ PFU of virus, a dose found to cause equal to 50% killing in CD1 mice in his experimental model. Our mice infected at this dose did not succumb to infection and had minimal weight loss (data not shown). The dose was subsequently increased to $2x10^3$ PFU per animal. At this dose, death was limited, with roughly 94% of mice surviving with more severe weight loss (Fig. 3). When sex was compared independent of genotypes, no difference in overall survival was seen despite the appearance of worsened phenotype in females (Fig. 3a). Differences in weight and sickness score between sexes were seen during peak infection (Fig. 3a). Separating for genotype, no differences were seen between wild type, heterozygous or homozygous mice in survival, weight loss or sickness score (Fig. 3b). Separating for sex and genotype did not yield differences in survival, weight loss or sickness score in males or females (Fig. 3c, d). Intriguingly, no homozygous mice succumbed to infection. The high rate of survival in mice overall limited the sensitivity of this paradigm and thus made it difficult to draw conclusions from this event. The lack of differences in weight or disease presentation (sickness score) suggests that the increased survival among homozygotes is reflective of lack of sensitivity. Still, the complete survival of these mice at a high number (n=30) warrants further exploration, either in survival at a higher dose or measuring the viral titre in the lungs of these mice to know if homozygotes provide a protective benefit. Of note, two studies have explored the role of ceramide metabolism in Influenza A cell culture infection, identifying roles for glucosylceramide (in *GBA1* knock out cells) and ceramide in inhibition of Influenza infectivity (Drews et al., 2019; Soudani et al., 2019). Conversely, two more publications demonstrated a reduction in Influenza infectivity when inhibiting or knocking out glucosylceramide synthase, which is responsible for the synthesis of glucosylceramide from ceramide (Drews et al., 2020; Vitner et al., 2021). Drews and colleagues describe the seemingly contradictory results as a product of sphingolipid

metabolism disruption, *i.e.*, disruption of glucosylceramide in either direction can affect infectivity of Influenza (Drews et al., 2020). A role for ceramide species in H1N1 inhibition could be relevant in H1N1 infection of p.D409V homozygotes, given their enzymatic deficiency. While increasing the viral dose or measuring viral titre are of a higher priority, should the trend in homozygous survival hold true, the study of lipid profiles before and after infection in p.D409V mice could point to a possible lipid-mediated mechanism in H1N1 infection in *Gba1* p.D409V homozygous mice.

Gba1's Role in a Serial Infection Paradigm

The high rate of survival in our H1N1 paradigm prompted us to infect mice with a second microbial challenge in a serial infection model. Surviving H1N1 mice were infected with *S. typhimurium* two weeks after H1N1 monitoring (four weeks after initial H1N1 infection) provided they regained 100% of their starting weight. The hypothesis was that *Gba1* mutant mice may have altered inflammation, despite complete physical recovery, leading to altered health outcomes following subsequent infection with *S. typhimurium.* When separated for genotype, no significant differences between the three genotypes were observed in bacterial load. No significant differences among genotypes were seen in males or females when separating for sex. The lack of differences between genotypes in *S. typhimurium* infection is consistent with what was observed in 6-month infected mice without H1N1 pre-infection. It appears as though H1N1 pre-infection is insufficient in eliciting differences in bacterial load in the conditions tested here. If repeating this experiment, a shorter time period between infections should be considered. It is possible that four-weeks post-infection was too late to take advantage of any altered inflammatory states between genotypes in response to H1N1 infection to elicit

differences in microbial load. A second possibility would be to employ a bacterial pathogen that causes pneumonia following viral H1N1 infection. Predisposition to bacterial pneumonia in Influenza patients is well appreciated (Small et al., 2010; Morris et al., 2017; Rowe et al., 2020) and has been shown in mice (Small et al., 2010). These alternative methods of serial infection may better inform us as to how *Gba1* affects inflammatory levels in response to H1N1 infection, and whether such exposure will confer altered susceptibility in subsequent bacterial infections.

