Master's Theses

Theses and Dissertations

2018

Investigating Autophagy, Extracellular Vesicles, and Glycobiology

Benjamin Cook
Loyola University Chicago

Follow this and additional works at: https://ecommons.luc.edu/luc_theses

Part of the Cell Biology Commons

LOYOLA UNIVERSITY CHICAGO

INVESTIGATING AUTOPHAGY, EXTRACELLULAR

VESICLES, AND GLYCOBIOLOGY

A THESIS SUBMITTED TO

THE FACULTY OF THE GRADUATE SCHOOL

IN CANDIDACY FOR THE DEGREE OF

MASTER OF SCIENCE

PROGRAM IN INFECTIOUS DISEASE AND IMMUNOLOGY

BY

BEN COOK

CHICAGO, ILLINOIS

AUGUST 2018

ACKNOWLEDGEMENTS

TABLE OF CONTENTS

LIST OF TABLES

LIST OF FIGURES

LIST OF ABBREVIATIONS

AD	Alzheimer's Disease
ALP	Autophagy-lysosome pathway
ANOVA	Analysis of variance
Asn	Asparagine
ATCC	American Type Culture Collection
CD-MPR	Cation-dependent MPR
CI-MPR	Cation-independent MPR
CMA	Chaperone mediated autophagy
CMV	Cytomegalovirus
CNS	Central nervous system
CSF	Cell-free supernatant
DMEM	Dulbecco's modified Eagle's Medium
DSP	Dual-split protein
ECM	Extracellular matrix
ER	Endoplasmic reticulum
ESCRT	Endosomal sorting complexes required for transport
EV	Extracellular vesicle
FBS	Fetal bovine serum
FITC	Fluorescein isothiocyanate

GCase	Glucocerebrosidase
GD	Gaucher's Disease
GFP	Green fluorescent protein
GlcCer	Glucosylceramide
HEK	Human embryonic kidney
LBA	Lectin binding assay
LSD	Lysosomal storage disease
LRRK2	Leucine-rich repeat kinase-2
MPR	M6P Receptor
mTOR	Mammalian target of rapamycin
MDM	Monocyte derived macrophage
MVB	Multivesicular body
MVE	Multivesicular endosome
M6P	Mannose 6-phosphate
N-glycan	Asparagine-linked glycan
NDS	Normal donkey serum
OGT	O-GlcNAcase
PBS	Phosphate-buffered saline
PD	Parkinson's Disease
PEI	Polyethylenimine
PINK1	PTEN induced putative kinase
P13K	Phosphatidylinositol-3-kinase

PI3KC3	Phosphatidylinositol-3-kinase, class 3
pLVX	Lenitviral vector
PMA	Phorbol myristate acetate
PTEN	Phosphate and tensin homolog
RPMI	Roswell Park Memorial Institute
RT	Room temperature
SEM	Standard error measure

CHAPTER ONE

OVERVIEW AND HYPOTHESIS

Many diseases are a result of the impairment of autophagy, an intracellular degradative process [1-3]. A crucial function of autophagic mechanisms is the degradation and recycling of cellular materials by the lysosome, an acidic organelle containing degradative enzymes [4]. Impaired lysosomal degradation leads to a class of diseases termed lysosomal storage disorders (LSDs) [1, 2].

There is evidence that impaired carbohydrate, aka glycan, degradation is the most common precipitate of these disorders [2]. Glycans are known to have various biological functions including modulating protein interactions, acting as specific intermediaries for cell-cell interactions, and regulating protein folding and trafficking [5, 6].

Similar to lysosomal dysfunction, another autophagic mechanism, extracellular vesicle (EV) secretion, has been implicated in a wide array of disease due to their ability to exchange materials intercellularly [7-14]. In our lab we are particularly interested in the role of extracellular vesicles in the spread of neurodegenerative diseases such as Parkinson's Disease (PD).

Given the correlation of autophagic impairment and glycan degradation with LSDs [1, 2] and EVs ability to exchange materials intercellularly [7-14] we predicted that changes in EV glycans—which are altered throughout the autophagic process sequentially [2, 15, 16] —play a vital role in EV trafficking and cargo selection.

The goal of this research is to elucidate mechanisms of both secretory and degradative autophagy. This will be accomplished through identifying changes in EV glycans upon perturbation of autophagic processes. Specifically, autophagy will be perturbed in two differing manners: induction of autophagy and disruption of Lysosomal degradation. Identifying changes in EV glycans may help to better understand autophagic mechanisms and EV trafficking. Improving the understanding of autophagic secretion may improve future diagnostic and therapeutic methods for treatment of LSDs such as PD.

The first aim of this research is to identify the presence and description of EV glycans. Additionally, we aimed to identify changes in EV glycans upon induction of autophagy as well as disruption of lysosomal degradation. Observed differences can be used to identify specific changes in glycans of autophagically secreted vesicles. These findings will contribute to the knowledge of glycobiology and autophagic function that can be applied to various cases of lysosomal disfunction such as PD.

CHAPTER TWO

REVIEW OF THE LITERATURE

Lysosomal Storage Disorders

LSDs are a class of disorders that involve an impairment in lysosomal degradative function [1, 17]. LSDs typical involve mutations in genes of hydrolytic enzymes but are also commonly caused by mutations in genes encoding lysosomal membrane proteins [3]. Elizabeth Neufeld demonstrated that cells lacking lysosomal enzymes were unable to degrade cellular components, leading to their accumulation in lysosomes [18]. She also demonstrated that exogenous lysosomal enzymes could restore lysosomal function when added to the cultures of cells with impaired lysosomal function [19].

There are currently over 50 identified inherited diseases that exhibit impaired lysosomal degradation of various macromolecules. More than half of these share a common feature; the substrate of the defective enzyme is a glycan species e.g. a glycoprotein, glycosaminoglycan, or glycolipid [17]. The frequent correlation of impaired glycan degradation and LSDs may be due to the need for an abundant number of specialized degradative enzymes specific to the many possible formations of glycan structures due to the multitude of glycan combinations and branching possibilities. This will be discussed further in following sections regarding glycobiology.

Symptoms of LSDs vary but are frequently caused by neurological impairment, which can include seizures, movement disorders, dementia, and loss of vision and

hearing [3]. There are also common peripheral symptoms in LSDs including hepatomegaly and splenomegaly [3]. The severity and progression of the symptoms of LSDs depend on the impaired enzyme and its substrate [17]. Development of LSD symptoms are typically inversely related to the age at which symptoms appear i.e. infantile LSD symptoms develop rapidly while adult LSD symptoms develop slowly [3].

While there are currently no cures for LSDs there are an increasing number of treatments being developed based upon two approaches: first, improving the availability of the deficient enzyme via methods such as bone marrow transplant, enzyme replacement therapy (ERT), small molecule pharmacological chaperones, and gene therapy and second, reducing the lysosomal load of the accumulating substrate via substrate reduction therapy [20-22].

Gaucher's Disease.

Gaucher's Disease (GD) is the most common LSD [23], caused by loss-of-function mutations in the glucocerebrosidase (GCase) gene, *GBA*, which encodes the GCase enzyme, a lysosomal enzyme that cleaves the β-glucosyl linkage of glucosylceramide (GlcCer) [24, 25]. Symptoms of GD are caused by accumulation of the GCase substrates GlcCer and glucosylsphingosine [26]. GD is divided into three subgroups according to the rate of disease progression and involvement with the central nervous system (CNS) [26, 27]. Type I is non-neurodegenerative and is characterized by hepatosplenomegaly, skeletal and hematopoietic system abnormalities [26]. Type II is neurodegenerative and progresses rapidly while type III is also neurodegenerative but progresses slowly [28]. ERT using methods such as a recombinant GCase targeting macrophage uptake receptors has proven effective for GD [23, 29]

Parkinson's Disease.

PD is the second most common neurogenerative disorder and the most common movement disorder [30]. It is a slowly progressing disorder which causes impaired motor and autonomic function [31-33]. Inherited forms of PD are caused by mutations in the gene which encodes the protein α-synuclein [30], which will be discussed further later. Clinical symptoms of PD are usually defined by motor disturbances, but there may be disturbances in several other functions of the nervous system [31-34].

The primary cause of PD symptoms is the degeneration of dopaminergic neurons due to neurotoxicity as a result of the aggregation of α-synuclein [31-34]. α-synuclein is a protein found abundantly in presynaptic nerve terminals [35]. While the role of α-synuclein is poorly understood it is suspected to play a role in maintaining synaptic vesicles in presynaptic nerves, regulating the release of dopamine, and synaptic membrane remodeling [36-38].

α-synuclein can spread from cell-to-cell in a prion-like manner, involving "seeding" by mutated or abnormally folded forms of the protein which then serve as a template for aggregation. The aggregation of α-synuclein forms cytoplasmic inclusions containing misfolded proteins, which are termed Lewy bodies [30, 39-42]. The mechanism by which α-synuclein is secreted by cells is not fully understood. There is growing evidence that it is secreted via unconventional secretion mechanisms [12, 43]. Not only is α-synuclein released from cells in EVs, but α-synuclein containing EVs are taken into recipient cells, leading to aggregation and neurotoxicity in those cells [12, 42]. In fact, evidence indicates that the uptake of α-synuclein within EVs occurs preferentially over non-vesicular α-synuclein [12]. Additionally, α-synuclein has been purified from EVs isolated from the

cerebrospinal fluid of PD patients [44], further supporting the model for cell-to-cell transfer of α-synuclein via EVs. These indications demonstrate that intercellular EV exchange is crucial to the pathogenicity and progression of PD. The mechanisms of EV biogenesis and secretion will be discussed in following sections [39].

There is evidence that mutant α-synuclein impairs intracellular degradation via inhibition of the autophagy-lysosome pathway (ALP) by binding to lysosomal receptors required for autophagic degradation [39]. ALP impairment has recently gained recognition as a major pathogenic event in neurodegenerative diseases, especially PD [45]. Mutations in the genes that are the most common causes of PD, such as leucine-rich repeat kinase-2 (*LRRK2*), parkin, and phosphatase and tensin homolog (PTEN)-induced putative kinase 1 (*PINK1*), are known to impair the ALP [45]. Another common PD mutation is in the *ATP132* gene, which is an essential lysosomal membrane protein. Mutations in this gene lead to impaired lysosomal membrane stability and acidification, reducing lysosomal degradation [45]. The ALP will be discussed, in depth, in later sections.

There are currently no treatments that will stop the progression of PD. Treatment with dopaminergic drugs can treat symptomatic motor dysfunctions with varying success [31, 32, 34]. As PD progresses, the effect of drugs, such as the dopamine prodrug Levodopa, have decreased effectiveness due to the loss of the cellular ability to store dopamine [31].

Given the high frequency of impaired glycan degradation when lysosomal function is compromised it is logical to expect that glycans will be altered by PD since PD is caused by impaired degradation of α-synuclein [45]. Indeed, exploratory studies have shown

changes in N-linked glycans in murine PD models [46]. In these studies researchers observed time-dependent changes in glycans which clustered in structurally-related groups, indicating that the observed changes were not random [46].

Link Between Gaucher's Disease and Parkinson's Disease.

As previously stated a hallmark of both GD and PD is impaired lysosomal function [47, 48]. Persons with GD are more likely to have PD than others due to a pathogenic loop between α-synuclein accumulation and the aforementioned GCase enzyme disfunction [47, 48]. In fact, mutations of GCase are the most frequent risk factor for PD in the general population [24]. Furthermore, there is evidence that altered metabolism of glycosphingolipids, such as GlcCer, contributes to synucleinopathies and neurodegeneration [49-51]. The link between impairments in the degradation of the β-glucosyl linkage within GlcCer and α-synuclein accumulation further supports the importance of glycans, glycosidases, and their role in protein quality control and intracellular degradation.

Glycobiology

Biological Functions of Glycans.

Glycans are known to have roles in a variety of eukaryotic biological functions including modulating protein interactions, acting as specific ligands for cell-cell interactions, and regulating protein folding and trafficking [5]. Glycans have significant roles within the nervous system with regards to development, regeneration and synaptic plasticity [6]. Also, glycans form a complex network of molecular communication by mediating interactions between recognition molecules at the cell surface and in the extracellular matrix (ECM) [6]. There is also growing evidence that glycans function in

vesicular cargo selection and trafficking [52, 53], indicating mechanistic roles in intracellular exchanges. As previously mentioned there is wide diversity of glycan structures due to various carbohydrates and branching diversity. This structural diversity provides a mechanism by which each glycan can have a very specific function based upon its unique structure [6].

N-linked Glycans.

Asparagine-linked glycans (N-glycans) are of particular interest for this research due to their importance in the regulation of the nervous system [54]. N-glycans were found to affect the function of synaptic proteins involved in the synaptic transmission as well as regulate multiple channel proteins [54]. Research has also shown that the structural features of glycans are key to their function [54]. They are glycans which are covalently attached to proteins at an asparagine (Asn) residue by an N-glycosidic bond. A common feature of all N-glycans is that they link to Asn via the acetylglucosamine carbohydrate, GlcNacβ1 [55]. The terminal epitopes of glycans are predominantly determined by N-glycan diversity [55] and N-glycans are crucial to the glycosylation-mediated mechanism of protein folding quality and control [56]. As previously mentioned N-glycans are also pivotal to processes by which lysosomal hydrolases are targeted for transit to the lysosome [57]. Research has shown that N-linked glycans play role in nervous system development via neural cell adhesion and axonal targeting functions [54]. Defects in N-glycan biosynthesis and degradation are commonly associated with nervous system, hepatic visual and immune system disfunction and congenital disorders [17].

Lysosomal degradation of glycoproteins with complex N-glycans is a sequential process that occurs in a specific order and in tandem with the degradation of the

proteinaceous portion of the glycoprotein [17]. During this process each glycan subunit is degraded by a specific glycosidase. The majority of glycans that enter the lysosome consist of only six carbohydrates linked in one or two anomeric conformations [17]. Most of what is known about the lysosomal degradation of glycans is based upon analysis of the accumulation of carbohydrates in tissues from persons with LSDs [17].

Lectins and Galectins.

The presence of cellular and nuclear membrane glycans which are orientated to the cytoplasm suggests that there are glycan binding proteins which participate in the intracellular function of glycans [58]. Indeed, there are various studies that have found intracellular processes mediated by glycan binding proteins [58, 59].These proteins have been designated as lectins [60].

Galectins are a type of lectin that have been termed S-type lectins due to their dependency on disulphide bonds for stability and their glycan binding ability [58, 59]. Galectins have been found to function in various biological roles but, specifically, galectins-3 and 8 have been proven to be part of autophagic mechanisms [61-63] .

Glycan Synthesis and Degradation.

Intracellular degradative and recycling mechanisms are required for the efficient catabolism of glycans [15]. Glycans are primarily degraded in lysosomes by glycan degrading enzymes, endoglycosidases and exoglycosidases [17]. Even though lysosomal degradation of glycans is not always complete, both partially and fully degraded glycan species can be returned to the Golgi compartment where the glycans can be elongated via glycan attaching enzymes such as glycosyltransferases and sialytransferases [17]. For example, if the protein portion of an endocytosed glycoprotein

was not degraded in the lysosome the protein may be returned to the Golgi complex to have the glycan portion reattached then returned to the cell surface [17]. A similar mechanism occurs in the case of partial glycan degradation of glycosphingolipids, producing glucosylceramide, and the complete glycan degradation of glycosphingolipids, producing sphingosine [17]. As previously mentioned, the ALP is pivotal to the degradation and recycling of glycans [16].

Impaired glucose metabolism has been linked to many neurodegenerative disorders [64-66]. A common feature between these disorders is the impaired O-GlcNAcylation of proteins [62, 67]. The impaired activity of the glycan modifying enzymes, O-Linked N-Acetylglucosamine Transferase (OGT) and O-GlcNAcase (OGA), has been linked to neurodegenerative disorders such as PD and Alzheimer's Disease (AD) [68], indicating further that impaired glycan degradation can contribute to neurodegeneration [67, 68]. This is additionally supported by evidence that decreasing O-GlcNAcylation increases side effects of neurodegeneration while increasing O-GlcNAc levels reduces plaque formation as well as cognition in murine model [69]. It has also been shown that O-GlcNAc modification blocks the aggregation and toxicity of α-synuclein [68]

Autophagy-Lysosome Pathway

The ALP is the eukaryotic intracellular process of degradation in which cytosolic materials are isolated then delivered to lysosomes for degradation [70]. The functions of degradative autophagy can be split into two groups: First, the recycling of nutrients during starvation; Second, maintaining cellular homeostasis by degrading damaged organelles, pathogens, and misfolded proteins [71]. The ALP is divided into three different pathways based upon their unique methods of cargo entrance into the lysosome [39]. Chaperone

mediated autophagy (CMA), microautophagy, and macroautophagy [72]. CMA is dependent upon the recognition and targeting of specific cargos to the lysosome by chaperon proteins [73]. Microautophagy is the process of direct invagination of nonspecific cytosolic cargo by an endosome or lysosome [74]. Lastly, there is macroautophagy, referred hereafter as autophagy, which involves the nonspecific engulfment of cytosolic components by autophagosomes which then fuse with lysosomes [72]. A simplified cartoon representing these mechanisms is shown in Figure 1 below.
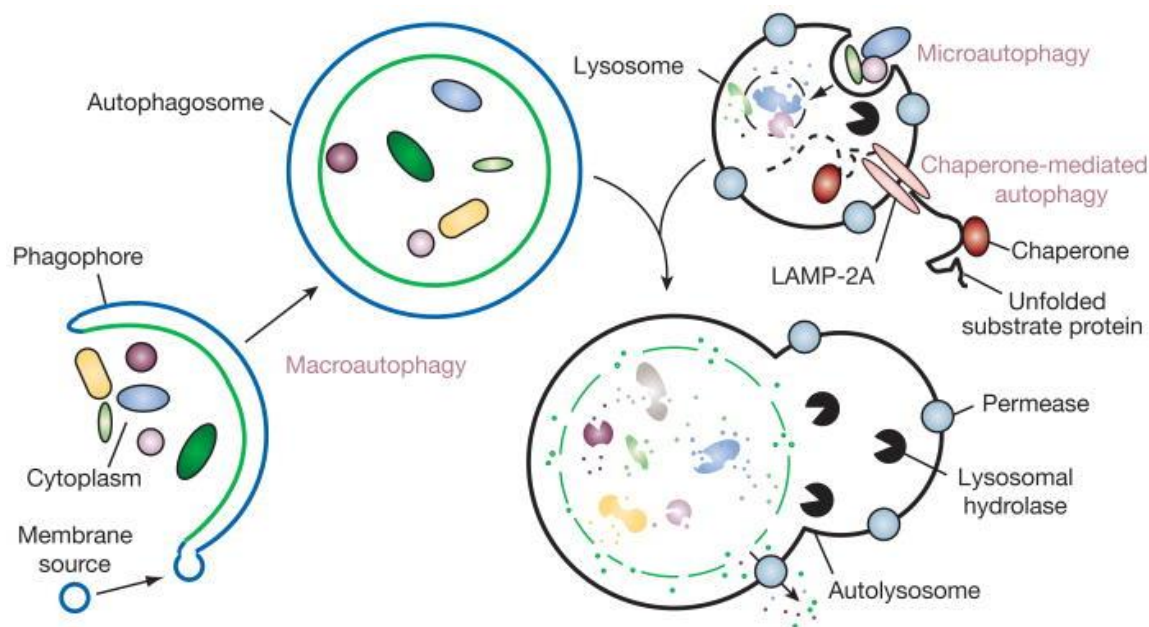
Figure 1. Different Types of Degradative Autophagy [77]. The three types of autophagy - macroautophagy, microautophagy, and chaperone-mediated autophagy – use different mechanisms to deliver cargo to lysosomes but all result in degradation of cargo for reuse within the cell.

As previously mentioned altered lysosomal function, and thereby autophagic function, have been implicated in various neurodegenerative diseases [75, 76]. In relation to PD, alterations in autophagy have been shown to affect the secretion of α-synuclein. This has been shown by various studies that have shown that promotion of autophagy

and inhibition of autophagosome-lysosome fusion increases α-synuclein secretion, impairment of autophagosome formation causes a decrease in α-synuclein secretion leading to intracellular α-synuclein accumulation [43].

Autophagosome Formation.

Autophagosome initiation begins with the formation of a double membraned, cup-shaped phagophore mediated by the scaffolding protein complexes ULK and Beclin-1 [77-80]. Each of these complexes serve an important purpose in the formation of the autophagosome: the ULK complex associates with membranes while the Beclin-1 complex induces the formation of a class of phosphorylating enzymes specific to autophagy, phosphatidylinositol-3-kinase class three (PI3KC3). [80]. After initiation, the phagophore is elongated by the ATG12-ATG5-ATG16 and ATG-LC3 complexes. During this process of elongation, the cytosolic contents that will be degraded are engulfed by the growing phagophore. Elongation is completed once the ends of the phagophore join, forming the double membraned autophagosome. The autophagosome is then able to fuse with an endosome to form an amphisome and/or a lysosome to form an autolysosome [80]. A detailed depiction of this molecular process can been seen in Figure 18 [70] in Appendix A.

Lysosomes.

Lysosomes are intracellular membrane-bound organelles that degrade cytoplasmic macromolecules using hydrolytic enzymes [4]. Lysosomes have a luminal pH of 4.5-5.0 which is produced and maintained by V-Type ATPase proton pump [81] and is required for the for the function of the hydrolytic enzymes within them [82]. Like many other enzymes, the lysosomal hydrolytic enzymes are glycoproteins that are synthesized

in the endoplasmic reticulum (ER) and traverse the ER-Golgi pathway [57]. Once in the Golgi compartment the hydrolytic enzymes are selectively trafficked to lysosomes, primarily via recognition of N-glycans containing mannose 6-phosphate (M6P) by P-type lectins, a class of glycan binding molecules which act as M6P receptors (MPR) [57]. Study of these MPRs has found two distinct transmembrane glycoproteins, the cation-independent MPR (CI-MPR) and cation-dependent MPR (CD-MPR), which are both required for efficient targeting of lysosomal enzymes [83].

Endosomes.

Endosomes are membranous organelles involved in the ALP [84] which originate from the trans-Golgi membrane [85]. Their role in the ALP has been shown to link endocytic, secretory and degradative pathways [86]. Endosomes are separated into three categories: Early endosomes, late endosomes, and recycling endosomes [44]. Both late and recycling endosomes begin as early endosomes however, they are differentiated by the fate of their cargo [87, 88]. The cargo of late endosomes is secreted or degraded while the cargo of recycling endosomes is not, so that the cargo can be reused within the cell [44, 87, 88].

Early endosomes mature into late endosomes before fusion with lysosomes [87, 89, 90] and are the major site for the entry of lysosomal hydrolases via the aforementioned CI-MPR, which leads to the fusion with, as well as the maintenance of degradative function of lysosomes [87]. A subgroup of late endosomes develops into multivesicular bodies (MVBs) which are formed by the inward invagination of the endosomal membrane [91]. The formation of MVBs is mediated by the well-defined mechanisms of the

endosomal sorting complexes required for transport (ESCRT), which facilitate membrane bending and scission reactions away from the cytoplasm and into the endosome [91].

Unconventional Secretion: Secretory Autophagy.

Although autophagy has been traditionally viewed as a degradative process, recent studies have shown that autophagic machinery is used in the unconventional secretion of leaderless cytosolic proteins, such as IL-1β [92-94], as well as RNA [8], cytokines [95], immune mediators [96], prion-like proteins [97], and, most pertinent to this body of research, EVs [98].

This unconventional secretion using autophagic machinery, secretory autophagy, occurs when an autophagosome or amphisome fuses with the plasma membrane to release their contents into the extracellular space rather than fusing with a lysosome and leading to degradation [96]. A cartoon of these divergent pathways is depicted in Figure 2 [95] below.

<div align="center">

Extracellular Vesicles

</div>

EVs are a heterogenous family of membrane-limited vesicles originating from the endosome or plasma membrane [99]. They are found in biological fluids such as stool, urine, breast milk, saliva, and cerebrospinal fluid [13]. EVs are not only important mediators of cell-to-cell communication in normal physiological processes but also a wide range of disease states such as inflammatory diseases [100], viral infections [7], breast cancer progression [9] and neurodegenerative diseases [11, 101]. EVs are separated into two subgroups based upon the origin of their membranes [14, 102].

Exosomes are EVs which are produced by the inward budding of an endosome, which produces an MVB, or multivesicular endosome (MVE), and are approximately

between 30nm and 150nm in diameter [14, 99, 102, 103]. Microvesicles are produced by the outward budding of the plasma membrane and are larger than exosomes, reaching diameters up to 1000 nm [14, 102, 103]. A simplified depiction of the difference between these processes is shown in Figure 3 [14] below. Research has indicated that EVs are linked to PD due to their role in the aggregation, secretion, and intercellular transfer of α-synuclein [104, 105]. Additionally, mutations associated with PD have been identified in proteins involved in EV biogenesis, trafficking, and fusion, such as LRRK2, SPS35, and ATP 13A2 [101, 106-110], supporting previously stated evidence that PD and autophagic mechanisms are intertwined.

Conclusion

Glycans are crucial to cellular processes due to their roles in vital mechanisms such as the trafficking and localization of glycans, protein quality control, intercellular communication, intracellular degradation, protein-protein interactions, and neural development. They have been found to be important in not only overall cellular homeostasis, but specifically in neurodegenerative diseases and LSDs. Likewise, the intercellular transfer of cargo in EVs has been implicated in various prion and neurodegenerative diseases. Many questions remain regarding the role of glycans in EV secretion and intercellular targeting and the relationship between impaired glycan degradation, secretory autophagy, and neurodegeneration.

The follow chapters will focus upon EV glycans, how these glycans change upon perturbation of autophagic processes, and the glycan definition of α-synuclein containing EVs.
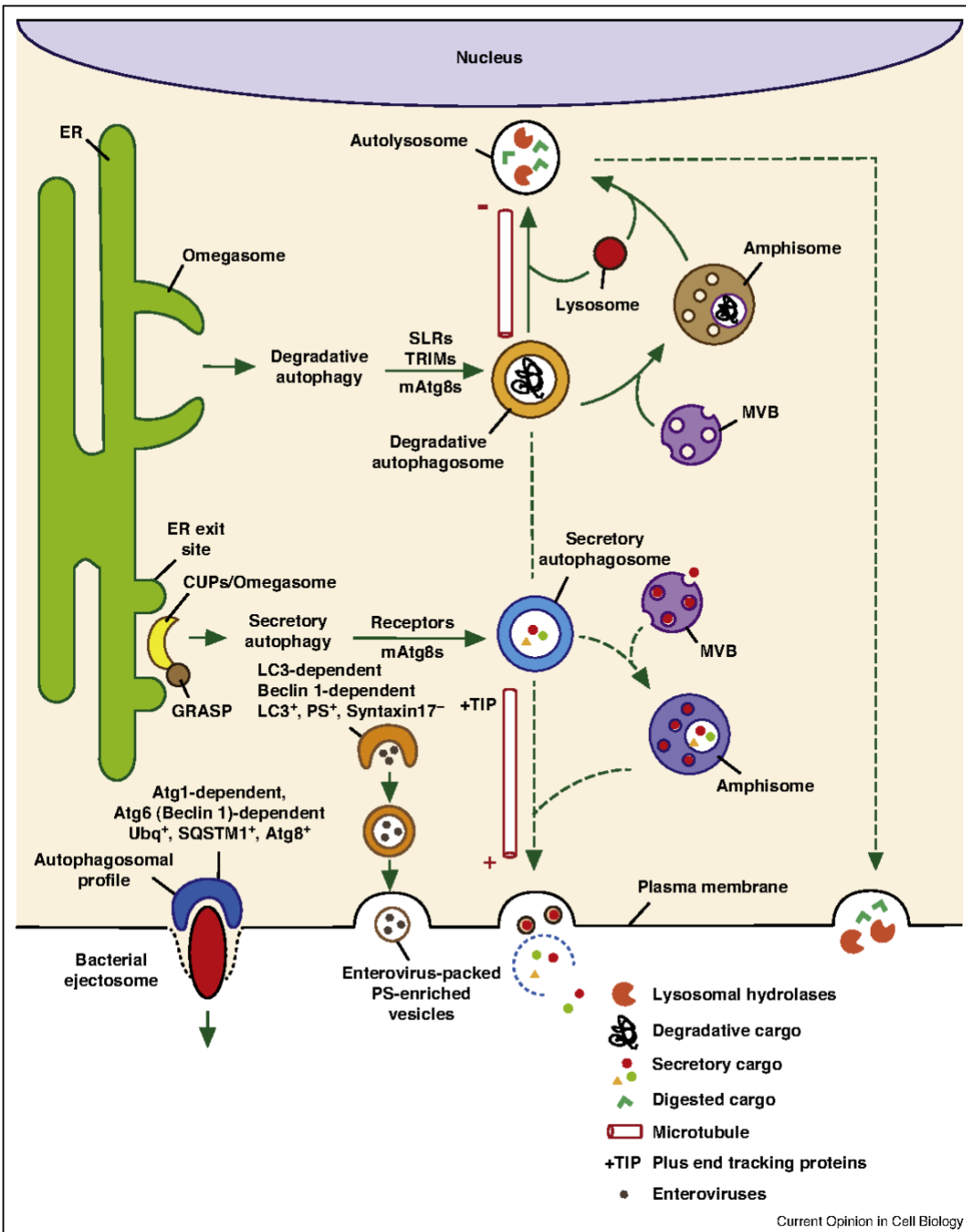
Figure 2. The Proposed Model For The Divergent Points Of Degradative Versus Secretory Autophagy [95]. Degradative amphisomes fuse with lysosomes while secretory amphisomes fuse with the plasma membrane to release their contents.
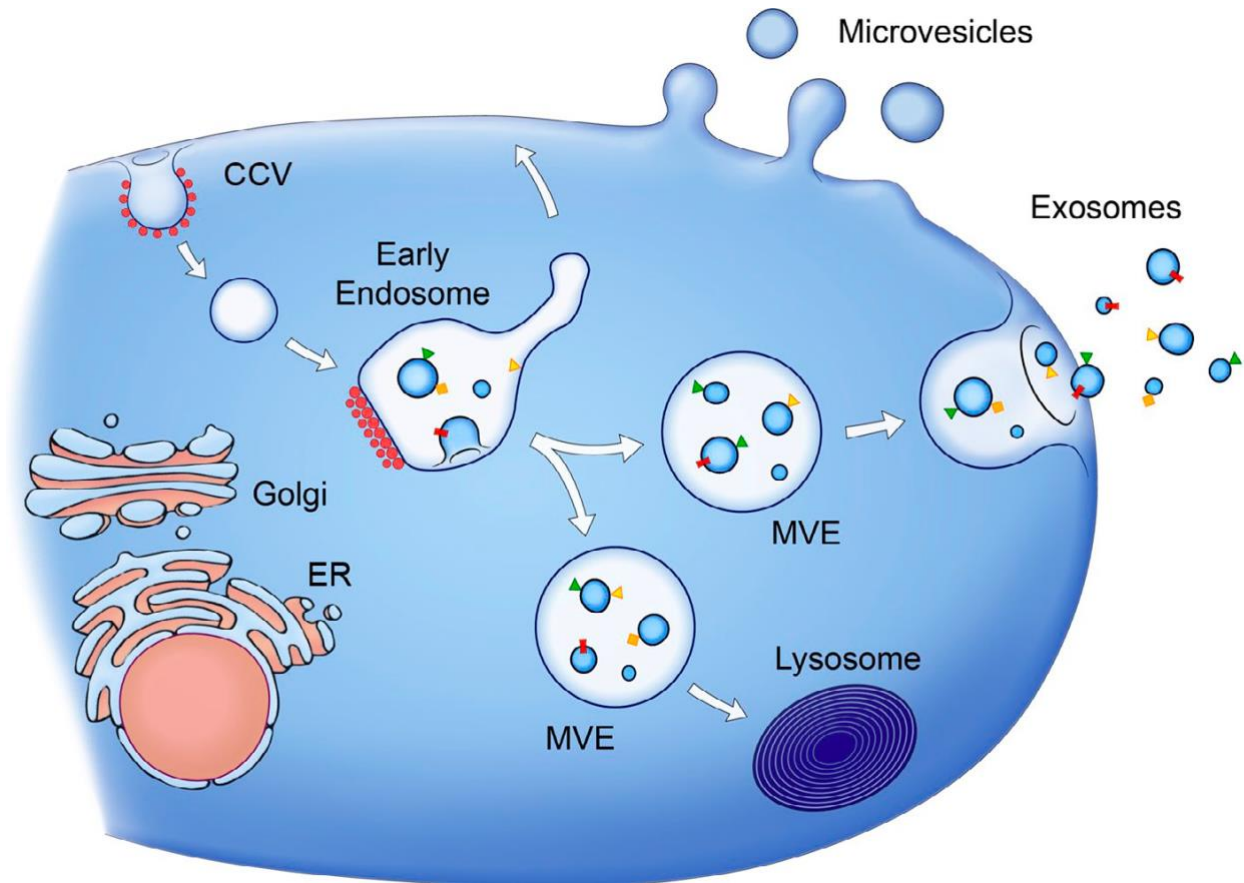
Figure 3 [14]. EV Biogenesis and Secretion. Microvesicles bud directly from the plasma membrane, whereas exosomes are represented by small vesicles of different sizes that are formed as the ILV by budding into early endosomes and MVEs and are released by fusion of MVEs with the plasma membrane.

CHAPTER 3

MATERIALS AND METHODS

The Novel Description of Extracellular Vesicle Glycans at The Individual Vesicle Level

Cell Culture.

The human monocyte cell-line THP-1 was acquired from the American Type Culture Collection (ATCC). Cells were cultured in an incubator at 37°C and 5% CO_2 in Roswell Park Memorial Institute (RPMI) 1640 media, supplemented with the addition of EV depleted 10% fetal bovine serum (FBS) (Hyclone), 10 μg/ml ciprofloxacin hydrochloride, 100 IU/ml penicillin, and 100 μg/ml streptomycin.

Expression of S15 mCherry Membrane Protein.