Gba1's Role in Neurotropic Viral Infection

Infection of p.D409V mutant animals with *S. typhimurium*, H1N1 or a serial infection involving both, yielded no distinguishable genotypic response using the outcome measures described in this work. These findings shifted our focus towards a neurotropic pathogen to assess the response of *Gba1* mutants in the context of the brain. For this, a nasal delivery route with VSV was employed because it causes lethal encephalitis in mice and infects olfactory structures (Nair et al., 2014; Fensterl et al., 2012; Huneycutt et al., 1994). In order to avoid difficulties such as those encountered in H1N1 survival, several doses were tested in wild type and heterozygous p.D409V mice using both sexes (Figure 4). The mortality seen here is higher than observed in other studies infecting C57Bl/6 mice with VSV, who observed 10-20% mortality and limited dose response (Zhou et al., 2007; Fensterl et al., 2012; Nair et al., 2014). Of note, none of these studies identify the sex of mice tested. As a pilot study, wild type, and heterozygous males were monitored for survival to VSV infection in three independent rounds of infections. From these initial dose finding studies, we learned that wild type and heterozygous mice appeared to survive equally well overall and independent of their sex (Fig. 4c).

To assess the impact of homozygous p.D409V mutations, cohorts comprised of littermates from all three genotypes were infected with $3x10^3$ PFU of VSV and monitored for survival and weight loss (Fig. 5). Independent of genotype, males showed a significantly reduced survival rate and lost significantly more weight than females starting at 7 DPI which continued throughout infection. This apparent sex difference is consistent with a previous report of increased susceptibility to VSV infection in male Balb/c and dm2 mice (Barna et al., 1996). Interestingly, when separated for genotype alone, heterozygous and homozygous mice survived significantly better than wild type animals. The increased survival in heterozygotes contradicts our pilot results, where heterozygotes exhibited similar survival rates to wild type mice. Looking at each individual round of infection (three in the pilot study, two that included homozygous littermates), better survival (although not individually significant) was seen in each experiment, with the exception of the dosing study, supporting the case for a heterozygous effect. Still, the differences seen under one condition (wild type, heterozygous and homozygous littermates studied), but not seen in another (wild type versus heterozygous littermates) emphasize the need to confirm this possible effect in a separate experiment, *e.g.,* viral load. Nonetheless, similar survival rates among heterozygous and homozygous mice could in theory be juxtaposed to overall PD and DLB development rates with *GBA1* mutations, where two mutant alleles do not appear to further increase PD risk compared to carrying a single mutation (Alcalay et al., 2014).

When separated for sex, heterozygous females showed increased survival rates compared to wild type mice, although not significantly, while homozygous females survived at a similar rate compared to wild type animals. Male heterozygous mice had a non-significant increase in rate of survival compared to wild type, while homozygous males faired significantly better than wild types, but not heterozygotes. These results, taken together with the survival in combined

sexes, suggest a possible beneficial role of heterozygous mutations in response to VSV infection. The role of a homozygous mutation status is more complex regarding a possible sex-bias, in that female homozygotes behave as wild types and male homozygotes confer added protection. A similar sex-bias has been reported in our *Lrrk2* study, where homozygous p.G2019S females show a sex-bias in rate of survival compared to wild type animals in response to reovirus infection (Shutinoski et al., 2019). A female sex-bias in human *LRRK2*-PD patients is observed, contrary to what is seen in the general population (Cilia et al., 2014). While the importance of sex in *GBA1*-PD is less well delineated, the presence of a male sex-bias in a cohort of DLB patients may be of relevance to what is seen in p.D409V mice here (Gámez-Valero et al., 2016).

To see whether differences in survival translated into altered viral load, VSV-infected mice were sacrificed at 2 DPI, which has been identified as a time point of peak titre in brain and lung in two independent studies of VSV infection in C57Bl6 mice (Fensterl et al., 2012; Nair et al., 2014). Olfactory bulbs, brains and lungs were collected as previously confirmed organs with detectable titre (Fensterl et al., 2012; Nair et al., 2014). Titres initially measured were variable and often below limit of detection (data not shown). It was discovered that during processing, flash freezing recently lysed tissues, followed by thawing and subsequent storage at -80^0C was an essential step. Brains and lungs were able to be titred following this method using the second hemisphere and other lung. Unfortunately, olfactory bulbs had to be combined due to their small size and titres were not able to be measured. A subsequent set of seven female mice were infected with VSV and the titre was measured in brain, lung (combined with previous group) and olfactory bulb (Fig. 7).