THP-1 cells were transfected to stably over-express S15-mCherry using a lentiviral viral vector (pLVX) containing our S15-mCherry construct and driven by a cytomegalovirus (CMV) promoter. Lentivirus was generated for transduction in human embryonic kidney (HEK) 293T cells transfected using the transfection reagent polyethylenimine (PEI) with equal parts concentration of VSV-g, ΔNRF or psPax2, and pLVX-CMV-S15-mCherry plasmid. 48 hours post-transfection lenti-viral particles were collected and purified from the 293T cells cultured media using a 0.45μm millipore syringe. The purified lenti-viral particles were then used to treat THP-1 cells. The treated THP-1 cells were then selected for those positive for the resulting S15 mCherry by supplementing the previously mention RPMI with 5 μg/ml puromycin (Hyclone).

Differentiation.

THP-1 cells were differentiated into monocyte derived macrophages by treatment with 100 nM phorbol myristate acetate (PMA) (Sigma) for 24 hours.

Media Collection.

After 24h PMA treatment media was changed with fresh, EV depleted, RPMI. After 24 hours of incubation at 37°C and 5% CO_2 media was collected and centrifuged to isolate the cell-free supernatant (CSF).

Immunofluorescent Staining and Preparation for THP-1 mCherry Extracellular Vesicles.

For the EV visualization, 2mL of CSF was spinoculated at 13°C for 2 hours at 1200 x g onto glass coverslips and subsequently fixed with a solution of 0.1 M PIPES with 3.7% formaldehyde (Polysciences) for 30 minutes. Immediately after fixation, vesicles were treated with 0.01% Saponin (Sigma-Aldrich) in a PBS block solution supplemented with 10% normal donkey serum (NDS), and 0.01% NaN_3 for 5 min. The coverslips were then individually subjected to 5 μg/ml of various biotinylated lectins (Vector Laboratories) (see Table 2 in appendix A for list) and incubated at 37°C for one hour. The coverslips were then treated with streptavidin conjugated to a fluorescein isothiocyanate (FITC) fluorophore (BD Pharmingen) diluted in phosphate-buffered saline (PBS), at a concentration of 1:400, for one hour at room temperature (RT). Finally, the coverslips were mounted on slides and allowed to dry for 24 hours.

Microscopic Observation and Analysis of mCherry Extracellular Vesicles at the Single Vesicle Level.

EV images were taken on a DeltaVision wide field fluorescent microscope (Applied Precision, GE) outfitted with a digital camera (CoolSNAP HQ; Photometrics), while using a 1.4 numerical aperture, and 60× objective lens. 10-15 images were taken from different locations on the cover-slip to create a representative population. The resulting collected images were deconvolved after their acquisition with the SoftWoRx deconvolution software (Applied Precision). The deconvolved images were then analyzed on Bitplane: Imaris software version 7.6.4, where the spots algorithm was built around the mCherry S15 signal (see Figure 4) and the maximum fluorescence intensity of FITC found within these spots was then analyzed. All acquired images were subjected to the same spots signal algorithm via the Batch Coordinator tool (Bitplane) to each respective signal. Images with statistical outlying levels of signal were subjected to individual analysis and excluded if deemed non-representative.

Figure 4. Representative image of spot algorithm built around the S15 construct. The S15 mCherry construct (red) while the gray indicates where a spot is made around the S15 signal. The left image shows a S15 mCherry channel alone. The right image shows a merge of the S15 mCherry channel with the S15 Spots algorithm.

Induction of Autophagic Mechanisms Increases Specific Extracellular Vesicle Glycans

Cell Culture.

The human monocyte cell-line THP-1 was acquired from the ATCC. Cells were cultured in an incubator at 37°C and 5% CO2 in RPMI 1640 media, supplemented with the addition of exosome depleted 10% FBS (Hyclone), 10 μg/ml ciprofloxacin hydrochloride, 100 IU/ml penicillin, and 100 μg/ml streptomycin.

Expression of S15 mCherry Membrane Protein.

THP-1 cells were transfected to stably over-express S15-mCherry using pLVX containing our S15-mCherry construct and driven by a CMV promoter. Lentivirus was generated for transduction in HEK 293T cells transfected using the transfection reagent PEI with equal parts concentration of VSV-g, ΔNRF or psPax2, and pLVX-CMV-S15-mCherry plasmid. 48 hours post-transfection lenti-viral particles were collected and purified from the 293T cells cultured media using a 0.45um Millipore syringe. The purified lenti-viral particles were then used to treat THP-1 cells. The treated THP-1 cells were then selected for those positive for the resulting S15 mCherry by supplementing the previously mention RPMI with 5 μg/ml puromycin (Hyclone).

Differentiation.

THP-1 cells were differentiated into monocyte derived macrophages by treatment with 100 ng PMA (Sigma) for 24 hours.

Autophagy-Inducing Drug Treatment.

After 24 hours of PMA treatment media was changed with fresh, EV depleted, RPMI with 100 μM rapamycin (ApexBio), a drug which inhibits the function of the

autophagy repressor mammalian target of rapamycin (mTOR), leading to increased autophagy function [111-113]. After an additional 24 hours of incubation at 37°C and 5% CO^2 media was collected and centrifuged to isolate the CSF.

Immunofluorescent Staining and Preparation for THP-1 mCherry Extracellular Vesicles.

For the EV visualization, 2mL of CSF was spinoculated at 13°C for 2 hours at 1200 x g onto glass coverslips and subsequently fixed with a solution of 0.1 M PIPES with 3.7% formaldehyde (Polysciences) for 30 minutes. Immediately after fixation, coverslips were treated with 0.01% Saponin (Sigma-Aldrich) in a PBS block solution supplemented with 10% NDS, and 0.01% NaN_3 for 5 min. The cover slips were then individually subjected to 5 μg/ml of various biotinylated lectins (Vector Laboratories) and incubated at 37°C for 1 hour. The coverslips were then treated with streptavidin conjugated to a FITC fluorophore (BD Pharmingen) diluted in PBS, at a concentration of 1:400, for 1 hour at RT. Finally, the coverslips were mounted on slides and allowed to dry for 24 hours.

Microscopic Observation and Analysis of mCherry Extracellular Vesicles at the Single Vesicle Level.

EV images were taken on a DeltaVision wide field fluorescent microscope (Applied Precision, GE) outfitted with a digital camera (CoolSNAP HQ; Photometrics), while using a 1.4 numerical aperture, and 60× objective lens. 10-15 images were taken from different locations on the cover-slip to create a representative population. The resulting collected images were deconvolved after their acquisition with the SoftWoRx deconvolution software (Applied Precision). The deconvolved images were then analyzed on Bitplane: Imaris software version 7.6.4, where the spots algorithm was built around the mCherry

S15 signal (see Figure 5) and the maximum fluorescence intensity of FITC found within these spots was then analyzed. All acquired images were subjected to the same spots signal algorithm via the Batch Coordinator tool (Bitplane) to each respective signal. Images with statistical outlying levels of signal were subjected to individual analysis and thrown out if deemed non-representative.
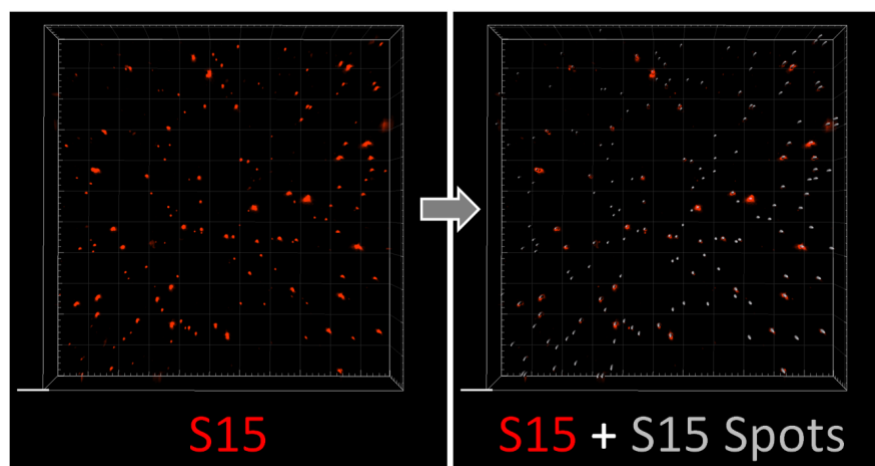
Impairment of Autopaghic Degradation Decreases Specific Extracellular Vesical Glycans

Cell Culture.

The human monocyte cell-line THP-1 was acquired from the ATCC. Cells were cultured in an incubator at 37°C and 5% CO^2 in RPMI 1640 media, supplemented with the addition of EV depleted 10% FBS (Hyclone), 10 μg/ml ciprofloxacin hydrochloride, 100 IU/ml penicillin, and 100 μg/ml streptomycin.

Expression of S15 mCherry Membrane Protein.

THP-1 cells were transfected to stably over-express S15-mCherry using pLVX containing our S15-mCherry construct and driven by a CMV promoter. Lentivirus was generated for transduction in HEK 293T cells transfected using the transfection reagent PEI with equal parts concentration of VSV-g, ΔNRF or psPax2, and pLVX-CMV-S15-mCherry plasmid. 48 hours post-transfection lenti-viral particles were collected and purified from the 293T cells cultured media using a 0.45μm Millipore syringe. The purified lenti-viral particles were then used to treat THP-1 cells. The treated THP-1 cells were then selected for those positive for the resulting S15 mCherry by supplementing the previously mention RPMI with 5 μg/ml puromycin (Hyclone).

Differentiation.

THP-1 cells were differentiated into monocyte derived macrophages by treatment with 100 nM PMA (Sigma) for 24 hours.

Lysosome-impairing Drug Treatment.

After 24 hours of PMA treatment media was changed with fresh, EV depleted, RPMI with 100 nM bafilomycin-A1 (Cayman Chemical Company), a drug which inhibits lysosomal degradation by preventing lysosomal acidification via impairment of the previously mentioned lysosome ATP proton pump, ATPase [114, 115]. After 24 hours incubation at 37°C and 5% CO_2 media was collected and centrifuged to isolate the CSF.

Immunofluorescent Staining and Preparation for THP-1 mCherry Extracellular Vesicles.

For the EV visualization, 2mL of cultured medium was spinoculated at 13°C for 2 hours at 1200 x g onto glass coverslips and subsequently fixed with a solution of 0.1 M PIPES with 3.7% formaldehyde (Polysciences) for 30 minutes. Immediately after fixation, vesicles were treated with 0.01% Saponin (Sigma-Aldrich) in a PBS block solution supplemented with 10% NDS, and 0.01% NaN_3 for 5 min. The cover slips were then individually subjected to 5 μg/ml of various biotinylated lectins (Vector Laboratories) and incubated at 37°C for 1 hour. The coverslips were then treated with streptavidin conjugated to a FITC fluorophore (BD Pharmingen) diluted in PBS, at a concentration of 1:400, for 1 hour at RT. Finally, the coverslips were mounted on slides and allowed to dry for 24 hours.

Microscopic Observation and Analysis of Extracellular Vesicles at the Single Vesicle Level.

EV images were taken on a DeltaVision wide field fluorescent microscope (Applied Precision, GE) outfitted with a digital camera (CoolSNAP HQ; Photometrics), while using a 1.4 numerical aperture, and 60× objective lens. 10-15 images were taken from different locations on the cover-slip to create a representative population. The resulting collected images were deconvolved after their acquisition with the SoftWoRx deconvolution software (Applied Precision). The deconvolved images were then analyzed on Bitplane: Imaris software version 7.6.4, where the spots algorithm was built around the mCherry S15 signal (see Figure 5) and the maximum fluorescence intensity of FITC found within these spots was then analyzed. All acquired images were subjected to the same spots signal algorithm via the Batch Coordinator tool (Bitplane) to each respective signal. Images with statistical outlying levels of signal were subjected to individual analysis and thrown out if deemed non-representative.

Trends in Extracellular Vesicle Glycan Changes Are Similar in α-synuclein EVs Cell Culture.

The SH-SY5Y human neuroblastoma cell-line was acquired from the ATCC. Cells were cultured in an incubator at 37C and 5% CO^2 in Dulbecco's modified Eagle's Medium (DMEM) containing phenol red (Invitrogen), supplemented with the addition of 10% FBS (Hyclone), 10 μ/ml ciprofloxacin hydrochloride, 100 IU/ml penicillin, and 100 μg/ml streptomycin.

Expression of α-Synuclein Dual Split Protein Construct.

SH-SY5Y cells were dually transduced by Kevin Burbidge M.S. to stably over-express both complements of our α-synuclein dual split protein construct (DSP) using two individual pLVXs containing either α-synuclein-DSP-A or α-synuclein-DSP-B, each driven by a CMV promoter, that combine to create green fluorescent protein (GFP). Lentivirus was generated for transduction in HEK 293T cells transfected using the transfection reagent PEI with equal parts concentration of VSV-g, ΔNRF or psPax2, and pLVX-CMV-Alpha-synuclein-DSP-A or pLVX-CMV-Alpha-synuclein-DSP-B plasmid. 24 hours post-transfection lenti-viral particles were collected and purified from the 293T cells cultured media using a 0.45µm millipore syringe. The purified lenti-viral particles were then used to treat SH-SY5Y cells and spinoculated at 13°C for 2 hours at 1200 x g. The treated cells were then selected for those positive for the resulting DSP constructs by supplementing the previously mention DMEM with 5 ug/ml puromycin. Cells were then sorted for green GFP intensity by flow cytometry for further selection, to ensure the cells were dually expressing both complements of the α-synuclein DSP.

Autophagy-altering Drug Treatments.

α-synuclein DSP expressing cells were grown in 15cm plates to approximately 80% confluence at which point plates were individually incubated for 24 hours in fresh RPMI and 100 µM rapamycin (ApexBio), 100nM bafilomycin-A1, or 0.1% DMSO.

Immunofluorescent staining and Preparation of SH-SY5Y Extracellular Vesicles.

For the EV visualization, 1mL of CFS was spinoculated at 13°C for 2 hours at 1200 x g onto glass coverslips and subsequently fixed with a solution of 0.1 M PIPES with 3.7%

formaldehyde (Polysciences) for 30 minutes. Immediately after fixation, vesicles were treated with 0.01% Saponin (Sigma-Aldrich) in a PBS block solution supplemented with 10% NDS, and 0.01% NaN_3 for 5 min. The cover slips were then individually subjected to 5 μg/ml of various biotinylated lectins (Vector Laboratories) and incubated at 37°C for 1 hour. The coverslips were then treated with streptavidin conjugated to a Cy3 fluorophore (BioLegend) diluted in PBS, at a concentration of 1:400, for 1 hour at RT. Finally, the coverslips were mounted on slides and allowed to dry for 24 hours.

Microscopic Observation and Analysis of α-synuclein Extracellular Vesicles at the Single Vesicle Level.

Extracellular vesicle images were taken on a DeltaVision wide field fluorescent microscope (Applied Precision, GE) outfitted with a digital camera (CoolSNAP HQ; Photometrics), while using a 1.4 numerical aperture, and 60× objective lens. 10-15 images were taken from different locations on the cover-slip to create a representative population. The resulting collected images were deconvolved after their acquisition with the SoftWoRx deconvolution software (Applied Precision). The deconvolved images were then analyzed on Bitplane: Imaris software version 7.6.4, where the spots algorithm was built around either the lectin signal or α-synuclein signal and the maximum fluorescence intensity found within these spots was then analyzed. All acquired images were subjected to the same spots signal algorithm via the Batch Coordinator tool (Bitplane) to each respective signal. Images with statistical outlying levels of signal were subjected to individual analysis and discarded if deemed non-representative.

Statistical Analysis

All graphs were generated and statistical analysis performed using GraphPad Prism version 7 (GraphPad Software, Inc.). All data shown was analyzed using two-way analysis of variance (ANOVA) with Sidak's multiple comparisons test and depicts the standard error of the mean unless otherwise specified.

CHAPTER FOUR

RESULTS

The Novel Description of Extracellular Vesicle Glycans

Due to the presence of membrane glycans [58] and the known roles of glycans in biological processes [5, 16, 52, 53, 56], we predicted that EVs would have a various membrane anchored and cargo glycans. In order to identify the presence of these glycans a lectin binding assay (LBA) was applied to EVs collected from the human cell line THP-1 monocyte derived macrophages (MDMs), which stably expressed an S15 mCherry membrane marker. Differing lectins bind to various glycan epitopes, allowing them to be used to indicate the presence of various glycans [116, 117]. Descriptions of lectin abbreviations and their suspected glycan specificities can be found in Table 2 (Vector Labs) in Appendix A. mCherry positive EVs were observed at the individual vesicle level via microscopy. A representative microscopic image of lectin binding is shown in Figure 5.

If EVs have various membrane anchored and cargo glycans, we expected to observe differential binding between the various lectins, indicated by differences in the intensity of the conjugated fluorophore. Indeed, we observed a range in the mean lectin intensities of THP-1 mCherry EVs (Figure 6). Not only did we observe high lectin intensities for some lectins and low intensities for others, but differences between the lectin intensities of mCherry EVs labeled with the same lectin (Figure 6).

29

LEL, RCA1, and WGA bound to mCherry EVs with a wide range of intensity but bound to nearly all EVs. Contrary to the lectins that bound nearly all mCherry EVs, the lectins DBA, GSL1, PNA, SJA, UEA1, and sWGA appeared to label very few mCherry EVs and, those that were labeled had similar intensities (Figure 6).
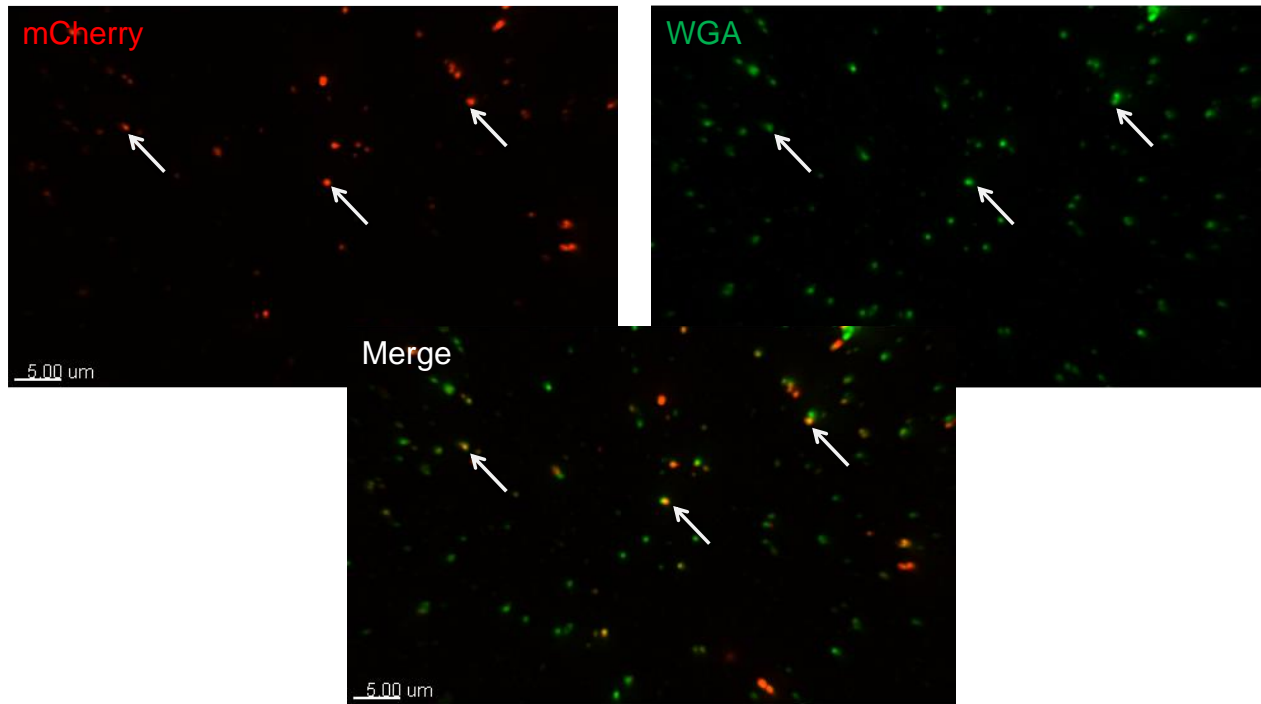
Figure 5. Representative Image of Lectin Staining and mCherry Marker. These microscopic images, taken at 60X magnification, demonstrate the membrane marker and lectin binding. The fluorescence of the mCherry membrane marker (top left), staining with the lectin WGA (top right), and a merge of the two channels (bottom) are depicted. Arrows indicate vesicles in which is mCherry and WGA colocalize.

To investigate these differences further we evaluated the percentage of mCherry EVs that were spositive for lectin binding (Figure 7). All mCherry negative EVs were excluded from our evaluation in order to avoid analysis of bovine derived EVs contained in the EV depleted FBS. Additionally, an LBA on fresh EV depleted media was performed as a negative control and no lectin binding was observed. There were some lectins, ConA,

WGA, RCA1 and LEL, which bound to nearly 100% of EVs. While there were other lectins,

DBA, sWGA, GLS1, PNA, SJA and UEA1, which bound to less than 5% of EVs (Figure

7).

THP-1 mCherry Extracellular Vesicles
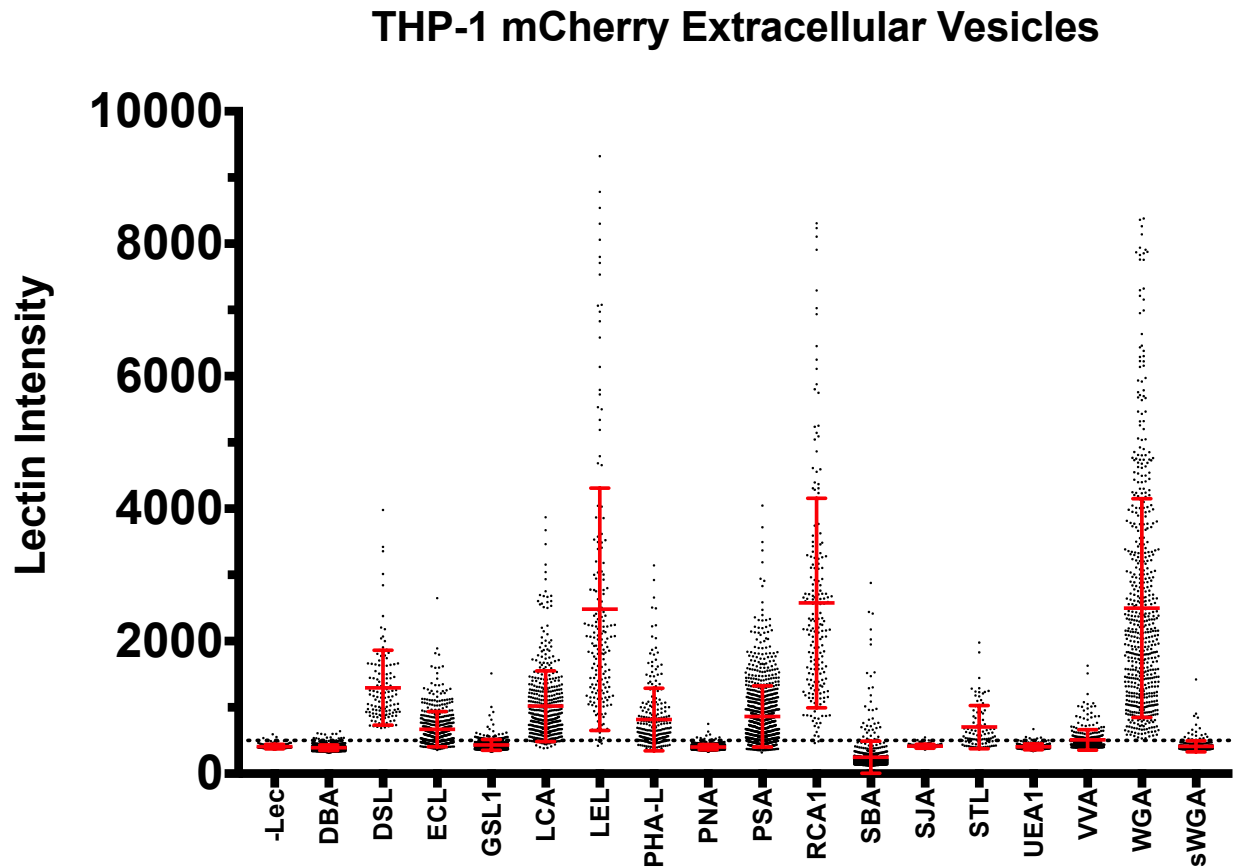
Figure 6. Lectin Binding of THP-1 mCherry Positive Extracellular Vesicles. Results from an LBA performed on THP-1 EVs. Each black dot represents an individually analyzed EV. The y-axis represents the intensity of the lectins listed on the x-axis. Error bars represent mean and standard deviation. The dotted horizontal line represents a negative lectin signal based up the background intensity of the negative control, which was EVs that had not been incubated with a lectin (-Lec). This graph is a individual representation of multiple experiments.
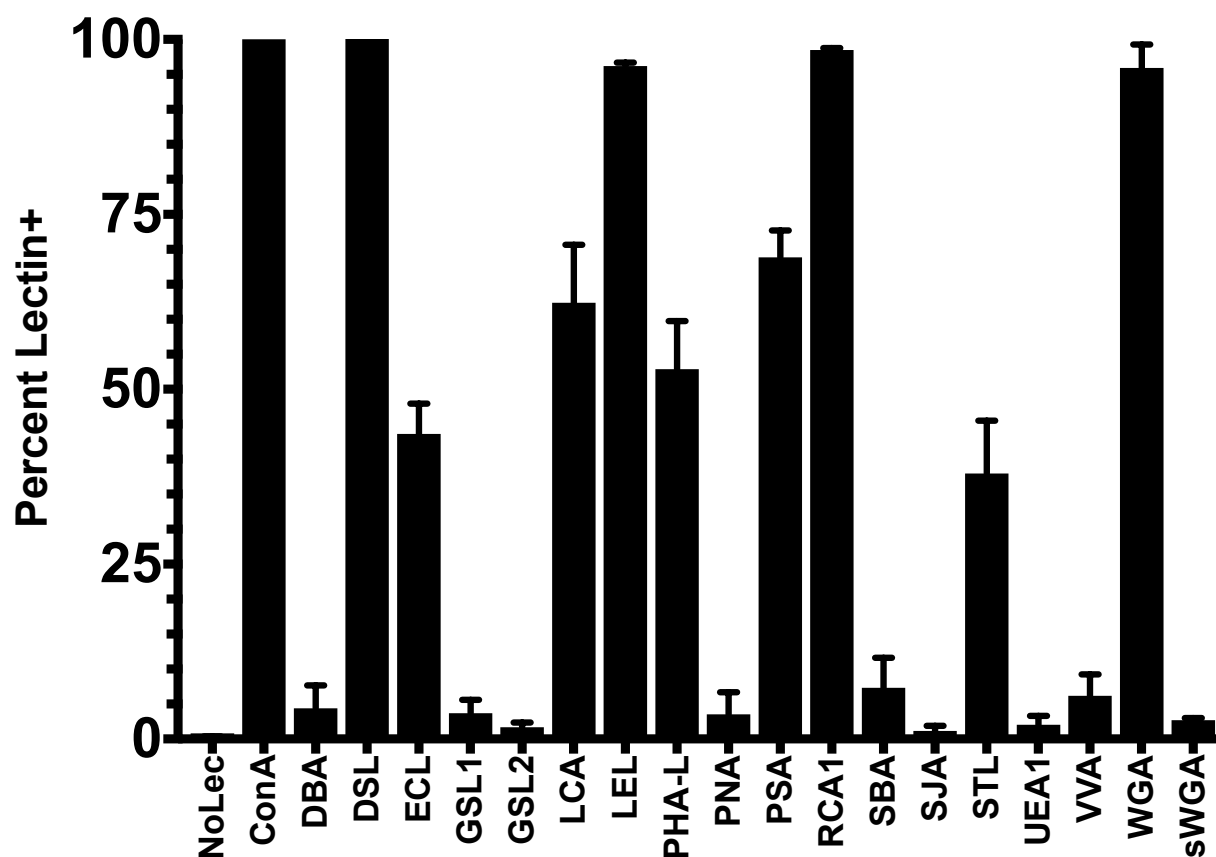
Figure 7. The Percentage of Lectin Positive THP-1 mCherry EVs. Lectin positive EVs were quantified by dividing the number of lectin positive mCherry vesicles by the overall number of mCherry vesicles found in the images from that condition, then multiplying that result by 100 to obtain a percentage of mCherry vesicles that were dual positive for the lectin and the mCherry S15 marker. Data shown depicts the means and SEM of multiple experiments.

Induction of Autophagic Mechanisms Increases Specific Extracellular Vesicles Glycans

Due to the aforementioned impact of autophagic mechanisms on glycan degradation, it is reasonable to predict that EV glycans, which are subjected to degradative processes, are altered through the autophagic process. Because of this we anticipated that EV glycans would change when autophagy is induced. When LBAs were

conducted on EVs secreted by THP-1 cells treated with the autophagy inducing drug rapamycin, we observed a general trend of increases in the percentage of glycan positive EVs (Figure 8). The lectins LCA, PNA, STL, and VVA exhibited significant changes of their EV presence upon induction of autophagy when compared to the EVs from untreated cell and there was notable increase in the presence of ECL, GSL1, SJA, and UEA1 as well (Figure 8).

Since autophagic mechanism can lead to the secretion of EVs [98] we predicted that induction of autophagy would lead to an increase in EV secretion. To investigate we quantified basal EV secretion and compared it to EV secretion when autophagy was induced using rapamycin (Figure 9). We observed a three to four-fold increase in EV secretion when autophagy was induced (Figure 9).

THP-1 mCherry Extracellular Vesicles
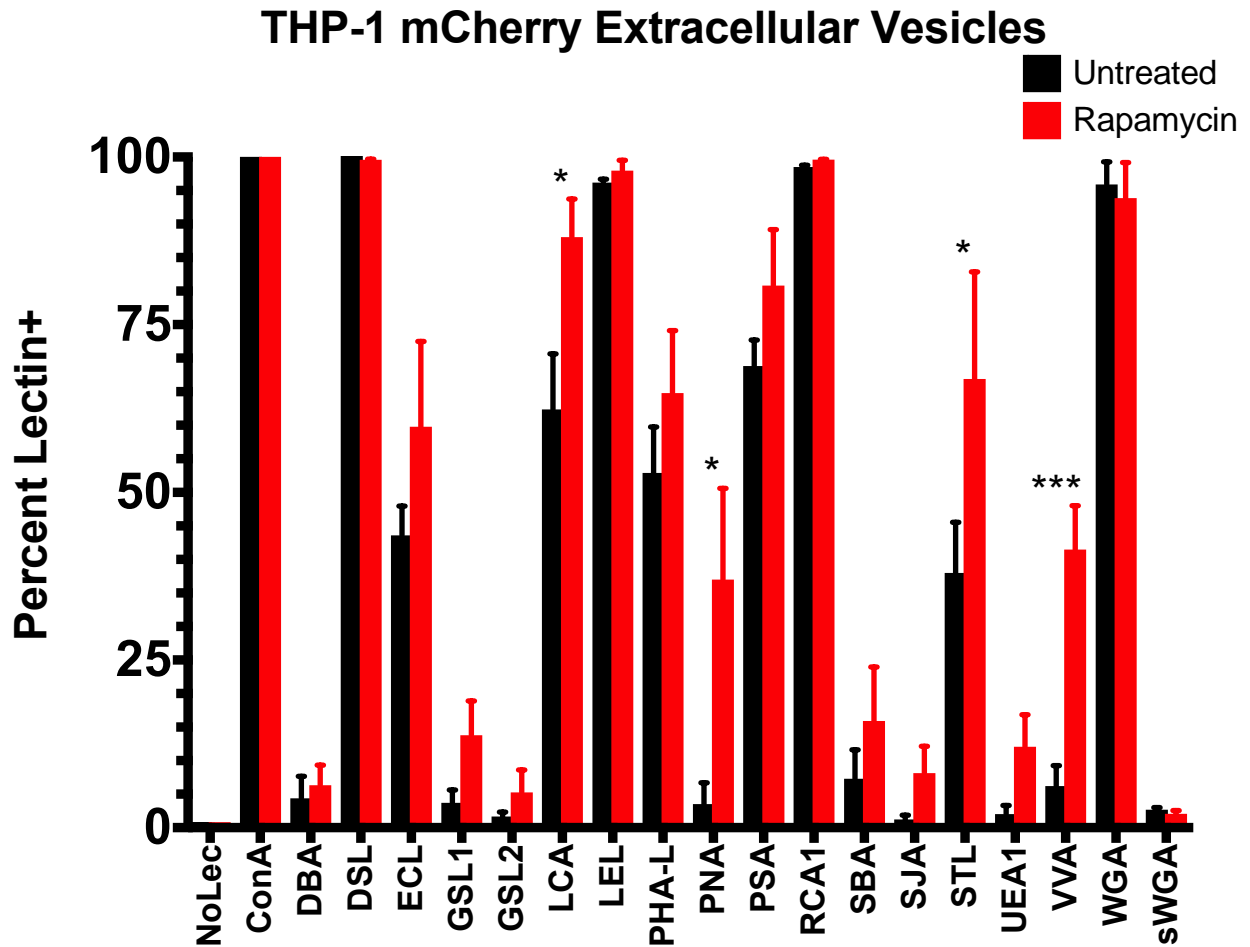
Figure 8. Changes in Lectin Positive Vesicles Upon Induction of Autophagy.
Comparison of LBAs performed on EVs collected from untreated and rapamycin treated
THP-1 cells. As in previous LBAs Lectin positive EVs were quantified by dividing the
number of lectin positive vesicles by the overall number of S15 positive vesicles found
in the images from that condition, then multiplying that result by 100 to obtain a
percentage of S15 vesicles that were dual positive for the lectin and the S15 marker.
Data shown depicts the means and SEM of multiple experiments.