Viral titre in the brain was not equal in males and females. Female brains with detectable titre typically showed roughly a ten-fold reduction compared to the average male brain, with

several females having titre that was below or near the detectable limit. Titre in male brains were much more consistent (Fig. 7a). This stark difference in viral load in the brain between sexes is consistent with the disparity in survival and suggests a possible sex-effect in viral dissemination and/or replication in the brain. No genotypic differences in the brain are seen in either sex, or when sexes are combined. This result does correspond to the survival trends seen in either sex. Viral titre in wild type males and female lungs were similar, unlike in the brain. Female heterozygous lungs exhibited significantly reduced titres compared to homozygotes but not compared to wild types. No genotypic differences were seen in the lungs of male mice or when sexes were pooled. Olfactory bulb titres in the seven female mice provided little insight into possible differences, with no differences observed between wild type and homozygous samples, and while the one heterozygous animal had a titre approximately 10-fold lower than wild type samples, it is impossible to conclude a genotypic effect with the current sample size.

The absence of differences in viral load in the brains of mice do not correspond to trends or differences seen in survival. It is possible that mutations in *Gba1* do not confer altered control of VSV infection, but rather are implicated in mechanisms that reduce neuronal cell death in response to VSV infection, similar to that what has been reported in nGD mice in response to neurotropic Sindbis infection (Melamed et al., 2020). Heterozygous females have lower titres in the lung, consistent with trends seen in female survival. This result suggests a sex- and genotype-dependent effect on viral load in this secondary target organ. One possible explanation for how this may translate to increased survival benefit in an infection model of encephalitis is the alteration of inflammatory mediators from the periphery that enter the brain, leading to reduced neuronal cell death. Titration of infected olfactory bulb across genotypes and in both sexes are of

the utmost importance as the first tissue infected in the mouse brain in VSV infection (Huneycutt et al, 1994).

Taken together, the effect of *Gba1* mutations in acute VSV infection are promising but remain inconclusive. A bona fide effect in *Gba1* heterozygous mice, specifically, in response to a neurotropic virus would carry added interest, considering the increased prevalence of a brain disease in mutation carriers, namely PD and DLB, and suggests that heterozygous mutations may confer a protective gain-of-function effect, wherein carrying a single mutation leads to better handling of the virus. In the males, the increased survival in homozygous mice suggest a beneficial sex-dependent function of two mutant alleles, which could be explained by a 'loss-of-function effect'.

GCase Activity in the Context of a Viral Infection

GCase activity was measured in mice infected with VSV to assess the effect infection has on enzymatic activity, or vice versa. Olfactory bulbs, brain and lung of VSV-infected mice were collected at 2 DPI. To maximize experimental output, these tissues were obtained from the same mice used for viral titre. Typically, organs for GCase activity measurement would be lysed in citric acid buffer; however, the detergent in this buffer would cause the inactivation of VSV prior to PFU analysis. To circumvent this issue, tissues were lysed in PBS and an aliquot was taken and diluted 1:4 in citric acid buffer. This ratio was determine based on cell culture work that employed a similar technique (Sanyal et al., 2020). GCase activity was subsequently measured and compared between mock and infected mice for each genotype and sex (Fig. 8). In most tissues, GCase activity was not significantly altered after infection. Interestingly, homozygous females had significantly reduced GCase activity after infection in the olfactory bulb. Given that

homozygous females behaved no differently compared to wild types in survival or viral load, it is possible that this reduction in activity is a consequence of infection rather than an anti-viral mechanism, *i.e.*, it is possible that VSV infection uses host proteins or lipids involved in GCase metabolism that could lead to altered GCase function.