THP-1 mCherry Extracellular Vesicles
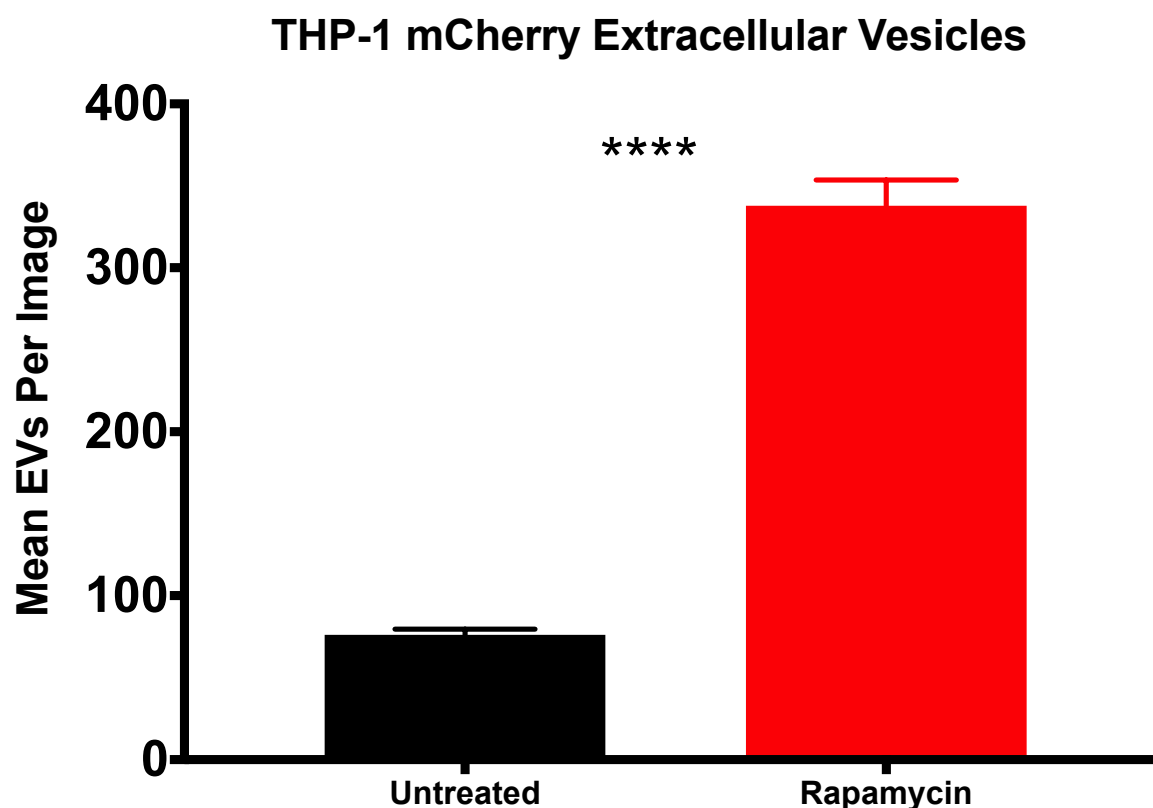
Figure 9. Comparison of Basal Extracellular Vesicle Secretion to Secretion Upon Induction of Autophagy. Comparison of basal EV secretion to EV secretion upon induction of autophagy via rapamycin. Graph represents the mean vesicles per image and SEM of multiple experiments. Statistics were analyzed using a two-tailed t-test with Welch's correction.

Impairment of Lysosomal Degradation Decreases Specific Extracellular Vesicle Glycans

We have shown that EV glycans increase when autophagy is induced. Knowing this, we wanted to investigate the impact that impairing lysosomal function would have on EV glycans. Since glycan degradation occurs in the lysosome [2] we predicted that disruption of lysosomal function would reduce EV glycans by either reducing glycan degradation or impairing glycan localization to EVs. When LBAs were conducted on EVs secreted by THP-1 cells treated with the lysosomal impairing drug bafilomycin-A1, we observed a general trend of decreases in the percentage of glycan positive EVs (Figure

10). The lectins LEL, PHA-L, and PSA demonstrated the most significant changes of their

EV presence when compared to the untreated EVs.

THP-1 mCherry Extracellular Vesicles
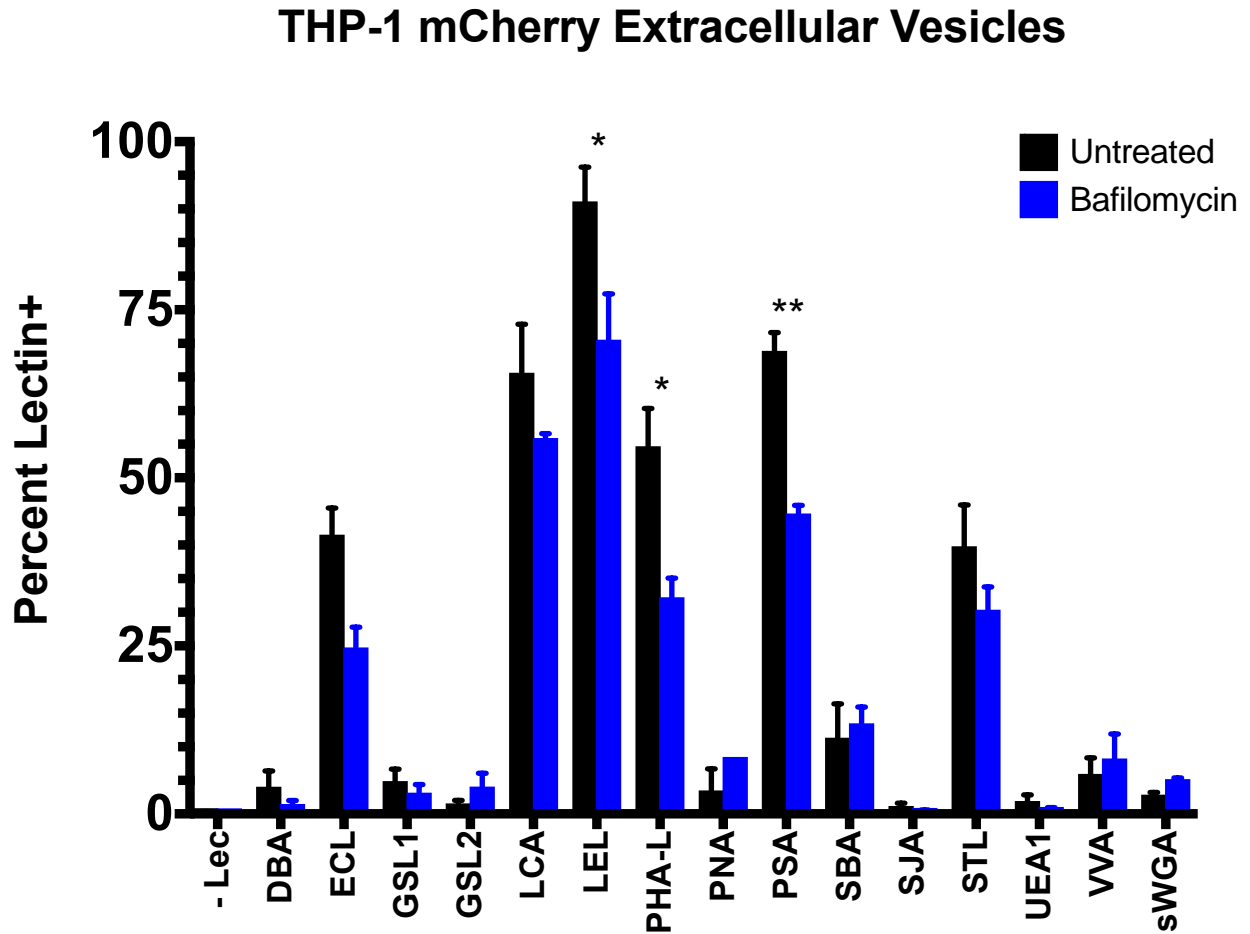
Figure 10. Changes of Extracellular Vesicle Glycans Upon Impairment of Lysosomal Degradation. Comparison of LBAs performed on EVs collected from untreated and bafilomycin-A1 treated THP-1 cells. As in previous LBAs Lectin positive EVs were quantified by dividing the number of lectin positive vesicles by the overall number of S15 positive vesicles found in the images from that condition, then multiplying that result by 100 to obtain a percentage of S15 vesicles that were dual positive for the lectin and the S15 marker. Data shown depicts the means and SEM of multiple experiments.

Since autophagic secretion mechanisms are intertwined with lysosomal function

[98] we predicted that EV secretion would increase upon disruption of lysosomal

degradation. We quantified EV secretion upon disruption of lysosomal function. We found

a three to four-fold increase in EV secretion upon treatment with bafilomycin-A1.

THP-1 mCherry Extracellular Vesicles
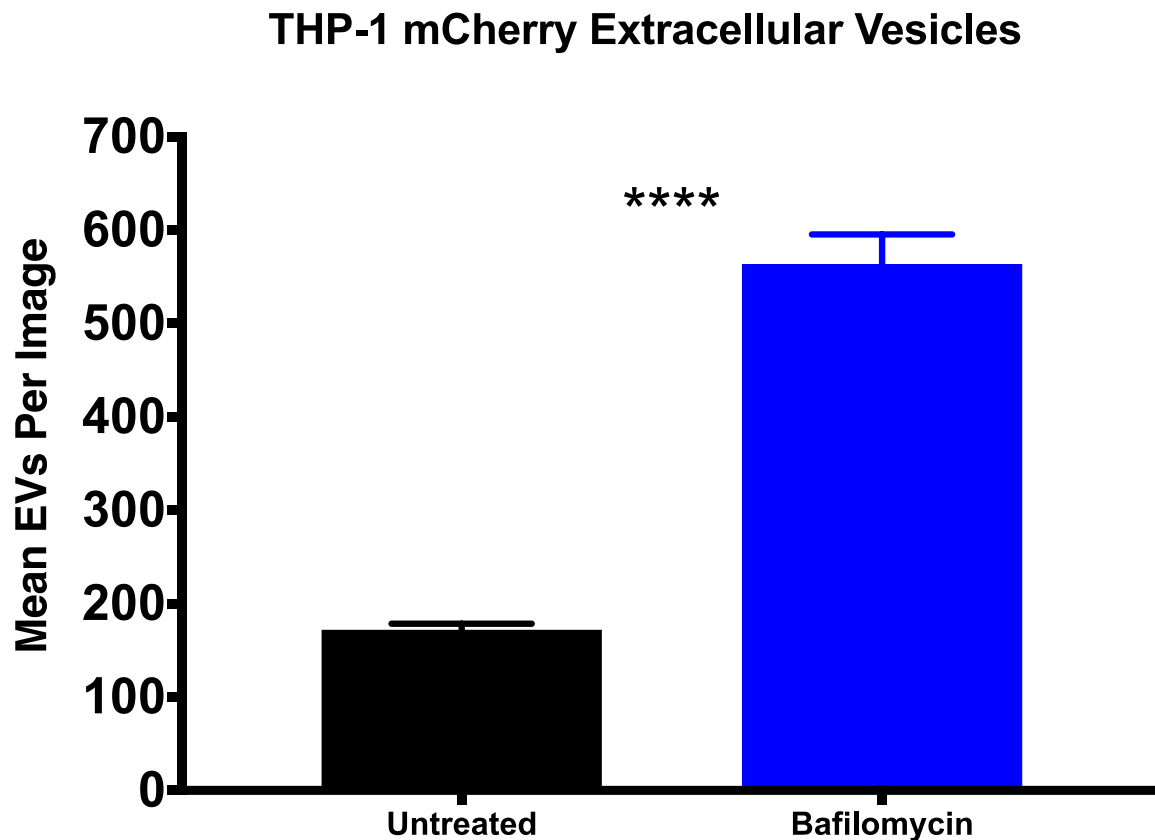
Figure 11. Changes in EV Secretion Upon Impairment of Lysosomal Degradation.
Comparison of basal EV secretion to EV secretion upon impairment of lysosomal
function via bafilomycin-A1. Graph represents the mean vesicles per image and SEM
of multiple experiments. Statistics were analyzed using a two-tailed t-test with Welch's
correction.

Trends in Extracellular Vesicle Glycans Changes Are Similar in Differing Cell Types

Knowing that EV glycans increase when autophagy is induced and decrease when

lysosomal function is impaired in THP-1 cells, we wanted to investigate these

mechanisms in an LSD model. We used the human neuroblastoma cell line SH-SY5Y

which was transfected to express a DSP α-synuclein. With this model we were able to

identify and characterize individual EVs containing α-synuclein. We quantified the basal percentage of glycan positive α-synuclein EVs (Figure 12&13) and the basal secretion of α-synuclein EVs (Figure 14&15) upon perturbation of autophagy using rapamycin and bafilomycin-A1. We found similar trends in increases of glycan positive α-synuclein EVs upon the induction of autophagy (Figure 12) as well as the three to four-fold increase of α-synuclein EV secretion (Figure 14) as previously observed. Likewise, impairment of lysosomal function resulted in a general reduction in glycan positive α-synuclein EVs (Figure 13) and a three to four-fold increase in α-synuclein EV secretion (Figure 15). Specifically, induction of autophagy lead to a significant increase of PHA-L and STL and a notable increase of ECL binding to EVs (Figure 12) while impairment of lysosomal function caused significant decreases in LCA, LAL, PHA-L, PSA, and STL positive α-synuclein EVs (Figure 13). Interestingly, the lectin ECL did not follow the trend of decreased presence upon impairment of lysosomal function but, instead, increased to a similar level to that of α-synuclein EVs from rapamycin treated cells (Figure 13).

While comparing basal α-synuclein EV secretion to the secretion of α-synuclein EVs upon impairment of lysosomal function it was discovered that the quantity of WGA EVs, which is typically used as a pan-EV marker, did not follow the trend of increasing three to four-fold (Figure 15). This lead us to investigate changes in the number of lectin positive EVs from both rapamycin and bafilomycin treated SH-SY5Y cells (Figure 16&17). We observed expected increases in the quantity of lectin positive vesicles from rapamycin treated cells except for STL, which was equivalent to the EVs from DMSO treated cells (Figure 16). Additionally, we observed slight increases in the quantity of lectin positive

vesicles for most lectins except for PHA-L, STL, and WGA, which had a decrease in the

amount of lectin positive EVs when treated with bafilomycin (Figure 17).
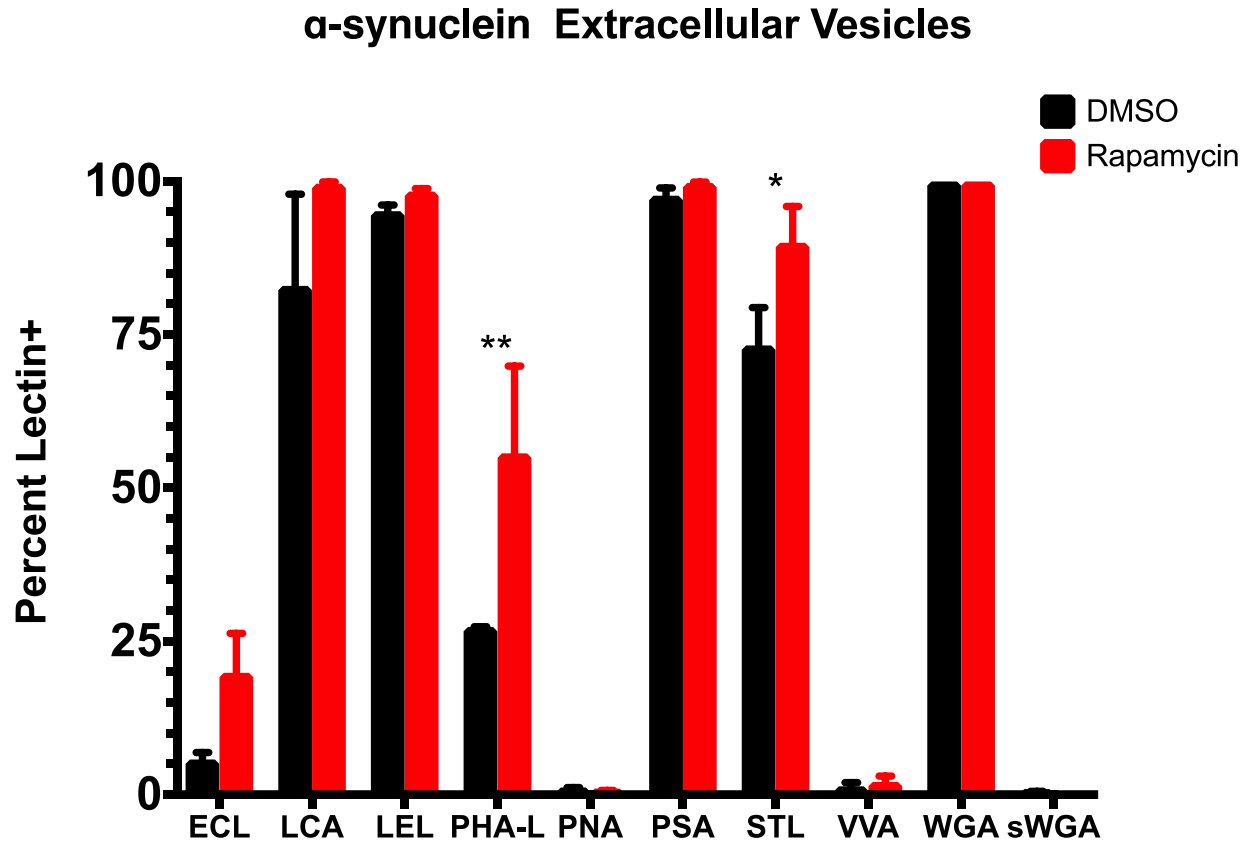
Figure 12. Glycan Changes of α-synuclein EVs Upon Induction of Autophagy. Comparison of LBAs performed on EVs collected from DMSO and rapamycin treated SH-SY5Y cells. As in previous LBAs Lectin positive EVs were quantified by dividing the number of lectin positive vesicles by the overall number of α-synuclein positive vesicles found in the images from that condition, then multiplying that result by 100 to obtain a percentage of α-synuclein vesicles that were dual positive for the lectin and the α-synuclein marker. Data shown depicts the means and SEM of multiple experiments.

α-synuclein Extracellular Vesicles
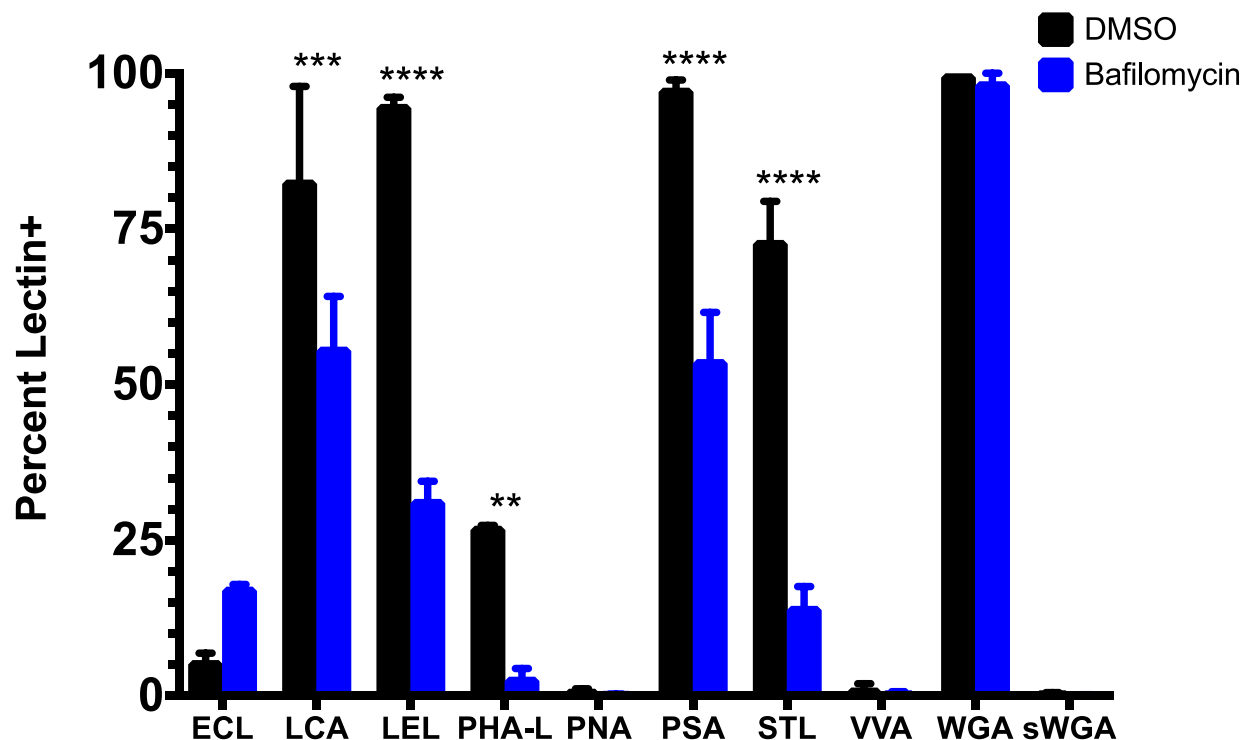
Figure 13. Glycan Changes of α-synuclein EVs Upon Disruption of Degradative Autophagy. Comparison of LBAs performed on EVs collected from DMSO and bafilomycin-A1 treated SH-SY5Y cells. As in previous LBAs Lectin positive EVs were quantified by dividing the number of lectin positive vesicles by the overall number of α-synuclein positive vesicles found in the images from that condition, then multiplying that result by 100 to obtain a percentage of α-synuclein vesicles that were dual positive for the lectin and the α-synuclein marker. Data shown depicts the means and SEM of multiple experiments.
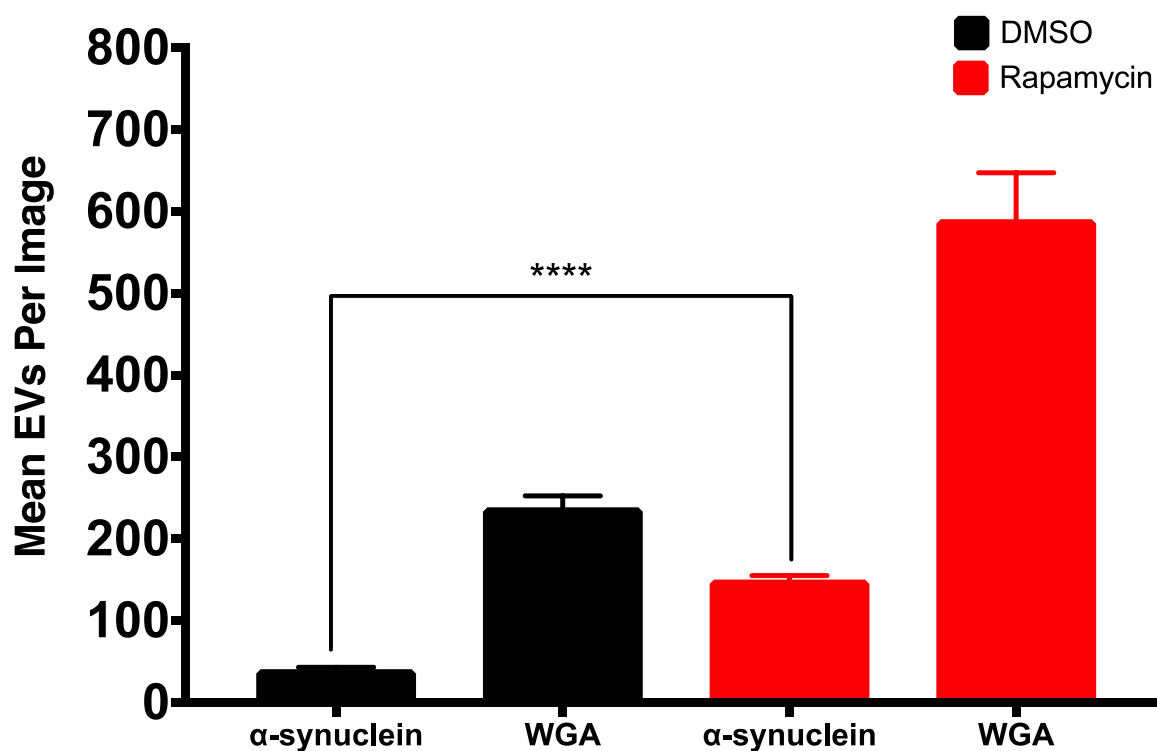
Figure 14. Changes in α-synuclein EV secretion Upon Induction of Autophagy.
Comparison of basal α-synuclein EV secretion to α-synuclein EV secretion upon induction of autophagy via rapamycin. α-synuclein EV secretion was compared to the secretion of WGA positive EVs in order to demonstrate that the increase of α-synuclein EVs is due to a general increase in autophagic secretion. Graph represents the mean vesicles per image and SEM of multiple experiments. Statistics were analyzed using a two-tailed t-test with Welch's correction.

SH-SY5Y Extracellular Vesicles
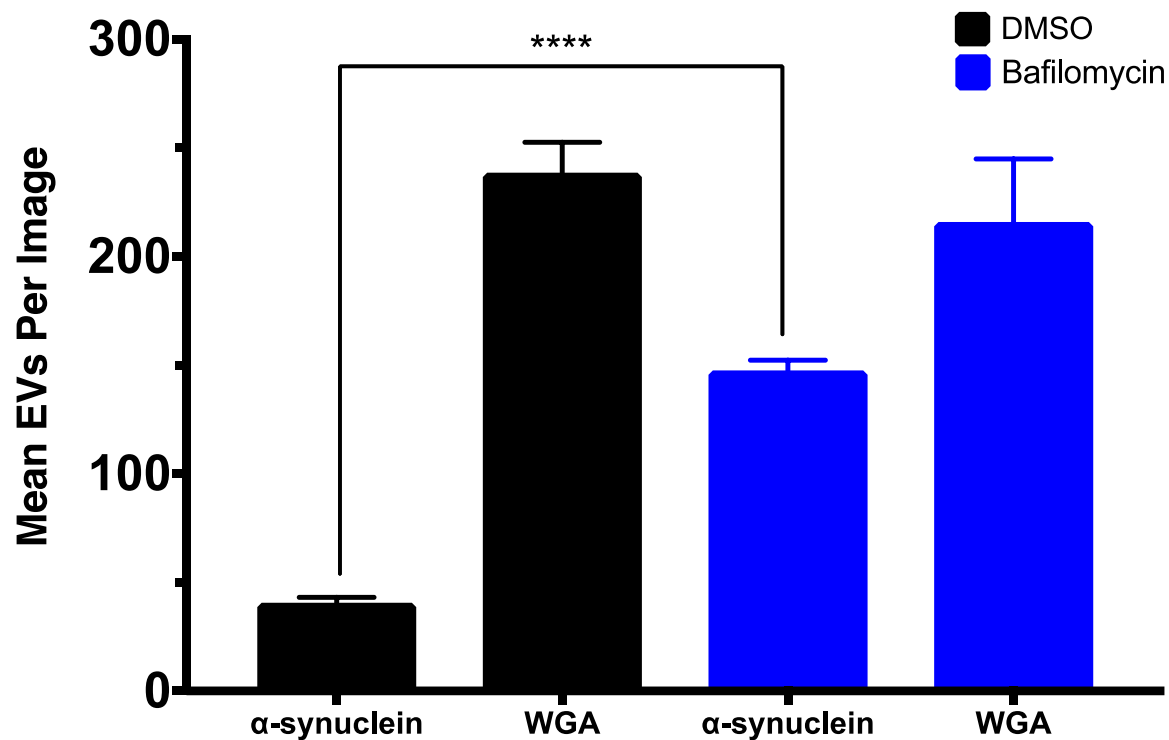
Figure 15. Changes in α-synuclein EV secretion Upon Disruption of Degradative of Autophagy. Comparison of basal α-synuclein EV secretion to α-synuclein EV secretion upon impairment of lysosomal function via bafilomycin-A1. α-synuclein EV secretion was compared to the secretion of WGA positive EVs in order to demonstrate that the increase of α-synuclein EVs is due to a general increase in autophagic secretion (discussed further in the results and discussion sections). Graph represents the mean vesicles per image and SEM of multiple experiments. Statistics were analyzed using a two-tailed t-test with Welch's correction.
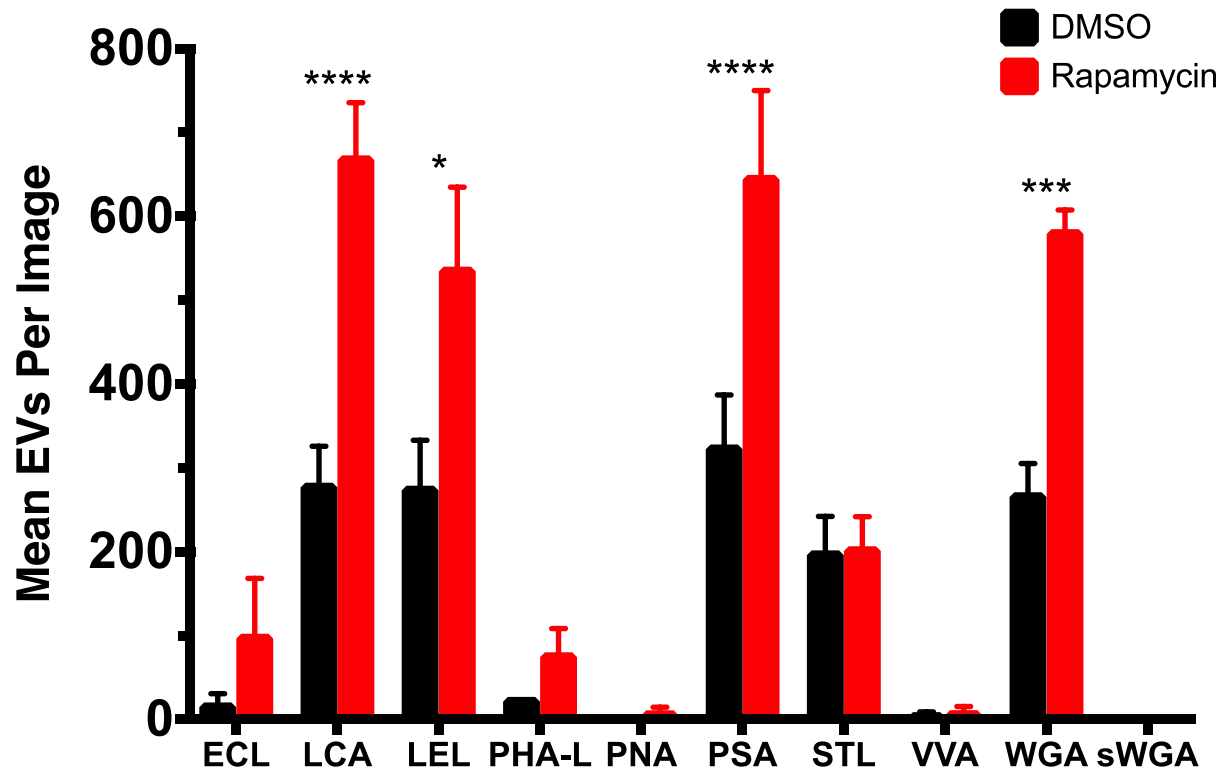
Figure 16. Changes in Lectin Positive EV Secretion Upon Induction of Autophagy. Comparison of LBAs performed on EVs collected from DMSO and rapamycin treated SH-SY5Y cells. Graph represents the mean of lectin positive vesicles per image and SEM of multiple experiments.
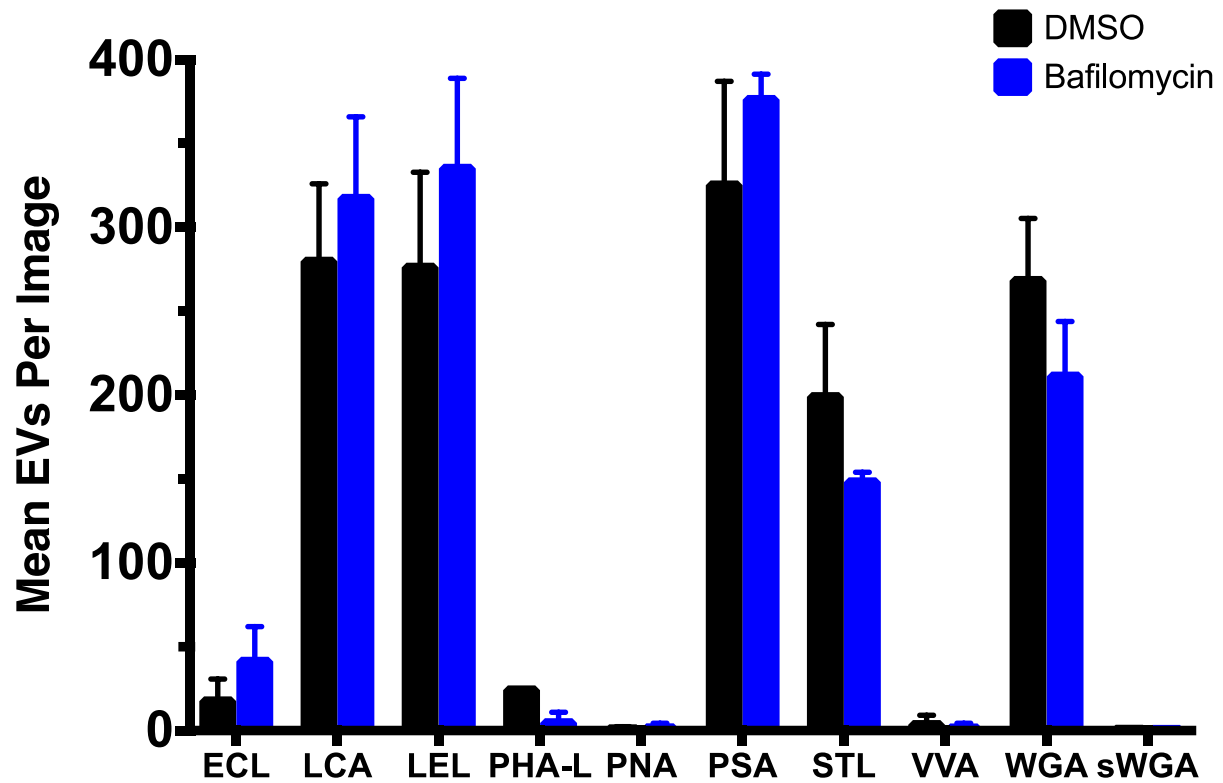
Figure 17. Changes in Lectin Positive EV secretion Upon Disruption of Degradative Autophagy. Comparison of LBAs performed on EVs collected from DMSO and bafilomycin-A1 treated SH-SY5Y cells. Graph represents the mean of lectin positive vesicles per image and SEM of multiple experiments.