Surprisingly, the apparent sex effect for GCase activity noted in Figure 1 was absent in mock-infected animals in this experiment. Differences in tissue processing include an extra freeze-thaw cycle in baseline readings and the 1:4 ratio of PBS:citric acid buffer in mock-infected samples. While these could affect absolute values of GCase, it is unlikely that sex effects would be masked. Mice used for mock-infected measurements differed in two ways from mice used for baseline readouts of GCase activity: in age and handling. Mice used for mock experiments were 11-13 weeks old (compared to 7 weeks at baseline). While a previous report showed no age-dependent differences in GCase activity at 4-, 8-, and 12-months, activities at earlier ages were not studied (Polinski et al., 2021). It is possible that changes in activity may be different in mice between sexes as they age in young adulthood. In humans, a Netherlands cohort reported age-dependent rises in sphingolipid concentration, with females having lower concentrations early in life compared to males, followed by a more rapid increase than males, leading to higher concentrations than males in later life (Muilwijk et al., 2021). If a similar mechanism were to occur in mice, it is possible that the loss of sex-dependent differences in GCase activity in 11-13-week-old mice may be explained by these changing concentrations of sphingolipids. Confirmation of age-dependent effects in our colony is ongoing, with the intention of measuring GCase activity and lipid profiles in 1-year-old p.D409V mice. Second, mock-infected control mice were transferred to a separate location in the animal facility and handled during mock infection prior to collection, both of which could induce stress in mice, although the

impact this may have on GCase activity is unknown. GCase activity in 7-week-old mock infected mice and baseline 11–13-week-old mice could help understand the lack of coherence between the two results.

Interestingly, activity in the olfactory bulb appeared to be nearly doubled compared to the rest of the brain (Fig. 8). Regional differences in GCase activity have been identified in mouse brain (Clarke et al., 2019), but to our knowledge this is the first identification of increased GCase activity in the olfactory bulb. Future assessment of mRNA and protein levels of *Gba1* and GCase will help substantiate this.

To assess genotype-dependent differences in GCase activity after infection, a ratio of infected-to-mock activity change was calculated and subsequently normalized to wild type activity, as a measure of relative increases or decreases in mutant mice compared to wild type mice (Fig. 9). In the brain, males showed a significantly increased ratio of infected-to-mock activity in heterozygous animals, with homozygous following a similar trend (although not significant). This effect was not recapitulated in the lungs or olfactory bulb. No differences were seen in female brains, but homozygous animals showed a significant decrease in mock-to-infected ratios in the lung and olfactory bulb compared to wild type mice. Neither of these findings point to a concrete anti-viral mechanism mediated by GCase activity alterations based on differences observed in survival. In males, while activity increases more closely reflect survival differences, it is counterintuitive that a beneficial effect in mutations which inherently exhibit reduction in enzymatic activity, would increase activity to combat infection. In females, the reductions in the lungs and olfactory bulbs of homozygous mice are not suggestive of an antiviral mechanism as homozygous females survived at a similar rate and had similar viral load in all organs measured compared to wild type. It is possible that these alterations in GCase

activity are instead a consequence of VSV infection, as proposed above. However, whether these changes in activity lead to downstream metabolic changes is of future interest. Both increases in activity in males and decreases in activity in females could lead to such changes, perhaps through different mechanisms, that both ultimately have negative downstream consequences rooted in homeostatic disruption. Immediate changes in mRNA synthesis rates and steady-state levels of α-synuclein concentrations following infection, levels of toxic (oligomeric) α-synuclein species and lipid profiles would be interesting to examine next to further probe the possible effects of infection versus *Gba1* metabolism and function. The possibility of these species being dysregulated along with GCase activity, as a result of infection, having been identified in *GBA1*-linked PD pathogenesis, would mesh nicely with our working hypothesis of gene-environment interactions, *i.e.*, changes mediated by mutated genes in response to microbial pathogen that may lead to pathological outcomes (Schlossmacher et al., 2017). Plasma has been obtained from male mice infected with VSV and lipids will be measured from these samples in collaboration with Dr. Steffany Bennett's team. Taken together, the role of GCase activity in VSV infection is unclear but warrants further exploration due to its implication and proposed mechanisms of action in both *GBA1*-linked PD and idiopathic PD.