CHAPTER FIVE

DISCUSSION

Evidence indicates that the cell-to-cell spread of neurodegenerative diseases, such as PD, are mediated by EVs [11, 101]. We have shown that alterations of autophagy cause similar glycobiological effects in different human cell lines We have identified changes in MDM EV glycans upon perturbation of autophagy that indicate mechanistic connections between degradative and secretory autophagy, EV biogenesis and secretion, and glycan synthesis and degradation. We propose that EV glycans serve as a signaling mechanism for EV cargo selection, EV secretion, and intercellular trafficking of EVs.

We found that some glycans are present on all EV populations while other glycans are present on some or no EV populations. When autophagy was induced we saw a general increase in the percentage of glycan positive EVs. This could be due to many different cellular mechanisms but we propose two different possibilities. First, that increasing autophagy increases the targeting and localization of certain glycans to EVs, potential as a signal for EV secretion. Second, that increased autophagy increases glycan processing, via exposure to degradative lysosomal enzymes, to produce a greater amount of glycan structures. The possible mechanistic role of EV glycans will discussed further below. We also observed an increase in vesicle secretion upon induction of autophagy supporting previous evidence that autophagic mechanisms lead to the

secretion of EVs [98]. When we disrupted lysosomal degradation, we saw a decrease in the percentage of glycan positive EVs. Similar to the autophagic induction results, we propose two differing possibilities. First, the decrease of EV glycans is a result of increased secretion of EVs that have not had the opportunity for glycans to be trafficked to the EVs ass an effect of the impairment of lysosomal degradation. Second, the decrease in EV glycans occurred because the EVs and their glycans were not exposed to the lysosomal degradative enzymes, resulting in EVs with unprocessed glycans.

Both proposals rely on a compensatory secretory mechanism that maintains cellular homeostasis when lysosomal degradation is impaired. The connections between the autophagic induction and impairment of lysosomal degradation proposals will be discussed later. We also observed an increase in EV secretion upon disruption of lysosomal degradation supporting the aforementioned secretory response to failed autophagic degradation, adding to evidence of the secretory autophagic pathway [95, 118], and indicating a link between LSDs and the cell-to-cell spread of pathogenic materials.

We have proposed two sets of potential explanations for the changes in EV glycans upon perturbation of autophagy. One explanation suggests that the trafficking of glycans to EVs increases upon induction of autophagy and decreases upon impairment of lysosomal function. The other explanation suggests that EV glycans increase or decrease due to the change of EV glycan exposure to degradative lysosomal enzymes. Again, both mechanism support evidence that secretory autophagy can maintain cellular homeostasis when degradative autophagy is impaired.

When experimenting on an LSD model, we found similar trends in changes of EV glycosylation however, the glycans that changed in each cell line were not identical. Table 1 shows the differences and commonalities of lectin binding changes between the examined THP-1 MDMs and SH-SY5Y neuroblastoma EVs. These similarities indicate a conserved mechanism of glycan processing while the differences may indicate cell type-specific mechanisms of glycan processing and EV cargo selection and trafficking.

When experimenting on neuroblastomas we found that the number of lectin positive vesicles generally increased upon induction of autophagy, except for STL, which remained constant. The homeostasis of the glycan that STL binds to may indicate a glycan that is pivotal to these mechanisms and is tightly regulated. Additionally, when we impaired lysosomal degradation in neuroblastomas we found that the amount of lectin positive vesicles increased slightly for most lectins. However, this was not due to a larger percentage of lectin positive EVs but was a result of overall increase in the autophagic secretion of EVs. Surprisingly though, there was a decrease in lectin positive EVs for some lectins. These lectins may indicate glycans that have mechanistic actions in lysosomal degradative mechanisms.

Given the known roles of glycans in protein folding, oligomerization, quality control, and trafficking [53], we predict that EV glycans also act in EV cargo selection and release. Additionally, we suspect that, among other glycans, the glycans which the lectins STL and PHA-L bind to are significant to autophagic secretion processes due to the recurrent and significant changes observed in those lectins' binding when autophagy was

perturbed. Significant changes in LCA, LEL, and PSA were also frequently observed in different cell lines (Table 1).

Overall, these findings lead us to insinuate a role of glycans in autophagic mechanisms, specifically EV cargo selection, secretion, and trafficking. Understanding autophagic mechanisms is crucial to the care of LSDs. Furthermore, we suspect that people with LSDs have differing glycans in their EV populations than unaffected persons. Given this we propose that EV glycan identification could be used as a biomarker of autophagic dysfunction. Similarly, if glycans are pivotal to autophagic secretion, the understanding of EV biogenesis, cargo selection, and trafficking, and the cell-to-cell spread of toxic materials can be greatly improved. All of which will also improve the understanding of cellular homeostasis and intracellular communication.

Lectin	THP-1 mCherry EVs	SH-SY5Y α-syn EVs
LCA	▲	▼
LEL	▼	▼
PHA-L	▼	▲ ▼
PNA	▲	
PSA	▼	▼
STL	▲	▲ ▼
VVA	▲	

Rapamycin
Bafilomycin

Table 1. Comparison of Changes in Lectin Binding Between Differing Cell Types.
Upon perturbation of autophagy differences and similarities in the lectin binding between cell types were observed.

Future Directions.

We have found that certain glycans are commonly altered by autophagic processes and we propose that those glycans influence EV cargo selection, targeting, and secretion. We have demonstrated that EV glycans can be evaluated at the individual EV level and that there are EV populations with differing glycans. Evidence indicates that autophagically derived EVs can be identified via autophagic markers such as LC3-II, SQSTM1, and FLOT1 [119]. We suspect that autophagically secreted EVs will exhibit glycans, which we refer to as a glycotype or glycome, specific to EVs produced via autophagic mechanisms. Given this information we suspect that there is an opportunity to identify glycans specific to EV populations based upon their cellular derivation. Additionally, we predict that EVs with glycans differing from the general EV population will have differing cargo as well as a colocalization with other glycans which we found to be commonly altered.

Future research also aims at the identification of the specific glycans that have been implicated by lectin binding. We are currently using spectrometric analysis to identify the EV glycans present upon each treatment. Furthermore, we will investigate mechanism by which glycans may have a functional role in EV cargo selection and trafficking. We plan to examine this via the introduction of varying glycosidases, with known enzymatic functions, to EVs prior to LBAs. We will then analyze the altered EVs for their content and ability for intercellular transfer.

Lastly, a comparison between the protein masses and volumes of EVs from the different treatments could further the understanding of EV cargo loading, EV biogenesis,

and secretory processes. We predict that EVs produced upon induction of autophagy will have a lower protein mass while EVs produced when lysosomal degradation is impaired will have a higher protein mass.

APPENDIX A
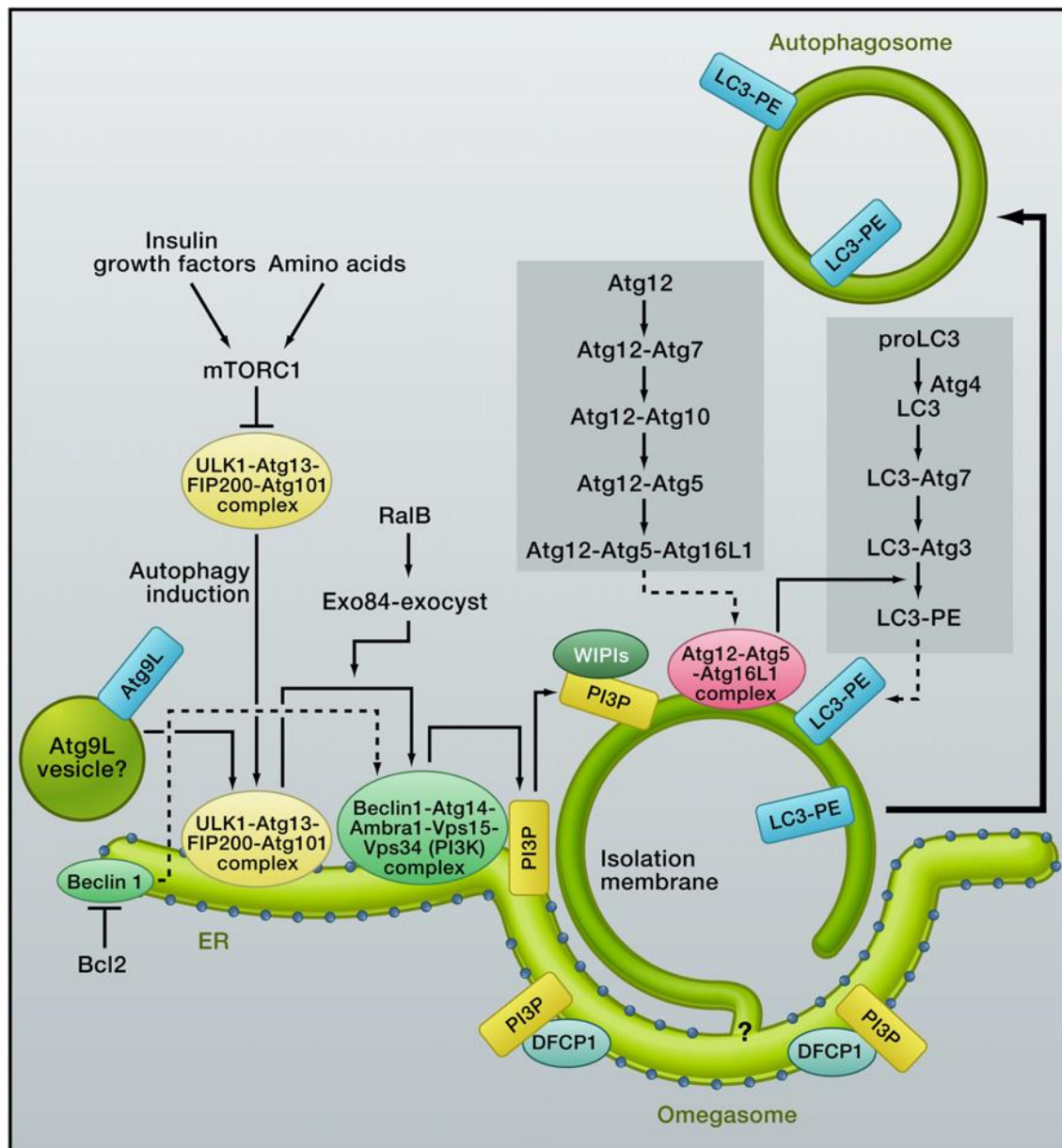
Figure 18.[70] Molecular Mechanisms of Autophagosome Formation. Depiction of the complex molecular mechanisms which lead to the formation of an autophagosome.

Lectin	Binding Specificity
ConA	α Man, α Glc
DBA	αGalNAc
DSL	(GlcNAc)$_{2-4}$
ECL	Galβ4GlcNAc
GSL-1	αGal
GSL-2	α or BGlcNAc
LCA	αMan, αGlc
LEL	(GlcNAc)$_{2-4}$
PHA-L	Galβ4GlcNAcβ6(GlcNAcB2Manα3)Manα3
PNA	Galβ3GalNAc
PSA	αMan, αGlc
RCA-1	Gal
SBA	αβGalNAc
SJA	βGalNAc
STL	(GlcNAc)$_{2-4}$
sWGA	GlcNAc
UEA-1	αFuc
VVA	GalNAc
WGA	GlcNAc

Table 2. Specificities. List of Lectins.

LIST OF REFERENCES

1. Neufeld, E.F., *Lysosomal storage diseases.* Annual review of biochemistry, 1991. **60**(1): p. 257-280.

2. Freeze, H.H., T. Kinoshita, and R.L. Schnaar, *Genetic Disorders of Glycan Degradation*, in *Essentials of Glycobiology*, A. Varki, et al., Editors. 2015: Cold Spring Harbor (NY).

3. Futerman, A.H. and G. Van Meer, *The cell biology of lysosomal storage disorders.* Nature reviews Molecular cell biology, 2004. **5**(7): p. 554.

4. De Duve, C., et al., *Tissue fractionation studies. 6. Intracellular distribution patterns of enzymes in rat-liver tissue.* Biochemical Journal, 1955. **60**(4): p. 604.

5. Varki, A. and P. Gagneux, *Biological Functions of Glycans*, in *Essentials of Glycobiology*, A. Varki, et al., Editors. 2015: Cold Spring Harbor (NY).

6. Kleene, R. and M. Schachner, *Glycans and neural cell interactions.* Nature Reviews Neuroscience, 2004. **5**(3): p. 195.

7. Alenquer, M. and M.J. Amorim, *Exosome Biogenesis, Regulation, and Function in Viral Infection.* Viruses, 2015. **7**(9): p. 5066-83.

8. Valadi, H., et al., *Exosome-mediated transfer of mRNAs and microRNAs is a novel mechanism of genetic exchange between cells.* Nature cell biology, 2007. **9**(6): p. 654.

9. Luga, V., et al., *Exosomes mediate stromal mobilization of autocrine Wnt-PCP signaling in breast cancer cell migration.* Cell, 2012. **151**(7): p. 1542-56.

10. Simons, M. and G. Raposo, *Exosomes–vesicular carriers for intercellular communication.* Current opinion in cell biology, 2009. **21**(4): p. 575-581.

11. Thompson, A.G., et al., *Extracellular vesicles in neurodegenerative disease - pathogenesis to biomarkers.* Nat Rev Neurol, 2016. **12**(6): p. 346-57.

12.   Danzer, K.M., et al., *Exosomal cell-to-cell transmission of alpha synuclein oligomers.* Molecular neurodegeneration, 2012. **7**(1): p. 42. 13. Vlassov, A.V., et al., *Exosomes: current knowledge of their composition, biological functions, and diagnostic and therapeutic potentials.* (0006-3002 (Print)).

14.   Raposo, G. and W. Stoorvogel, *Extracellular vesicles: exosomes, microvesicles, and friends.* J Cell Biol, 2013. **200**(4): p. 373-383.

15.   Seino, J., et al., *Basal Autophagy Is Required for the Efficient Catabolism of Sialyloligosaccharides.* J Biol Chem, 2013. **288**(37): p. 26898-907.

16.   Rini, J.M. and J.D. Esko, *Glycosyltransferases and glycan-processing enzymes.* 2017.

17.   Freeze, H.H., *Genetic disorders of glycan degradation.* 2009.

18.   Hickman, S. and E.F. Neufeld, *A hypothesis for I-cell disease: defective hydrolases that do not enter lysosomes.* Biochemical and biophysical research communications, 1972. **49**(4): p. 992-999.

19.   Fratantoni, J.C., C.W. Hall, and E.F. Neufeld, *Hurler and Hunter syndromes: mutual correction of the defect in cultured fibroblasts.* Science, 1968. **162**(3853): p. 570-572.

20.   Bruni, S., et al., *Update on treatment of lysosomal storage diseases.* Acta Myologica, 2007. **26**(1): p. 87.

21.   Parenti, G., et al., *New strategies for the treatment of lysosomal storage diseases.* International journal of molecular medicine, 2013. **31**(1): p. 11-20.

22.   Beck, M., *New therapeutic options for lysosomal storage disorders: enzyme replacement, small molecules and gene therapy.* Human genetics, 2007. **121**(1): p. 1-22.

23.   Mistry, P.K., E.P. Wraight, and T.M. Cox, *Therapeutic delivery of proteins to macrophages: implications for treatment of Gaucher's disease.* The Lancet, 1996. **348**(9041): p. 1555-1559.

24.   McNeill, A., et al., *Ambroxol improves lysosomal biochemistry in glucocerebrosidase mutation-linked Parkinson disease cells.* Brain, 2014. **137**(5): p. 1481-1495.

25.   Brady, R.O., J. Kanfer, and D. Shapiro, *The metabolism of glucocerebrosides I. Purification and properties of a glucocerebroside-cleaving enzyme from spleen tissue.* Journal of Biological Chemistry, 1965. **240**(1): p. 39-43.

26. Balwani, M., et al., *Type 1 Gaucher disease: significant disease manifestations in "asymptomatic" homozygotes.* Archives of internal medicine, 2010. **170**(16): p. 1463-1469.

27. Grabowski, G.A., *Phenotype, diagnosis, and treatment of Gaucher's disease.* The Lancet, 2008. **372**(9645): p. 1263-1271.

28. Tajima, A., et al., *Clinical and genetic study of Japanese patients with type 3 Gaucher disease.* Molecular genetics and metabolism, 2009. **97**(4): p. 272-277.

29. Grabowski, G.A., *Gaucher disease and other storage disorders.* (1520-4383 (Electronic)).

30. Goedert, M., *Alzheimer's and Parkinson's diseases: The prion concept in relation to assembled Aβ, tau, and α-synuclein.* Science, 2015. **349**(6248): p. 1255555.

31. Sigurlaug, S., *The clinical symptoms of Parkinson's disease.* Journal of Neurochemistry, 2016. **139**(S1): p. 318-324.

32. Chaudhuri, K.R., D.G. Healy, and A.H. Schapira, *Non-motor symptoms of Parkinson's disease: diagnosis and management.* The Lancet Neurology, 2006. **5**(3): p. 235-245.

33. Politis, M., et al., *Parkinson's disease symptoms: the patient's perspective.* Movement Disorders, 2010. **25**(11): p. 1646-1651.

34. Chaudhuri, K.R. and A.H. Schapira, *Non-motor symptoms of Parkinson's disease: dopaminergic pathophysiology and treatment.* The Lancet Neurology, 2009. **8**(5): p. 464-474.

35. Bendor, J.T., T.P. Logan, and R.H. Edwards, *The function of α-synuclein.* Neuron, 2013. **79**(6): p. 1044-1066.

36. Diao, J., et al., *Native α-synuclein induces clustering of synaptic-vesicle mimics via binding to phospholipids and synaptobrevin-2/VAMP2.* Elife, 2013. **2**.

37. Bodner, C.R., C.M. Dobson, and A. Bax, *Multiple tight phospholipid-binding modes of α-synuclein revealed by solution NMR spectroscopy.* Journal of molecular biology, 2009. **390**(4): p. 775-790.

38. Fevrier, B., et al., *Cells release prions in association with exosomes.* Proceedings of the National Academy of Sciences of the United States of America, 2004. **101**(26): p. 9683-9688.

39.   Pan, T., et al., *The role of autophagy-lysosome pathway in neurodegeneration associated with Parkinson's disease.* Brain, 2008. **131**(8): p. 1969-1978.

40.   Chu, Y. and J.H. Kordower, *The prion hypothesis of Parkinson's disease.* Current neurology and neuroscience reports, 2015. **15**(5): p. 28.

41.   Kordower, J.H., et al., *Lewy body–like pathology in long-term embryonic nigral transplants in Parkinson's disease.* Nature medicine, 2008. **14**(5): p. 504.

42.   Steiner, J.A., E. Angot, and P. Brundin, *A deadly spread: cellular mechanisms of α-synuclein transfer.* Cell death and differentiation, 2011. **18**(9): p. 1425.

43.   Ejlerskov, P., et al., *Tubulin polymerization-promoting protein (TPPP/p25α) promotes unconventional secretion of α-synuclein through exophagy by impairing autophagosome-lysosome fusion.* Journal of Biological Chemistry, 2013. **288**(24): p. 17313-17335.

44.   Grant, B.D. and J.G. Donaldson, *Pathways and mechanisms of endocytic recycling.* Nature reviews Molecular cell biology, 2009. **10**(9): p. 597.

45.   Dehay B Fau - Dehay, B., et al., *Lysosomal Impairment in Parkinson's Disease.* (0885-3185 (Print)).

46.   McCarthy, D. and H. Asada, *Discovery of Changes in N-linked Glycans Associated with Parkinson's Disease*. 2013, The Michael J. Fox Foundation: Pine Brook, New Jersey, United States.

47.   Deng, H., X. Xiu, and J. Jankovic, *Genetic convergence of Parkinson's disease and lysosomal storage disorders.* Molecular neurobiology, 2015. **51**(3): p. 1554-1568.

48.   Mazzulli, Joseph R., et al., *Gaucher Disease Glucocerebrosidase and α-Synuclein Form a Bidirectional Pathogenic Loop in Synucleinopathies.* Cell, 2011. **146**(1): p. 37-52.

49.   Bembi, B., et al., *Gaucher's disease with Parkinson's disease Clinical and pathological aspects.* Neurology, 2003. **61**(1): p. 99-101.

50.   Katsel, P., C. Li, and V. Haroutunian, *Gene expression alterations in the sphingolipid metabolism pathways during progression of dementia and Alzheimer's disease: a shift toward ceramide accumulation at the earliest recognizable stages of Alzheimer's disease?* Neurochemical research, 2007. **32**(4-5): p. 845-856.

51.   Mielke, M.M., et al., *Plasma ceramide and glucosylceramide metabolism is altered in sporadic Parkinson's disease and associated with cognitive impairment: a pilot study.* PLoS One, 2013. **8**(9): p. e73094.

52.   Hara-Kuge, S., et al., *Vesicular-integral membrane protein, VIP36, recognizes high-mannose type glycans containing α1→ 2 mannosyl residues in MDCK cells.* Glycobiology, 1999. **9**(8): p. 833-839.

53.   Helenius, A. and M. Aebi, *Intracellular functions of N-linked glycans.* Science, 2001. **291**(5512): p. 2364-2369.

54.   Scott, H. and V.M. Panin, *N-glycosylation in regulation of the nervous system*, in *Glycobiology of the Nervous System*. 2014, Springer. p. 367-394.

55.   Stanley, P. and R.D. Cummings, *Structures common to different glycans.* 2017.

56.   Freeze, H.H., J.D. Esko, and A.J. Parodi, *Glycans in glycoprotein quality control.* 2009.

57.   Figura, K.v. and A. Hasilik, *Lysosomal enzymes and their receptors.* Annual review of biochemistry, 1986. **55**(1): p. 167-193.

58.   Wiederschain, G.Y., *Essentials of glycobiology.* Biochemistry (Moscow), 2009. **74**(9): p. 1056-1056.

59.   Barondes, S.H., et al., *Galectins: a family of animal beta-galactoside-binding lectins.* Cell, 1994. **76**(4): p. 597-8.

60.   Liener, I., *The lectins: properties, functions, and applications in biology and medicine*. 2012: Elsevier.

61.   Chauhan, S., et al., *TRIMs and galectins globally cooperate and TRIM16 and galectin-3 co-direct autophagy in endomembrane damage homeostasis.* Developmental cell, 2016. **39**(1): p. 13-27.

62.   Fahie, K. and N.E. Zachara, *Molecular Functions of Glycoconjugates in Autophagy.* Journal of Molecular Biology, 2016. **428**(16): p. 3305-3324.

63.   Kimura, T., et al., *Dedicated SNAREs and specialized TRIM cargo receptors mediate secretory autophagy.* (1460-2075 (Electronic)).

64. Chen, Z. and C. Zhong, *Decoding Alzheimer's disease from perturbed cerebral glucose metabolism: implications for diagnostic and therapeutic strategies.* Progress in neurobiology, 2013. **108**: p. 21-43.

65. Skundric, D.S. and R.P. Lisak, *Role of neuropoietic cytokines in development and progression of diabetic polyneuropathy: from glucose metabolism to neurodegeneration.* Journal of Diabetes Research, 2003. **4**(4): p. 303-312.

66. Mazziotta, J.C., et al., *Reduced cerebral glucose metabolism in asymptomatic subjects at risk for Huntington's disease.* New England Journal of Medicine, 1987. **316**(7): p. 357-362.

67. Yuzwa, S.A., et al., *Increasing O-GlcNAc slows neurodegeneration and stabilizes tau against aggregation.* Nature chemical biology, 2012. **8**(4): p. 393.

68. Marotta, N.P., et al., *O-GlcNAc modification blocks the aggregation and toxicity of the protein α-synuclein associated with Parkinson's disease.* Nature chemistry, 2015. **7**(11): p. 913.

69. Hart, G.W. and Y. Akimoto, *The O-GlcNAc modification.* 2009.

70. Mizushima, N. and M. Komatsu, *Autophagy: renovation of cells and tissues.* Cell, 2011. **147**: p. 728-41.

71. Klionsky Dj Fau - Klionsky, D.J., *The molecular machinery of autophagy: unanswered questions.* Journal of Cell Science, 2005. **118**(1): p. 7-18.

72. Klionsky, D.J. and Y. Ohsumi, *Vacuolar import of proteins and organelles from the cytoplasm.* Annual Review of Cell and Developmental Biology, 1999. **15**: p. 1-32.

73. Kaushik, S. and A.M. Cuervo, *Chaperone-mediated autophagy: a unique way to enter the lysosome world.* Trends in cell biology, 2012. **22**(8): p. 407-417.

74. Li, W.-w., J. Li, and J.-k. Bao, *Microautophagy: lesser-known self-eating.* Cellular and Molecular Life Sciences, 2012. **69**(7): p. 1125-1136.

75. Menzies, F.M., A. Fleming, and D.C. Rubinsztein, *Compromised autophagy and neurodegenerative diseases.* Nature Reviews Neuroscience, 2015. **16**(6): p. 345.

76. Nixon, R.A., *The role of autophagy in neurodegenerative disease.* Nature medicine, 2013. **19**(8): p. 983.

77.   Mizushima, N., et al., *Autophagy fights disease through cellular self-digestion.* Nature, 2008. **451**(7182): p. 1069.

78.   Levine, B. and D.J. Klionsky, *Development by self-digestion: molecular mechanisms and biological functions of autophagy.* Developmental cell, 2004. **6**(4): p. 463-477.

79.   Klionsky, D.J. and S.D. Emr, *Autophagy as a regulated pathway of cellular degradation.* Science, 2000. **290**(5497): p. 1717-1721.

80.   Klionsky, D.J., *Autophagy: from phenomenology to molecular understanding in less than a decade.* Nature reviews Molecular cell biology, 2007. **8**(11): p. 931.

81.   Mindell, J.A., *Lysosomal acidification mechanisms.* Annual review of physiology, 2012. **74**: p. 69-86.

82.   Lüllmann-Rauch, R., *History and morphology of the lysosome*, in *Lysosomes*. 2005, Springer. p. 1-16.

83.   Munier-Lehmann, H., F. Mauxion, and B. Hoflack, *Function of the two mannose 6-phosphate receptors in lysosomal enzyme transport*. 1996, Portland Press Limited.

84.   Gruenberg, J. and K.E. Howell, *Membrane traffic in endocytosis: insights from cell-free assays.* Annual review of cell biology, 1989. **5**(1): p. 453-481.

85.   Ghosh, P. and S. Kornfeld, *The GGA proteins: key players in protein sorting at the trans-Golgi network.* European journal of cell biology, 2004. **83**(6): p. 257-262.

86.   Mallard, F., et al., *Early/recycling endosomes-to-TGN transport involves two SNARE complexes and a Rab6 isoform.* The Journal of cell biology, 2002. **156**(4): p. 653-664.

87.   Stoorvogel, W., et al., *Late endosomes derive from early endosomes by maturation.* Cell, 1991. **65**(3): p. 417-427.

88.   Morelli, A.E., et al., *Endocytosis, intracellular sorting, and processing of exosomes by dendritic cells.* Blood, 2004. **104**(10): p. 3257-3266.

89.   Luzio, J.P., et al., *Lysosome-endosome fusion and lysosome biogenesis.* J Cell Sci, 2000. **113**(9): p. 1515-1524.

90.   Futter, C.E., et al., *Multivesicular endosomes containing internalized EGF-EGF receptor complexes mature and then fuse directly with lysosomes.* The Journal of cell biology, 1996. **132**(6): p. 1011-1023.

91.   Raiborg, C. and H. Stenmark, *The ESCRT machinery in endosomal sorting of ubiquitylated membrane proteins.* Nature, 2009. **458**(7237): p. 445.

92.   Rabouille, C., V. Malhotra, and W. Nickel, *Diversity in unconventional protein secretion*. 2012, The Company of Biologists Ltd.

93.   Rubartelli, A., et al., *A novel secretory pathway for interleukin-1 beta, a protein lacking a signal sequence.* The EMBO journal, 1990. **9**(5): p. 1503-1510.

94.   Andrei, C., et al., *The secretory route of the leaderless protein interleukin 1β involves exocytosis of endolysosome-related vesicles.* Molecular biology of the cell, 1999. **10**(5): p. 1463-1475.

95.   Ponpuak, M., et al., *Secretory autophagy.* Current Opinion in Cell Biology, 2015. **35**: p. 106-116.

96.   Manjithaya, R. and S. Subramani, *Role of autophagy in unconventional protein secretion.* Autophagy, 2010. **6**(5): p. 650-651.

97.   Nilsson, P., et al., *Abeta secretion and plaque formation depend on autophagy.* Cell Rep, 2013. **5**(1): p. 61-9.

98.   Duran Jm Fau - Duran, J.M., et al., *Unconventional secretion of Acb1 is mediated by autophagosomes.* (0021-9525 (Print)).

99.   Johnstone Rm Fau - Adam, M., et al., *Vesicle formation during reticulocyte maturation. Association of plasma membrane activities with released vesicles (exosomes).* (0021-9258 (Print)).

100.   Buzas, E.I., et al., *Emerging role of extracellular vesicles in inflammatory diseases.* Nat Rev Rheumatol, 2014. **10**(6): p. 356-64.

101.   Kong, S.M., et al., *Parkinson's disease-linked human PARK9/ATP13A2 maintains zinc homeostasis and promotes α-Synuclein externalization via exosomes.* Human molecular genetics, 2014. **23**(11): p. 2816-2833.

102.   Abels, E.R. and X.O. Breakefield, *Introduction to Extracellular Vesicles: Biogenesis, RNA Cargo Selection, Content, Release, and Uptake.* Cellular and Molecular Neurobiology, 2016. **36**(3): p. 301-312.

103.   György, B., et al., *Membrane vesicles, current state-of-the-art: emerging role of extracellular vesicles.* Cellular and molecular life sciences, 2011. **68**(16): p. 2667-2688.

104. Lee, H.-J., S. Patel, and S.-J. Lee, *Intravesicular localization and exocytosis of α-synuclein and its aggregates.* Journal of Neuroscience, 2005. **25**(25): p. 6016-6024.

105. Stuendl, A., et al., *Induction of α-synuclein aggregate formation by CSF exosomes from patients with Parkinson's disease and dementia with Lewy bodies.* Brain, 2015. **139**(2): p. 481-494.

106. Shin, N., et al., *LRRK2 regulates synaptic vesicle endocytosis.* Experimental cell research, 2008. **314**(10): p. 2055-2065.

107. Tang, F.-L., et al., *VPS35 in dopamine neurons is required for endosome-to-Golgi retrieval of Lamp2a, a receptor of chaperone-mediated autophagy that is critical for α-synuclein degradation and prevention of pathogenesis of Parkinson's disease.* Journal of Neuroscience, 2015. **35**(29): p. 10613-10628.

108. Sullivan, C.P., et al., *Retromer disruption promotes amyloidogenic APP processing.* Neurobiology of disease, 2011. **43**(2): p. 338-345.

109. Zavodszky, E., et al., *Mutation in VPS35 associated with Parkinson's disease impairs WASH complex association and inhibits autophagy.* Nature communications, 2014. **5**: p. 3828.

110. Park, J.-S., et al., *Parkinson's disease-associated human ATP13A2 (PARK9) deficiency causes zinc dyshomeostasis and mitochondrial dysfunction.* Human molecular genetics, 2014. **23**(11): p. 2802-2815.

111. Thoreen, C.C. and D.M. Sabatini, *Rapamycin inhibits mTORC1, but not completely.* Autophagy, 2009. **5**(5): p. 725-726.

112. Sarkar, S., et al., *Rapamycin and mTOR-independent autophagy inducers ameliorate toxicity of polyglutamine-expanded huntingtin and related proteinopathies.* Cell death and differentiation, 2009. **16**(1): p. 46.

113. Cao, C., et al., *Inhibition of mammalian target of rapamycin or apoptotic pathway induces autophagy and radiosensitizes PTEN null prostate cancer cells.* Cancer research, 2006. **66**(20): p. 10040-10047.

114. Yamamoto, A., et al., *Bafilomycin A1 prevents maturation of autophagic vacuoles by inhibiting fusion between autophagosomes and lysosomes in rat hepatoma cell line, H-4-II-E cells.* Cell structure and function, 1998. **23**(1): p. 33-42.

115.   Klionsky, D.J., et al., *Does bafilomycin A1 block the fusion of autophagosomes with lysosomes?* 2008, Taylor & Francis.

116.   Kuno, A., et al., *Evanescent-field fluorescence-assisted lectin microarray: a new strategy for glycan profiling.* Nature methods, 2005. **2**(11): p. 851.

117.   Prescher, J.A. and C.R. Bertozzi, *Chemical technologies for probing glycans.* Cell, 2006. **126**(5): p. 851-854.

118.   Jiang, S., et al., *Secretory versus degradative autophagy: unconventional secretion of inflammatory mediators.* J Innate Immun, 2013. **5**(5): p. 471-9.

119.   Minakaki, G., et al., *Autophagy inhibition promotes SNCA/alpha-synuclein release and transfer via extracellular vesicles with a hybrid autophagosome-exosome-like phenotype.* Autophagy, 2018: p. 1-22.

VITA

The author, Ben, was born in Phoenix, AZ on March 21, 1986 to Capt. Glen and Melody Cook. He served as a Field Medical Service Technician for the U.S. Marine Corps Infantry. Ben was married to Meryl Sullivan in May 2011 and they had a daughter, Emery, in January 2013. While continuing his service in the U.S. Navy Reserve, he attended Northeastern Illinois University in Chicago, Illinois where, in May 2013, he earned a Bachelor's of Arts, cum laude, in Sociology. After graduation, Ben worked as a Certified Brain Injury Specialist in a post-acute brain injury rehabilitation center. During this tenure, Ben decided to pursue an advanced degree and matriculated into the Loyola University Chicago Stritch School Infectious Disease and Immunology Graduate Program. Upon completion of his master's degree, Ben will further his education in a Ph.D. program in medical sciences at University of Illinois, Chicago.