Gba1's Influence on α-Synuclein Levels During Viral Infection

Gba1 mutants have been demonstrated to alter α-synuclein levels in mice (Sardi et al, 2011; Cullen et al, 2011). Additionally, our group and others have shown upregulation of α-synuclein after viral infection (Beatman et al., 2015; Shutinoski et al., 2019). With this in mind. total α-synuclein concentrations were measured in the olfactory bulbs of male VSV-infected mice (female OBs were not sufficiently available to obtain GCase activity as well as α-synuclein

measurement) (Fig. 10). Levels of α-synuclein were not altered in mock-infected animals. Previous studies demonstrating differences between p.D409V mutants typically did so in older mice (Sardi et al., 2011; Cullen et al., 2011). When examined at 2 DPI, concentrations of total murine α-synuclein were not significantly different between genotypes. Additionally, when assessing infected-to-mock changes in α-synuclein concentration between p.D409V mutant mice compared to wild type mice, no significant differences were detected. A possible confounding factor (in the lack of any observed difference) could be the high abundance of α-synuclein in the adult mammalian brain (estimated to be ~0.1% of the CNS proteome) and its long half-life (estimated to be greater than one week) (Mollenhauer et al., 2008). Hence, the short interval between viral infection and measurement of α-synuclein concentration could theoretically result in a net change in α-synuclein (in either direction), which our ELISA system may not have been sensitive enough to detect. Future studies will also address whether post-translational modifications of α-synuclein and conformer changes were induced by infection, which our total α-synuclein-based quantification assay would not have been able to identify.

Further Probing of Gba1 Function: Next Steps

Taken together, the results shown here indicate a possible role for heterozygous *Gba1* p.D409V mutations in survival of neurotropic VSV infection, and a sex-biased role for homozygous mice in males. GCase activity appears to be altered in VSV infection in an organ- and sex-dependent manner, the mechanism of which is unknown. GCase activity across brain, lung and spleen also appear to be sex-dependent, and lipid analysis may shed further light on these differences.

First and foremost, a handful of experiments highlighted above should be completed prior to advancing this project. The first involves understanding the loss of sex dependent effects in mock-treated animals by measuring activity in mock-treated 7-week-olds and activity at baseline in 11–13-week-olds to rule out an effect of age and/or handling. This, along with lipid profile analysis, should shed light on the significance of sex-dependent alterations in the context of *Gba1* and GCase. Second, repetition of *S. typhimurium* infection and viral titre measurements in H1N1-infected mice (possibly at an increased viral dose) should be performed to confirm that mutations in *Gba1* do or do not cause altered microbial outcomes in these paradigms. Third, VSV titration at 2 DPI should be re-performed in males and females in the olfactory bulbs of mice, to better understand how p.D409V mutations impact viral load in this target organ and how that may translate to survival results. In addition to absolute GCase activity, which has been measured in this study, the resulting changes in lipid homeostasis is a critical link to underlying mechanisms. Plasma has been collected for this purpose in collaboration with Dr. Bennett's team.

If current results hold true, there are multiple avenues that can be taken with this project that are not mutually exclusive. First, mechanistic exploration into the effects of *Gba1* function(s) in VSV infection. For example, isolation of resident macrophages from the lung and brain (microglia) can performed to understand how GCase activity changes in response to infection and how GCase activity alterations (e.g., through pharmalogical agents) can affect VSV replication and cell death.

Second, additional challenges with new pathogens, carrying different genetic makeups and methods of action in infectivity. For example, to date we have not tested DNA viruses in our infection paradigms (Shutinoski et al., 2019; Tomlinson et al., 2017). This could be

accomplished to further distinguish whether the effect of *Gba1* mutations is pathogen specific and/or subcellular compartment-specific, (i.e., in response to VSV and other RNA viruses or enveloped neurotropic infections) e.g., by using Herpes Simplex virus (Lin et al., 1997; Lang et al., 2020). Pathogens that simultaneously infect olfactory neurons and the gastrointestinal tract could be employed, consistent with the Braak and Del Tredici "dual-hit" model. For example, type-3 (Dearing) Respiratory-Enteric-Orphan virus, a double stranded RNA virus that we have shown is affected by mutations in murine *Lrrk2* (Shutinoski et al., 2019).

Third, the downstream impact of *Gba1* mutations in microbial infection ought to be explored. Understanding how *Gba1* mutations affect subtle biochemical outcomes in response to infection, such as lipid homeostasis, α-synuclein processing, mitochondrial function and inflammatory processes. These readouts may provide insight as to how environmental factors, such as microbial infection, may initiate or contribute to *Gba1*-associated pathological changes. While the survival differences in VSV likely would lead to the prioritization of these readouts in this model, they can also be carried out in infection paradigms that do not elicit differences, which could add a layer of understanding: namely, to answer 'how severe does an infection need to be to create changes in homeostasis of proteins and lipids that lead to pathological outcomes?'

Implications of Gba1's Host Response on GBA-PD Pathogenesis

In this work, we have identified a possible role for *Gba1* in host response to neurotropic infection. How this effect of *Gba1* mutations on neurotropic infection relates to PD may not be immediately obvious, *i.e.*, how could increases in survival rates among *Gba1* mutants contribute to eventual neurodegeneration in PD? In our *Lrrk2* mouse studies, p.G2019S mutant female mice infected with reovirus had a lower viral load compared to their wild type littermates.

Surprisingly, this did not translate to increases in survival, with p.G2019S mutant female mice exhibiting reduced survival rates compared to wild type mice. From this we reasoned that the pro-inflammatory state, while effective in viral clearance, was detrimental to brain health leading to death (Shutinoski et al., 2019). A similar mode of action could be considered in *Gba1* mice in considering its role in host response to infection in PD pathogenesis, *i.e.*, it is possible that *Gba1* mutation carriers incite altered inflammatory processes which in the short-term help in combatting infection but subsequently initiate or contribute to PD pathology. Additionally, if changes in GCase activity are associated with changes in lipid species in response to acute infection, this altered lipid metabolome could be implicated in PD pathogenesis.

Confirmation of the *Gba1*-mediated effects in host response to acute infection seen here is a top priority, as reviewed above. The eventual goal is to identify how this possible role of *Gba1* is implicated in PD and *GBA1*-PD. This link will likely involve aging mice, where chronic, sustained changes and brain health outcomes are measured after insult whether through acute, chronic or repeated infections. While they did not yield significant differences in the outcomes tested in this study, the usage of systemic infections should not be excluded in studies assessing the impact of *Gba1*-pathogen interactions in PD pathogenesis given the importance of systemic inflammation in PD and the ubiquitous expression of *GBA1*.

Conclusions

Typical PD is a multifactorial disease, well appreciated to be contributed to by several converging factors. Included is one's genetic susceptibility and environmental exposures. The Braak and Del Tredici dual-hit hypothesis, wherein a microbial pathogen may cause initial disease pathology in olfactory and gut neurons that can progress towards the brain during PD

pathogenesis, has driven us towards modeling PD through environmental pathogens, such as virulent pathogens, leading to infection in mice carrying concrete mutations in PD risk genes (Hawkes et al., 2007). For the purpose of this thesis, we sought out to identify a possible role for *Gba1* in acute infection. Mice carrying *Gba1* p.D409V mutations were infected with a selection of pathogens affecting the spleen (*S. typhimurium),* lung (H1N1) and brain (VSV). Thus far, results indicate that mutations in *Gba1* affect outcomes in a neurotropic VSV infection paradigm. Additionally, it appears that this effect, along with GCase activity, acts in a sex-dependent manner, which to our knowledge has not been previously described. GCase activity also appears to be altered in response to VSV infection in a sex- and organ-dependent manner, with the nature of such changes remaining to be fully delineated.

In summary, this thesis serves as a useful starting point in exploring the role of microbial infections on *Gba1* biology in experimental animals, and vice versa, in better understanding acute as well as chronic tissue changes that are associated with *GBA1*-linked diseases in humans.

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